

The effects of fungal strains with biocontrol potential on the motility and reproduction of *Meloidogyne incognita*

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

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Graduation May 2023

26988569

ACKNOWLEDGEMENTS

First of all, I want to honour and thank our Heavenly Father for this opportunity and for all the strength and support He has given me to be able to accomplish this achievement. It was once again such a big realization of how great our God truly is.

To Prof Driekie Fourie, words can't describe how much I appreciate all the effort and work you put in to help me with this dissertation. I will forever be grateful to you for guiding me through each and every step of the way.

Dr Gerhard du Preez, my co-supervisor, a great thanks to you for all the inputs I so gratefully received from your side. It made a big contribution towards the process of writing this dissertation.

Dr Gerhard Engelbrecht, thank you for your guidance and mentorship during this assessment period. Be it with practical work or the write up of this dissertation, you were always there to give insightful inputs. Thank you for this.

To my friends and family, thank you for the endless support and motivation you gave me when I so very much needed it. I would like to give a special thanks to my mother and Ané Loggenberg in this regard for always being there when times were tough.

A big thanks to Andermatt Plant Health Products (Pty) Ltd. and AgriSETA for sponsorship of this study in order to improve research in this field. It would not have been possible without you. Gilmore Pambuka, thank you also for all your guidance in the practical aspects of this study. I greatly appreciate it.

ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) pose a global threat to grain crop production since it causes major yield losses which results in less food sources for animals and humans. Since classical nematicides are continually withdrawn from markets, safer nematode management alternatives are gaining popularity. This includes the use of biological agents, viz. fungi and bacteria in particular, for the management of major nematode pests such as *Meloidogyne* spp. During this study strains of *Trichoderma asperellum*, *Trichoderma asperellum* kd, *Trichoderma harzianum*, *Trichoderma virens* and *Clonostachys rosea* together with two formulated products containing spores of *C. rosea* (CR) and *T. asperellum* strain kd (Eco-T®) as active ingredients and supplied by Andermatt Plant Health Products (Pty) Ltd. were tested for their adverse effects on the biology of *M. incognita*. *In vitro* trials were conducted to determine the effects of the spores of the fungal isolates and formulated products on the motility of second-stage juveniles (J2), while *in vivo* trials investigated their effects on female reproduction. The first set of *in vitro* studies, during which the fungal strains were propagated on potato dextrose agar, resulted in non-optimal growth and spore production. Follow-up *in vitro* trials using a bran-based substrate as growth medium for the fungal strains, resulted in the highest concentrations of 10^8 spores/ml being the most effective (>70%) in reducing J2 motility of all strains. Unfortunately the efficacy of the two formulated products in reducing J2 motility was inferior (<30%). *In vivo* glasshouse experiments, conducted for all five strains applied at concentrations of 10^7 and 10^8 spores/ml, as either seed treatments or direct soil applications, were able to reduce *M. incognita* life-stage densities 48 days after planting and inoculation between 10% - 45%; *T. harzianum* having the lowest and *C. rosea* having the highest reduction respectively. Reduction in *M. incognita* root galling ranged from 15% - 47% with *T. harzianum* having the lowest and *T. virens* having the highest. Insignificant decreases of *M. incognita* J2 numbers in soil as well as increases in plant growth parameters were obtained for soybean in this South African study. Similarly to the *in vitro* results obtained in this study, the efficacy of the two formulated products in reducing *M. incognita* life-stage numbers and root galling were mostly insignificant, while similar trends were evident for non-significant increases in plant growth parameters. Field experiments should be done to evaluate the fungal strains and formulated products under natural, prevailing environmental conditions, while improved formulation and seed-coating approaches should be investigated to optimize the efficacy of these biological products showing potential bionematicide activity.

Keywords: *Clonostachys rosea*, direct soil application, *in vitro*, *in vivo*, *Meloidogyne incognita*, seed treatment, soybean, *Trichoderma* spp.

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CHAPTER 1 INTRODUCTION

Grain crops are important because they provide about half the calories humans consume and the production thereof has increased substantially since the 1990's – especially for soybean (*Glycine max* L. Merr.) and maize (*Zea mays* L.) which are being used in rotation on a big scale in South Africa (Paterson *et al.*, 2005; Gianessi, 2013). Sustainable production of grain crops is essential due to an increase in human population threatening food security. This applies both regionally and globally and especially to a great extent in developing countries such as sub-Saharan Africa (SSA) - including South Africa (SA). This is a contributing factor to daily constraints on food availability for the population (Amalu, 2002; Kumar & Kalita, 2017). Not only is sustainable food production important for humans but also for animals, since 80% of land is used for animal grazing and a third of the world's cereal production is fed to animals (FAO, 2006; Alexandratos & Bruinsma, 2012; Smith & Gregory, 2012).

The production of grain crops is hampered by what we call a 'yield gap' in which the actual crop yield deviates from the potential crop yield because of inefficient management (Neumann *et al.*, 2010). Low soybean and maize production yields in SSA can be attributed to several challenges including declining soil fertility, decreased crop productivity, reduced N₂-fixation caused by biological and environmental factors and erratic or low rainfall patterns (Sileshi *et al.*, 2010; Chianu *et al.*, 2011; Buah *et al.*, 2017; Acevedo-Siaca & Goldsmith, 2020). On top of this, management practices such as tilling, application of synthetic fertilizers, overuse of chemicals against pests and diseases (causing water pollution) and crop rotation (which do not suppress but rather promote pest infections such as plant-parasitic nematodes) degrade soil health (Sileshi *et al.*, 2010; Bazuin *et al.*, 2011; Kihara *et al.*, 2012; Van der Laan *et al.*, 2017; Engelbrecht *et al.*, 2020). According to Sileshi *et al.* (2010), the use of inorganic fertilizers gave rise to bigger yield gaps than those of organic supplements. The illusion that SSA is land rich is countered with the fact that this land has poor and degraded soil quality (Vlek *et al.*, 2008; Zingore *et al.*, 2015). According to Vlek *et al.* (2008), SSA's lands require optimized management practices to restore high potential in agricultural regions. To improve sustainable food production while reducing the unfavourable impact on the environment, the advancement towards conservation agriculture is becoming more popular (Swanepoel *et al.*, 2017; Loggenberg, 2021).

General insect, fungal, bacterial, and viral pests attacking soybean in SA include the soybean leaf miner (*Proaerema modicella*), rust disease fungus (*Phakopsora pachyrhizi*), bacterial tan spot or bacterial wilt (*Curtobacterium flaccumfaciens*) and soybean blotchy mosaic virus (*Cytorhabdovirus* spp.) (Lamprecht *et al.*, 2010; Fourie *et al.*, 2015; Pawlowski & Hartman, 2019). The taro caterpillar (*Spodoptera litura*), fungal stalk and ear rot (*Fusarium* spp.) and maize streak

disease (*Xanthomonas vasicola*) (Chilaka *et al.*, 2012; Karamura *et al.*, 2015; Rahmawati *et al.*, 2020) are pests of maize. On top of these pest infections the ubiquitous appearance of plant-parasitic nematodes poses a great constraint on agricultural crop production (Fourie *et al.*, 2015). *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949 is one of the most predominant and economically important species concerning root-knot nematodes globally, but especially in SSA on soybean and other rotation crops such as maize (Fourie *et al.*, 2015; Cox *et al.*, 2019; Wram & Zasada, 2019). *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne enterolobii* Yang and Eisenback, 1983 and *Meloidogyne javanica* (Treub, 1885) Chitwood 1949 have also been found in maize and soybean production areas in SSA (Fourie *et al.*, 2015; Pretorius, 2018; Collet, 2020). Traditionally, chemical control of plant-parasitic nematodes has been practiced in SSA including SA, relying on synthetic chemical nematicides combined with either monoculture cropping systems or wheat-based crop rotations, giving rise to higher plant-parasitic nematode populations – especially root-knot nematodes (Atandi *et al.*, 2017; Flower *et al.*, 2019). According to Agbenin (2011), biological control is more stable, more enduring, and environmentally friendly than chemical control and therefore is proposed as an attractive substitute to mitigate plant-parasitic nematode damage to crops.

Development of biological control products utilizing fungi and bacteria has formed part of mainstream research to control plant-parasitic nematodes in roots of maize and soybean (Engelbrecht *et al.*, 2020; Poveda *et al.*, 2020; Ahmad *et al.*, 2021; Karuri, 2022). For example, commercial products (mainly containing *Bacillus* spp.) such as BioYield® (combination of *Bacillus amyloliquefaciens* strain IN937a and *Bacillus subtilis* strain GB03) (Gustafson LLC, USA) (Kloepper *et al.*, 2004; Burkett-Cadena *et al.*, 2008); Nemix® (*Bacillus* spp.) (AgriLife/CHR Hansen, Brazil) (Hallmann *et al.*, 2009) and VOTiVO® (*Bacillus firmus* GB – 126) (Bayer CropScience, Germany) (Wilson & Jackson, 2013) are used as environmentally safe bacterial alternatives (Xiang *et al.*, 2017a). Poncho®VOTiVO® is a biocontrol product also manufactured by BASF, utilising *B. firmus* I-1582 as a seed coat on maize and soybean seeds (Haarith *et al.*, 2020; BASF, 2021). According to Pacheco *et al.* (2020), certain *Trichoderma* spp. were able to produce promising control for *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev & Schuurmans-Stekhoven, 1941 in soybean. *Purpureocillium lilacinum* and *Purpureocillium chlamydosporia* have also been found to reduce rhizosphere colonization of plant-parasitic nematodes when applied to infested maize (Baheti *et al.*, 2017).

This study focused on and aimed to study the potential effect of fungal strains on *M. incognita* second-stage juvenile (J2) motility (*in vitro* assays) and female reproduction (*in vivo*) utilizing spores of isolates of *Trichoderma* spp. and *Clonostachys rosea* to determine whether it can be used as biocontrol agents to protect crops and enable sustainable production thereof.

The objectives of the study were:

- i. To determine the effect of four *Trichoderma* isolates and *C. rosea* and two formulated products (CR and Eco-T®) on the motility of *M. incognita* J2 in *in vitro* well-plate experiments (initial and repeat).
- ii. To determine the efficacy of spores, as soil-applied and seed treatments, of four *Trichoderma* isolates and *C. rosea* in reducing *M. incognita* densities in soybean rhizospheres in glasshouse experiments (initial and repeat).
- iii. To determine the efficacy of two commercialized products containing *Trichoderma asperellum* kd and *C. rosea* in reducing *M. incognita* densities in soybean rhizospheres in initial and repeat glasshouse experiments (soil-applied and seed treatments).

Hypotheses for the study:

- i. Spores of endemic *Trichoderma* spp. and *C. rosea* fungal isolates, as well as those of two formulated products (CR and Eco-T®) will reduce *M. incognita* J2 motility.
- ii. Spores of endemic *Trichoderma* fungal isolates and those of *C. rosea*, as well as the application of formulated products of *C. rosea* and *T. asperellum* strain kd, respectively, will reduce *M. incognita* densities in soybean rhizospheres.

CHAPTER 2 LITERATURE REVIEW

Despite the fact that the world committed to ending hunger, malnutrition and food insecurity in the year 2015, we are still off-track to achieve this objective (FAO, 2020). An increase of up to 70% in global food production has to commence in order to feed the entire populace of the world by the year 2050 (Hassan *et al.*, 2013). Grain crops play a very important role as a food source since 50% of the global population's calorie intake consists of cereal grains (Awika, 2011). Table 2-1 indicates the top countries with global food production figures of the important grain crops, namely maize (*Zea mays* L.) and soybean (*Glycine max* L. Merr.) for the year 2018 since these two crops are commonly used in rotation. However, since this study focused on soybean, no reference will further be made to maize. Soybean is a very important leguminous crop concerning food production since it consists of about 40% protein and 20% oil (Chung & Singh, 2008). According to Oyedunmade (2003), soybean is considered as a cheap source of protein in the human diet as well as livestock feeding.

The promotion of more sustainable agricultural practices has mainly been due to the negative impacts associated with conventional food production (Pimentel *et al.*, 2005; Pelletier *et al.*, 2008). Conventional farming includes the application of high dosages of synthetic chemicals paired with heavy tillage practices (Gomiero *et al.*, 2011; Sheoran *et al.*, 2019). In SSA (sub-Saharan Africa) the implementation of alternative agricultural practices such as biological control is still low, but increasing due to the need for such practices to be manageable, productive, and profitable (De Ponti *et al.*, 2012; Seufert *et al.*, 2012; Crowder & Reganold, 2015; Muller *et al.*, 2017; Seufert & Ramankutty, 2017; Meemken & Qaim, 2018; Schader *et al.*, 2021). Most South African farmers still utilize soil tillage, monocropping and fallow periods which are all considered as unsustainable practices (Haarhoff *et al.*, 2020). The proposition for less degrading management practices such as biological control is, however, growing in terms of its adoption by South African producers (Findlater *et al.*, 2019; Haarhoff *et al.*, 2020).

Since there is a high production of maize and soybean in the global top producing countries (Table 2-1), elimination of threatening plant pathogens becomes significant. Apart from the adverse impact of plant-parasitic nematodes on crop production, bacterial, viral, fungal pathogens and insects are other diseases and pests that hinder successful agricultural produce (Lamprecht *et al.*, 2010; Chilaka *et al.*, 2012; Fourie *et al.*, 2015; Karamura *et al.*, 2015; Pawlowski & Hartman, 2019; Rahmawati *et al.*, 2020).

Table 1-1: Global food production figures for maize and soybean in top producing countries, with special reference to sub-Saharan Africa for the year 2018 (FAOSTAT, 2020).

Crop	Area	Country (Top producers)	2018 Area harvested (Million ha)	2018 Production (Million MT)
Maize	International	USA	33, 079	392,450
		China (Mainland)	42,130	257, 173
		Brazil	16, 121	82, 288
		Argentina	7, 138	43, 462
		Ukraine	4, 564	35, 801
	sub-Saharan Africa	South Africa	2, 318	12, 510
		Nigeria	4, 853	10, 155
		Egypt	0, 935	7, 300
Soybean	International	USA	35, 657	123, 664
		Brazil	34, 771	117, 887
		Argentina	16, 318	37, 787
		China (Mainland)	7, 970	14, 189
		India	11, 400	13, 786
	sub-Saharan Africa	South Africa	0, 787	1, 540
		Nigeria	0, 780	0, 758
		Zambia	0, 191	0, 302

2.1 Soybean production in South Africa: a concise overview

The farmer's decision to produce soybean in South Africa (SA) is mainly affected by temperature, rainfall, and floods (Kephe *et al.*, 2020). Foyer *et al.* (2019) suggested that high-yielding soybean cultivars with sustainable yield traits, are able to compensate for yield reduction due to climate change, should be the focus of research. Temperature and rainfall are considered two main factors that limit soybean yields (Vollmann *et al.*, 2000; Ohnishi *et al.*, 2010; Gawęda *et al.*, 2020). Temperature ranges under which soybean is produced in SA generally ranges between 25 °C to 32 °C and have the potential to increase and decrease, because of global warming, between 3.4 °C to 3.8 °C for the high and low temperatures, respectively (Ajadi *et al.*, 2011; Harrison *et al.*, 2011; Adisa *et al.*, 2018; Mbangiwa *et al.*, 2019; Ahmad *et al.*, 2020). Due to global warming an increase in average temperature, drought, extreme events, and the change in amount of rainfall is a great challenge to grain production in SA (Masipa, 2017). Most farmers in SA rely on rain-fed farming making them incredibly vulnerable to drought because of climate change. With an average annual rainfall of 600 mm occurring in drier regions of SA, it is difficult to predict the outcome of rain-fed farming since factors such as evaporation also play a significant role (Masupha & Moeletsi, 2018).

Synthetic nitrogen fertilizers are predominantly used in dryland production in SA and give rise to emissions of nitrous oxide which contributes to climate change (Tongwane *et al.*, 2020). The application of synthetic fertilizers to grain crops has many complications such as the fact that it is expensive and contaminating underground and surface water sources (Musokwa *et al.*, 2019; Igiehon & Babalola, 2021). According to Igiehon & Babalola (2021), this contamination tends to increase the risk of blue baby syndrome in infants and stomach cancer in adults. The use of organic rather than synthetic fertilizer has potential to ameliorate the threat of climate change and food insecurity as well as the detrimental effects on the environment (Hammed *et al.*, 2019). To use a safe alternative for these damaging chemicals, organic fertilizers such as *Rhizobium/Bradyrhizobium* and mycorrhizal fungi as well as commercially available products have been tested for their ability to improve growth, seed quality, yield, and drought tolerance of soybean (Onyenali *et al.*, 2020; Igiehon & Babalola, 2021). Microbial bio-pesticides derived from microorganisms such as bacteria, fungi and viruses with actives attacking target pests are also applied to commercial crops (Kumar *et al.*, 2021). Investigation of the effect of microbial inoculants (*Pseudomonas* spp.) have been evaluated in SA on maize and resulted in an increase of plant height and girth (Alori *et al.*, 2019), while limited and usually non-published information on their effect on nematode assemblages exist (Personal communication, Driekie Fourie, NWU, April 2022).

From the year 1996, South Africa encountered a change from using glyphosate-sensitive to glyphosate-tolerant soybean cultivars in which the latter is currently dominating the local market (Fourie *et al.*, 2001; Mbatyoti *et al.*, 2020). About 85% of maize and soybean in SA are genetically modified and therefore sprayed with herbicides (Albrecht, 2017). According to Khojely *et al.* (2018), in 2017 the area under soybean cultivation in SA consisted of 90% glyphosate-tolerant cultivars. Dlamini *et al.* (2014), also stated that 80% of soybean seed purchased in SA are Roundup Ready® cultivars (DAFF, 2010). It is reported that over 50% of maize and 90% of soybean grown in SA is glyphosate tolerant (Dlamini *et al.*, 2014; James, 2015; ISAA, 2017; Viljoen *et al.*, 2019). Another genetically modified alternative brought to and used in SSA is Bt-maize resulting in a 56% reduction in insecticide use globally (Brookes & Barfoot, 2018; Ala-Kokko *et al.*, 2021).

Apart from insects, bacteria, fungi, and viruses attacking commercialized grain crops globally, plant-parasitic nematodes pose a great global threat (Oerke & Dehne, 2004; Sikora *et al.*, 2005; Moens *et al.* 2009; Ntidi *et al.*, 2012). Some key target species infecting and damaging for example soybean in SA are *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev & Schuurmans-Stekhoven, 1941 and *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 which can also appear in combination with *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Bekker *et al.*, 2016; Coyne *et al.*, 2018). Soybean-cropping systems are, however, in most South African fields adversely affected by root-knot nematodes (McDonald *et al.*, 2017; Briedenhann, 2018; Rashidifard *et al.*, 2021) and therefore the focus will be primarily on this genus.

2.2 Plant-parasitic nematodes

The phylum Nematoda consists of approximately 1 million species including human, animal, and plant parasites of which only 25% (~23 000) have been described as parasites of animals and plants (Blaxter & Koutsovoulos, 2015; Xu *et al.*, 2020). According to Blaxter & Koutsovoulos (2015), most terrestrial plants, animals and humans are associated with at least one parasitic nematode species during their lifetime. Approximately a third of the global human population experience nematode parasitism in their lives (Blaxter & Koutsovoulos, 2015). Respiratory and gastrointestinal disease caused by the parasite *Strongyloides stercoralis* Bavay, 1876 with inadequate weight gain as symptom, and thelaziasis caused by the parasite *Thelazia callipaeda* Railliet & Henry, 1910, are examples of nematodes that parasitize vertebrate animals as opposed to entomopathogenic nematodes (*Heterorhabditis* Poinar, 1976), and those that are obligate mycophages (*Bursaphelenchus* Fuchs, 1937 spp.), which are nematode parasites of invertebrates. Moreover, medical conditions such as anaemia (*Ancylostoma duodenale* Dubini,

1843), African river blindness (*Onchocerca volvulus* Leukart, 1983) and elephantiasis (*Wuchereria bancrofti* (Cobbold, 1877) Seurat, 1921) are some of the diseases caused in humans by parasitic nematodes (Wasmuth *et al.*, 2008; Blaxter & Koutsovoulos, 2015; Vidal *et al.*, 2021).

Plant-parasitic nematodes are major concern to global food security since they cause an estimated damage of \$US 80 billion to crop yields annually (Decraemer & Hunt, 2006; Keating *et al.*, 2010; Nicol *et al.*, 2011; Montarry *et al.*, 2021; Pulavarty *et al.*, 2021). In conjunction with this, nematode control continues having high financial costs as part of agricultural management practices (Toju & Tanaka, 2019). Amongst all genera of plant-parasitic nematodes, root-knot nematodes (*Meloidogyne* Goeldi, 1892) are the most devastating to the agricultural industry worldwide (Jones *et al.*, 2013; Castagnone-Sereno *et al.*, 2019), followed by cyst (*Globodera* Skarbilovich, 1959 and *Heterodera* A Schmidt, 1871) and lesion nematodes (*Pratylenchus* Filipjev, 1936). The soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) cause up to 60% yield loss when susceptible soybean cultivars are planted (Rocha *et al.*, 2021). *Pratylenchus thornei* Sher & Allen, 1954 is recognized as one of the most economically important root-lesion nematodes and is widely distributed in chickpea (*Cicer arietinum*, L.) production areas globally (Channale *et al.*, 2021). Spiral nematodes (*Scutellonema* spp. Andrásy, 1958) are also ubiquitous polyphagous, herbivorous nematodes and of economic importance to many food crops (Nicol *et al.*, 2011; Maina & Ng'endo, 2020). Another example of an economically important plant-parasitic nematode genus is *Helicotylenchus* Steiner, 1945, which causes plant growth reduction by feeding on plant roots (Daramola *et al.*, 2020).

2.2.1 Root-knot nematodes: classification, basic biology, and damage

Globally the economic importance of *Meloidogyne* spp. and *Pratylenchus* spp. is of great concern as these genera cause enormous yield losses to soybean and maize crops (Kawanobe *et al.*, 2020; Mo *et al.*, 2021). The soybean cyst nematode also attacks soybean crops in many countries (Argentina, Asia, Brazil, Canada, China, Korea, USA) including SSA, but have not yet been reported in SA (Wrather *et al.*, 2010; Mwangi *et al.*, 2015; Allen *et al.*, 2017; Niragire *et al.*, 2019; St-Marseille *et al.*, 2019; Mburu *et al.*, 2020; Huang *et al.*, 2021; Kang *et al.*, 2021; Personal communication, Driekie Fourie, NWU, November 2022).

The classification of the genus *Meloidogyne* is as follows (Van den Berg *et al.*, 2017):

Phylum Nematoda (Rudolphi, 1808) Lankester, 1877

Class Secernentea von Linstow, 1905

Subclass Diplogasteria Maggenti, 1982

Order Tylenchida Thorne, 1949

Suborder Tylenchina Chitwood, 1950

Superfamily Tylenchoidea Örley, 1880

Family Heteroderidae Filipjev & Schuurmans
Stekhoven, 1941

Subfamily Meloidogyninae Skarbilovich,
1959

Genus *Meloidogyne* Goeldi, 1892

Like other plant-parasitic nematode genera, *Meloidogyne* is an obligate parasite and therefore needs living plants to develop and reproduce (Oka *et al.*, 2000). Above ground symptoms caused by root-knot nematodes are not always easy to recognize in an infested soybean field and can be confused with water- or nutrient deficiency, lack of/ or limited fertilizer application, waterlogging due to heavy rains, and others as demonstrated in Figure 2-1 (Oka *et al.*, 2000). Upon the entry of root-knot nematodes into roots of host plants, they facilitate the possible entry of other plant pathogens such as bacteria and fungi, which cause other diseases to arise in crops (Oka *et al.*, 2000; Berry *et al.*, 2017). Root-knot nematodes are polyphagous meaning that they do not only feed in roots of flowering crops, but also crops such as grasses and trees by forming root galls (Oka *et al.*, 2000).



Figure 2-1: Damage symptoms (root galls) and damage caused by *Meloidogyne incognita* in soybean roots / fields (Driekie Fourie, NWU).

The root-knot nematode life cycle starts with a sedentary female forming an egg mass and releasing the eggs (Mashela *et al.*, 2017). These eggs develop into first-stage juveniles (J1) which again develop into infective, migratory second stage juveniles (J2) that hatch and penetrate the host's roots (Mashela *et al.*, 2017). The J2 then develops into the third and fourth stage juveniles (J3 & J4) which then finally develops into either males or females and the cycle repeats (Mashela

et al., 2017). The complete life cycle of *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Figure 2-2) ranges from 25 to 30 days, whereas the infective J2 takes one to five days to develop (Kavitha et al., 2011). Therefore, the first few days when a seedling emerges, it is in great danger and vulnerability to root-knot nematode infection (being exposed to viable, infective *M. incognita* J2s), representing the most crucial time for it to be protected.

Root-knot nematodes are economically the biggest concern in SA (Ngobeni et al., 2011; Rashidifard et al., 2019), particularly for soybean. Globally this genus parasitizes a wide range of agricultural crops, flowers, grasses, and weeds and has a broad host range with a worldwide distribution (Trudgill & Blok, 2001; Castagnone-Sereno et al., 2010; Fourie et al., 2017). *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne hapla* Chitwood, 1949, *M. incognita* and *M. javanica* are considered four of the most common species, of which *M. incognita* is the most economically important in SA and the focus of this study (Riekert, 1996; Ngobeni et al., 2011; Rashidifard et al., 2019; Mbatyoti et al., 2021). According to Castagnone-Sereno (2012) and (Rashidifard et al., 2019), *Meloidogyne enterolobii* Yang & Eisenback, 1983 is an emerging pest more devastating than its counterparts and infects a wide range of crops and weeds

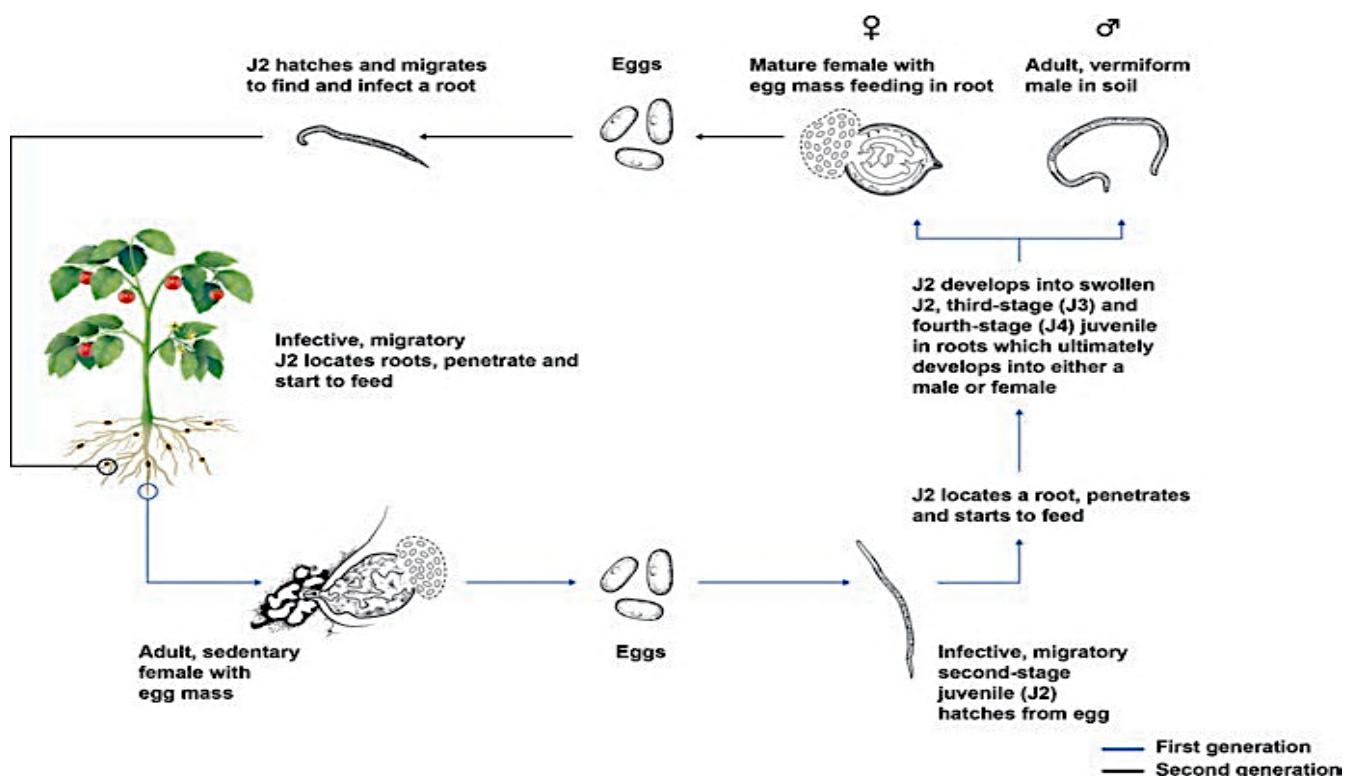


Figure 2-2: The life cycle of the genus *Meloidogyne* (Hannes Visagie, North-West University, South Africa).

A study done in South Africa on soybean infected with *M. incognita*, inoculating approximately 20 000 J2 and eggs per soybean seed at planting, resulted in a yield loss of up to 70% (Fourie *et al.*, 2010). The management of root-knot nematodes on soybean cultivars include methods such as host plant resistance, crop rotation and chemical control (Fourie *et al.*, 2017). According to Jones (2017), biological control of nematodes is the use of natural enemies such as protozoa, bacteria, and fungi to infect and control nematodes. Organic amendments (manure) and crop rotation with allelopathic plants are also considered as biological control of nematodes (Jones, 2017). Sharon *et al.* (2001), considers *Trichoderma harzianum* as an effective biological control agent of *M. incognita*, *M. javanica* and *M. arenaria* on a variety of crops. Ample research is available on the control of plant-parasitic nematodes using fungal bioagents, although not all these studies were effective (Mankau, 1980; Izuogo & Abiri, 2015). Although biological control of nematodes (*Meloidogyne* specifically) may not be an immediate effective alternative for chemical nematicides, it is still considered an integral part of IPM (integrated pest management) (Kerry, 1997; Chinheya, 2015).

2.3 Management of *Meloidogyne* species that infect and damage soybean crops

The damage caused to soybean by root-knot nematode parasitism is dependent on the species involved, population density, cultivar susceptibility and drought stress or extreme rainfall to which the host is subjected (Koenning, 2015). Globally IPM of root-knot nematodes consists of chemical control, genetic host plant resistance (GHPR), cultural control (primarily crop rotation) and recently an increase in using biological control agents (Fourie *et al.*, 2015; Xiang *et al.*, 2017b; Lopes *et al.*, 2020). Similarly, countries in SSA utilized chemical control, GHPR and cultural control as primary management for plant-parasitic nematodes (Onkendi *et al.*, 2014; Fourie *et al.*, 2017), but in recent years the market for biological control agents has been increasing (Xiang *et al.*, 2017a). The use of biological nematicides is expected to become more important in the future to replace synthetic nematicides (Desaeger *et al.*, 2020), or at least partially replace them.

2.3.1 Chemical control: traditional synthetic products as well as those derived from living organisms

Chemical control makes use of traditional toxic synthetic nematicides (Khalil & Selim, 2021; El-Ashry *et al.*, 2022) to control root-knot nematodes globally, especially in the United States of America (USA) and Brazil that are two of the biggest soybean-producing countries (Wrather *et al.*, 2001). Basamid® G (active ingredient/a.i. doxamet) (Jardim *et al.*, 2018) and ILeVO® (active ingredient fluopyram) (Dhital, 2020) are two of the conventional more toxic commercialized nematicides used in the USA on soybean crops. Marshal® 400 (a.i. carbosulfan) is a known nematicide widely used in Brazil on both maize and soybean (Ebene *et al.*, 2019). Limited

research is available for the efficacy of carbosulfan specifically on soybean, but this product has been tested on many other crops such as cowpea (*Vigna unguiculata* L. Walp.) (Verma *et al.*, 2009), chickpea (*C. arietinum*) (Meher *et al.*, 2008), okra (*Abelmoschus esculentus* L. Moench) (Sekhar & Khan, 2010) and sunflower (*Helianthus annuus*, L.) (Prasad & Archana, 2004) against different root-knot nematode species resulting in high efficacy. Poncho®VOTiVO® (Briedenhann, 2018) and ILeVO® (a.i. fluopyram) (Dhital, 2020) are commercialized bio-nematicides (softer product) on soybean in the USA, combining a synthetic insecticide (Poncho1) and *Bacillus firmus* (Abd-Elgawad & Askary, 2018; BASF, 2021). It has been found that the active ingredient of Poncho®VOTiVO® namely *B. firmus*, colonizes root-knot nematode egg masses and eventually destroy the eggs which reduces the number of infective J2s and root galling (Keren-Zur *et al.*, 2000; Wilson & Jackson, 2013). Another 'softer', more environmentally friendly product Avicta® 500 FS (a.i. abamectin), is applied as seed treatment instead of soil application and has become a main control strategy in the United States of America (USA) and Brazil (Briedenhann, 2018; Engelbrecht *et al.*, 2020; Syngenta, 2022). This product contains naturally occurring or semi-synthetic macrocyclic, lactones (endectocides) which are produced from the fermentation products of the soil-inhabiting actinomycete, *Streptomyces avermectilis* (Fent, 2014). A total of 80% reduction in *M. incognita* root galling was found by Cabrera *et al.* (2009) together with a 99% J2 mortality after a two-hour *in vitro* exposure to abamectin (Faske & Starr, 2006), pointing this product to having a potentially high efficacy.

Although there are a range of products available for the control of plant-parasitic nematodes in SA, it is highly unlikely to be profitable to apply to soybean crops (Briedenhann, 2018). In 2014 ILeVo® (Bayer CropScience; fluopyram as a.i.), had been registered against *M. incognita* (Faske & Hurd, 2015; Engelbrecht *et al.*, 2020). Velum® 1GR (a.i. fluopyram) in turn, was only recently registered on soybean against root-knot nematodes in SA (Bayer, 2021; Engelbrecht *et al.*, 2021). This product is a valuable tool that will benefit farmers since they had to rely on cultural control practices in the field until now (Fourie, 2022).

2.3.2 Genetic host plant resistance

Genetic host plant resistance involves the use of root-knot nematode resistant cultivars which is more convenient than chemical control, cost effective and forms a big part of integrated pest management (Haq *et al.*, 2021). According to Sikora *et al.* (2018a), it is important to use resistant cultivars in conjunction with other IPM practices to avoid the development of virulent populations that are encountered specifically with *M. incognita*. The USA and Brazil are two countries that especially uses GHPR as control method of plant-parasitic nematodes (Clifton *et al.*, 2018; Machado *et al.*, 2022). Numerous soybean resistant cultivars are available for the use against root-knot nematodes in the USA and Brazil (Sikora *et al.*, 2018a). 'Bragg' (Ha *et al.*, 2004),

'Forrest' (Luzzi *et al.*, 1994), 'Benning' (Boerma *et al.*, 1997), 'Boggs' (Boerma *et al.*, 2000), 'G93-9009' (Luzzi *et al.*, 1996), 'Haskell' (Boerma *et al.*, 1994), and 'Lee 74' (Ha *et al.*, 2004) are all soybean resistant cultivars available for the use against different *Meloidogyne* species (Centintas *et al.*, 2008). Machado (2021) also states that many soybean cultivars are available resistant to *M. incognita* of which most are derived from the cultivar Bragg. According to Centintas *et al.* (2008), a few soybean genetic resistant cultivars are available for use against *M. incognita* in Brazil (Luzzi *et al.*, 1987; Luzzi *et al.*, 1994). Another soybean cultivar - MG/BR 46 Conquista was considered resistant against *M. javanica* with a Brazilian study showing a reduction of the number of eggs per root (Puerari *et al.*, 2013).

Coming to SA, only a limited amount of resistant soybean cultivars is available (Fourie *et al.*, 1999; Fourie *et al.*, 2006; Chinheya *et al.*, 2017). This specifically relates to resistance against the root-knot nematode *M. incognita* (Fourie *et al.*, 2013), where Windham & Williams (1988) found that several soybean cultivars were resistant to *M. javanica* and *M. incognita* race 1 and 4 but did not show resistance against *M. incognita* race 2. In 2006 a study conducted by Fourie *et al.* showed that soybean LS5995 resistant cv. were able to reduce giant cell induction, development and feeding caused by *M. incognita*. Fourie *et al.* (1999), also found that the soybean cultivar 'Gazelle' has superior genetic resistance against *M. javanica*. Previously, updated information was available on resistant soybean cultivars because of screenings continuously taking place (Windham & Williams, 1988; Fourie *et al.*, 1999; Fourie *et al.*, 2017), but this is not the case anymore since no new information has been produced since 2018 (Mbatyoti, 2018).

2.3.3 Cultural control

Most damaging levels of root-knot nematodes that occur in a field can be attributed to the continuous planting of the same crop or other crop hosts for several years (Sikora *et al.*, 2018a). Globally, cultural control of plant-parasitic nematodes is mainly based on crop rotation but includes tillage, planting time, application of organic amendments and sanitization (Dababat & Fourie, 2018). According to Sikora *et al.* (2018), graminaceous crops are less susceptible hosts and can be rotated with soybean together with summer planting to reduce *Meloidogyne* juvenile populations. For thorough eradication of root-knot nematode infestations both preventive and corrective practices, including the washing of equipment to remove contaminated soil and freeing fields from weeds which are good hosts, are needed (Sikora *et al.*, 2018).

The use of crop rotation in SA is a common practice although this is generally not effective on soybean because the crop(s) (mainly maize, dry bean, sunflower, wheat) typically used in rotation with soybean are susceptible to *M. incognita* and *M. javanica* (Fourie *et al.*, 2017). Also, crop

rotation and the use of cover crops are hampered because of the feeding habit and wide distribution of *Meloidogyne* spp. (Oka *et al.*, 2000; Rabie, 2017). A study in Nigeria found that soybean rotated with sorghum resulted in lower *M. javanica* population densities (Jada & Muhammad, 2017; Fourie & De Waele, 2020). Two other studies conducted by Fourie *et al.* (1999; 2006), showed a 99% reduction in *M. javanica* population densities by rotating soybean cv. A7119 with carrot cv. Chanteney Karoo (*Daucus carota* L.) (Mashela *et al.*, 2017). According to Fourie *et al.* (2017), it is important to utilize poor hosts of root-knot nematodes (nematode-resistant cultivars) in soybean-based cropping systems for effective control.

2.3.4 Biological control

The three main reasons for introduction of biological control into IPM is to reduce (I) environmental as well as health risks; (II) management costs in the production of agricultural crops, and (III) The continuous de-registration of synthetically derived nematicides from international markets, has caused the necessity for developing and implementing more effective alternative control methods (Toju & Tanaka, 2019). The integration of especially bacteria and fungi into biological control methods has been popular in the development of some commercial products (Engelbrecht *et al.*, 2020). Biocontrol agents and essential oils may also be effective for management of *M. incognita*, while reducing risk to the environment. However, the biocontrol efficacy is usually unstable under field conditions (Ji *et al.*, 2020). Unlike herbicides, insecticides, and fungicides, for which safer products have been available for decades, nematicides seem to have become stagnant in the 1960's and 1970's, an age when regulatory requirements were in their infancy (Desaeger *et al.*, 2020), limiting registration of alternative products.

Globally the use of biological control has not yet been adopted by commercial growers. However, biological seed treatments with bacteria on soybean crops are being used in the USA, and Sikora *et al.* (2018) suggests that it could have a major impact on the production of food legumes. The use of seed treatments containing biological agents is a popular and preferred approach (Fourie & De Waele, 2020). According to Sikora (2022), more effective biological nematicides needs to be developed that include the following aspects: (i) contain natural predators of nematodes (nematode-trapping fungi), (ii) capable of being mass produced by industry and (iii) should survive multiple seasons and cropping cycles. Brazilian soybean producers utilize synthetically derived nematicides combined with biological control agents either as seed treatments or, when not compatible, a chemical is applied as seed treatment and, a biological treatment is done in-furrow (i.e., nematophagous fungi or rhizobacteria). The initial response is then brought about by the nematicide, followed by the long-term response of the biological agent developing as a living microorganism (Soares & Nascimento, 2022). Like Brazil, the USA utilizes chemical seed

treatments together with biological control agents to combat nematodes and improve yield (Sikora, 2022).

In SA the use of biological control against *Meloidogyne* spp. is also popular amongst soybean production and a few commercial products have been made available (Fourie & De Waele, 2020). VotIVO® (a.i. *B. firmus*) and CLARIVA® (a.i. *Pasteuria nishizawae*) are examples of these products and followed the registration of Avicta® 500 FS (a.i. avermectin produced by *Streptomyces avermitilis*) (Viljoen *et al.*, 2019). More examples of biological products registered in SA for commercial use include: Trichoplus (a.i. *Trichoderma asperelloides*) registered by BASF South Africa (Pty) Ltd; Eco-T® (a.i. *Trichoderma asperellum* strain kd) registered by Andermatt-PHP (Pty) Ltd; Tri-Cure SP (a.i. *T. harzianum*) N Laboratories (Pty) Ltd; Incite (a.i. *Bacillus methylotrophicus*) N Laboratories (Pty) Ltd and DiPel DF (a.i. *Bacillus thuringiensis*) Valent BioSciences (Agri-Intel, 2021). Unfortunately, only a few bionematicides are registered – specifically for soybean production – in SA. This study focused on fungi as biocontrol for root-knot nematodes of which more information follows below.

2.4 Fungi as biological control agents

The detrimental effects of fungal biological control agents (on the environment, non-target populations etc.) are minimal, and therefore the commercialization thereof has received increasing attention (Goettel *et al.*, 2001). Fungi (especially *Trichoderma* spp.) are used as a living active ingredient in many products for the safe and effective regulation of pest populations such as plant-parasitic nematodes, insects, weeds, and plant pathogens (Goettel *et al.*, 2001; Woo *et al.*, 2014). An example of such a product is the biological control product TrichoPlus® which contains different *Trichoderma* species and is registered in SA on a variety of crops namely: maize, potato (*Solanum tuberosum*, L.), bean (*Phaseolus vulgaris*, L.), strawberry (*Fragaria x ananassa*, Duchesne), cucumber (*Cucumis sativus*, L.), tomato (*Solanum lycopersicum*, L.) and flowers (Woo *et al.*, 2014). This product is used for the control of pathogens such as *Rhizoctonia solani*, *Pythium* spp., *Sclerotinia* spp., *Fusarium* spp. and *Phytophthora* spp. (De Bary, 1876) that causes damping off and pathogenic root diseases (BASF, 2017). *Trichoderma* strains are also known for controlling plant-parasitic nematodes. For example, it has been found that *T. harzianum* hyphae was highly capable of parasitizing eggs of *Caenorhabditis elegans* Maupas, 1900 through confrontation assays on synthetic media agar plates (Szabó *et al.*, 2012). *Trichoderma* spp. are also found to manage insects and other pathogens. Shakeri & Foster (2007) found that *T. harzianum* acts as an insecticide against mealworms (*Tenebrio molitor*) when ingested or applied to the cuticle surface. Another example is where a *T. harzianum* spore suspension was able to reduce a cockroach population applied either as a spray or as food additive (Abdul-Wahid & Elbanna, 2012).

A great deal of research has been done regarding the biological control of plant-parasitic nematodes utilizing different fungal strains on numerous grain, leguminous, vegetable and fruit crops. Some examples of these crops are soybean (Stirling, 2014), sunflower (Dawar *et al.*, 2008; Mohammed *et al.*, 2014), carrot (*Daucus carota* subsp. *sativus* (Hoffm.) Schübl. & G. Martens) (Prasad *et al.*, 2014), tomato (Chinheya, 2015), cucumber (Zhang *et al.*, 2015), bell pepper (*Capsicum annuum*, L.) (Meyer *et al.*, 2001), okra (Dawar *et al.*, 2008), eggplant (*Solanum melongena*, L.) (Osman *et al.*, 2018) and guava (*Psidium guajava*, L.) (Jindapunnapat *et al.*, 2013). Some of the various plant-parasitic nematode genera that fungal biocontrol research has been done on include: cyst nematodes, *Globodera* (Contina *et al.*, 2017) and *Heterodera* (Zhang *et al.*, 2014); root-knot nematodes *Meloidogyne* (El-Nagdi *et al.*, 2019); false root-knot nematodes *Nacobbus* Thorne, 1944 (Sosa *et al.*, 2018); lesion nematodes *Pratylenchus* (De Oliveira *et al.*, 2021) and the reniform nematode *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Bokhari, 2009). Of these genera, root-knot nematodes are generally considered to be the most threatening to soybean crops in most soybean production areas worldwide (Perry & Moens, 2011; Fourie *et al.*, 2015). However, the cyst nematode *H. glycines* is another highly destructive nematode pest in soybean fields in certain parts of the world, e.g Brazil (Sikora *et al.*, 2018), China (Cheng *et al.*, 2012; Shi & Zeng, 2013; Sikora *et al.*, 2018) and USA (Sikora *et al.*, 2018), but luckily has not yet been reported in SA (Fourie *et al.*, 2015; Fourie *et al.*, 2017).

Information on the type of fungi that should be considered for use as a fungal biological control agent is listed in Table 2-2.

Table 2-2: Desirable characteristics for the classification of proper fungal biocontrol agents used against *Meloidogyne* spp.

Three types of fungi used as biocontrol	Desirable characteristics	Examples of fungi effective against <i>Meloidogyne</i> spp.
Endoparasitic fungi	Obligate parasite with limited saprophytic phase completing life cycle within host (Siddiqui & Mahmood, 1996)	<i>Hirsutella rhossiliensis</i> (nematophagous fungi) with ability to reduce nematode densities (Viaene & Abawi, 2000; Siddiqui & Mahmood, 1996)
Predacious fungi	Attack, capture and kill nematodes by forming traps (adhesive traps/knobs and non-constricting/constricting rings). Also feed on other fungi in soil or organic matter (Siddiqui & Mahmood, 1996; Rosenheim <i>et al.</i> , 1995)	<i>Arthrobotrys dactyloides</i> (Mankau, 1980)
Opportunistic fungi	Attack formed egg masses and parasitized developing eggs (Siddiqui & Mahmood, 1996). Reduce penetration and development of root-knot nematodes (Ashraf & Khan, 2005)	<i>Paecilomyces lilacinus</i> (Ashraf & Khan, 2005)

Additionally, a good fungal biological control agent should be (i) highly parasitic to nematodes, (ii) primarily focused on attacking nematodes without it being pathogenic to plants and animals, (iii) growing at suitable pH and temperature ranges, (iv) reared on artificial media, (v) having a known mode of action, and (vi) formulated in a usable form by producers (Kim & Riggs, 1992; Siddiqui & Mahmood, 1996; Druzhinina *et al.*, 2011). For example, it has been found that *Paecilomyces lilacinus* grows well between 15 °C and 30 °C (with 25-30 °C resulting in optimal growth) and is adaptable to a wide range of soil pH resulting in a competitive organism in agricultural soils (Siddiqui & Mahmood, 1996). For a biocontrol agent to be successful, it must also have the ability to compete and persist in the specific environment placed in and provide continued benefits to the crops it is applied to (Harman, 2000; Sariah *et al.*, 2005; Al-Hazmi & TariqJaveed, 2016).

This study focused on the use of different *Trichoderma* strains as well as *Clonostachys rosea* endemic to South Africa. Ample research is available on the biological control of the root-knot nematode, *M. incognita* utilizing species of these two fungal genera (Cristóbal-Alejo *et al.*, 2021; Mahapatra *et al.*, 2021). The genus *Trichoderma* often comes from diverse habitats and can be found in the same locality in the rhizosphere, as most plant-parasitic nematodes (Verma *et al.*,

2007; Vinale *et al.*, 2008; Chinheya, 2015). *Trichoderma* is known to colonize rhizospheres of roots rapidly, produce a great number of spores and release numerous enzymes which makes them an effective nematode antagonist (Verma *et al.*, 2007; Vinale *et al.*, 2008). Since the various strains of *Trichoderma* differ in ability to colonize plant roots, the most effective strains colonize roots and bring about benefits for at least the duration that annual crops are grown (Harman, 2000).

Except for *Trichoderma* spp. being used as fungal biological control to combat root-knot nematodes (Mahaptra *et al.*, 2021), *Clonostachys* is also to a lesser degree known to act as a biological control agent for numerous plant pathogens including fungi, nematodes and insects (Sun *et al.*, 2020). The most notorious strain of the *Clonostachys* genus is *C. rosea* (Ten Hoopen *et al.*, 2010). This fungal strain was originally known as *Gliocladium roseum* and was later renamed *Clonostachys rosea* mainly because of differences in morphology, ecology, teleomorph/sexual reproduction stage and DNA sequence data between these two genera (Schroers *et al.*, 1999; Toledo *et al.*, 2006). Like the *Trichoderma* genus, *Clonostachys* is distributed in various habitats globally, but has the highest occurrence in soil (Sun *et al.*, 2020). *Clonostachys rosea* is, however, not considered as a biocontrol agent exclusively, but also aids in the biodegradation of plastic waste, biotransformation of bioactive compounds, fermentation, and acts as a bioenergy source (Sun *et al.*, 2020).

Except for limited research on the biocontrol efficacy of *C. rosea* on nematodes, little knowledge about its role as an entomopathogenic fungus for insect control exists. However, Ten Hoopen *et al.* (2010) found that *C. rosea* was able to provide control of leafhoppers (*Eurymeloides bicincta*) in Argentina.

2.5 *Trichoderma* as biological control agent of *Meloidogyne*

The genus *Trichoderma* is considered economically important since it acts as a source of enzymes, antibiotics, plant growth promoters (PGP), decomposers of xenobiotics and as commercial biofungicides (Daniel & Filho, 2007). *Trichoderma* is important worldwide in industries and medicine, and it can also cause diseases in particularly immuno-compromised humans (Daniel & Filho, 2007; Druzhinina *et al.*, 2008). Research done by Druzhinina *et al.* (2008) proved that *Trichoderma longibrachiatum* is, for example, capable of causing invasive human mycoses.

An important discovery in terms of agriculture, however, is that *Trichoderma* has been found to release siderophores that promotes plant growth and inhibits iron uptake to other microorganisms to control plant pathogens (Contreras-Cornejo *et al.*, 2020). Therefore, *Trichoderma* is of great

economic importance (Monte & Llobell, 2003; Daniel & Filho, 2007) to producers and agricultural industries, especially in recent times with the search for more environmentally friendly management tactics.

To promote plant growth, *Trichoderma* strains produce plant-growth-promoting compounds (Contreras-Cornejo *et al.*, 2009; Studholme *et al.*, 2013) including volatile metabolites, secretion of hydrolytic enzymes, production of siderophores and solubilization of phosphates (López *et al.*, 2019). Research testing the PGP compound 6-pentyl- α -pyrone (produced by *Trichoderma* spp.) on soybean crops found that this metabolite was able to successfully enhance stem length, number of pods, plant weight and some yield parameters of plants (Marra *et al.*, 2019).

In general, *Trichoderma* spp. as a biological control agent of root-knot nematodes is considered effective. This is especially the case for *T. harzianum* (Bokhari, 2009; Al-Hazmi & TariqJaveed, 2016). It has been shown that testing of *Trichoderma* spp. against *Meloidogyne* spp. was promising in a variety of locations worldwide under varying environmental conditions (Table 2-3) (Sahebani & Hadavi, 2008; Affokpon *et al.*, 2011; Mascarin *et al.*, 2012; Jindapunnapat *et al.*, 2013; Al-Hazmi & TariqJaveed, 2016). However, according to Al-Hazmi & TariqJaveed (2016), during the research conducted for *Trichoderma* spp. and its effect on root-knot nematodes, certain aspects were neglected such as the parasitic potential of the fungus in correlation with its inoculum densities to reduce the *Meloidogyne* spp. population densities.

Table 2-3: Studies showing different *Trichoderma* spp. that were used as biocontrol agents of *Meloidogyne* spp. for a range of crops and across different countries.

<i>Trichoderma</i> spp.	Target <i>Meloidogyne</i> spp.	Crop	Country	Effect on nematode life cycle	Reference
<i>Trichoderma harzianum</i>	<i>Meloidogyne</i> spp.	<i>In vitro</i>	Iraq	High second-stage juvenile (J2) mortality percentage and low J2 hatching	Lafta & Kasim (2019)
<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Eggplant	Egypt	Decrease in population density as well as number of root galls and egg masses	Osman <i>et al.</i> (2018)
<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Tomato	Italy	Salicylic acid-signalling pathway and ethylene production; restricting nematode reproduction and development	Leonetti <i>et al.</i> (2017)
<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Chickpea	India	Improved nutrient uptake by host plant; decreased root-knot gall formation and production of eggs	Rizvi <i>et al.</i> (2018)

Table 2-3 cont: Studies showing different *Trichoderma* spp. that were used as biocontrol agents of *Meloidogyne* spp. for a range of crops and across different countries.

<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Soybean	Nigeria	Neutralization of organic compounds in soil – higher nutrient uptake and ability of plants to resist nematodes	Izuogo & Abiri (2015)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Eggplant	Pakistan	Egg parasitism	Murslain <i>et al.</i> (2014)
<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Carrot	India	Production of chitinase enzyme causing premature hatching of J2	Prasad <i>et al.</i> (2014)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Tomato	Iran	Decrease in number of galls, egg masses and eggs per egg mass. J2 hatching inhibition and mortality. Proteolytic activity	Jamshidnejad <i>et al.</i> (2013)
<i>Trichoderma harzianum</i>	<i>M. enterolobii</i>	Guava	Thailand	Inhibit development of J2 densities	Jindapunnapat <i>et al.</i> (2013)
<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Cucumber	Brazil	Contaminate eggs and immotilize J2	Mascarin <i>et al.</i> (2012)
<i>Trichoderma harzianum</i>	<i>M. incognita</i>	Sunflower	Egypt	Endochitinase production which adversely affects the eggshell of nematode	Mohamed <i>et al.</i> (2011)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Tomato	Iran	Reduction in number of galls, eggs and egg masses	Naserinasab <i>et al.</i> (2011)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Sunflower & okra	Pakistan	Parasitism of nematode eggs and nematodes, as well as production of nematocidal compounds	Dawar <i>et al.</i> (2008)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Tomato	Iran	Penetrate nematode egg mass matrix and decreased J2 hatching. Increase in extracellular chitinase activity and inducing plant defence mechanisms leading to systemic host plant resistance	Sahebani & Hadavi (2008)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Tomato	Pakistan	Antibiotic production	Siddiqui & Shaukat (2004)
<i>Trichoderma harzianum</i>	<i>M. javanica</i>	Tomato	Israel	J2-immotilization and colonize eggs and egg masses	Sharon <i>et al.</i> (2001)
<i>Trichoderma longibrachiatum</i>	<i>M. incognita</i>	Cucumber	China	Reduction of J2s and production of protease enzyme	Zhang <i>et al.</i> (2015)
<i>Trichoderma virens</i>	<i>M. incognita</i>	Bell pepper	USA	Reduction in egg numbers and J2 numbers per root	Meyer <i>et al.</i> (2001)
<i>Trichoderma viride</i>	<i>M. incognita</i>	Okra, eggplant & tomato	Egypt	Parasitize nematode eggs; cease egg embryogenesis/ deplete egg content & sporulate nematode juveniles	Ashoub <i>et al.</i> (2009)
<i>Trichoderma viride</i>	<i>M. incognita</i>	Okra	Not indicated	Reduce J2 infection in roots of host plant	Baheti (2005)
<i>Trichoderma viride</i>	<i>M. incognita</i>	Tomato	New Delhi	Toxicity and inhibition of J2 hatching	Goswami & Mittal (2004)
<i>Trichoderma asperellum & Trichoderma atroviride</i>	<i>M. javanica</i>	<i>In vitro</i>	Israel	Binding of antibodies to bodies of J2, which causes conidial attachment to nematodes	Sharon <i>et al.</i> (2009)

Table 2-3 cont: Studies showing different *Trichoderma* spp. that were used as biocontrol agents of *Meloidogyne* spp. for a range of crops and across different countries.

<i>Trichoderma asperellum</i> & <i>Trichoderma viride</i>	<i>M. incognita</i>	Tomato	India	Reduction in number of galls per plant, umber of eggs and larvae/egg mass and final nematode population	Kumari <i>et al.</i> (2020)
<i>Trichoderma album</i> & <i>Trichoderma harzianum</i>	<i>M. incognita</i>	Tomato	Egypt	Production of secondary metabolites and direct parasitism of eggs	Radwan <i>et al.</i> (2012)
<i>Trichoderma harzianum</i> & <i>Trichoderma asperellum</i>	<i>M. javanica</i>	Banana (<i>Musa</i> L.) cv. Grande Naine	Brazil	<i>In vitro</i> J2 mortality test. Reduction in reproduction factor and population density	Almeida <i>et al.</i> (2022)
<i>Trichoderma harzianum</i> & <i>Trichoderma asperellum</i>	<i>M. incognita</i>	Tomato	Spain	Reduction in number of eggs/egg masses, nematode infectivity and reproduction	Pocurull <i>et al.</i> (2020)
<i>Trichoderma harzianum</i> & <i>Trichoderma viride</i>	<i>M. javanica</i>	Tomato	Saudi Arabia	Direct and indirect effects on reproduction and host response	Al-Hazmi & TariqJaveed (2016)
<i>Trichoderma harzianum</i> & <i>Trichoderma viride</i>	<i>M. incognita</i>	Tomato	Germany	Reduction in number of galling, egg masses and immotilize J2	Dababat & Sikora (2007)
<i>Trichoderma harzianum</i>, <i>Trichoderma viride</i>, <i>Trichoderma koningii</i> & <i>Trichoderma asperellum</i>	<i>M. javanica</i>	<i>In vitro</i>	Egypt	Egg-hatching, egg parasitism, J2 immobilisation and production of secondary metabolites	Hemeda & El Deeb (2019)
<i>Trichoderma viride</i>, <i>Trichoderma harzianum</i>, <i>Trichoderma hamatum</i>, <i>Trichoderma koningii</i> & <i>Trichoderma pseudokoningii</i>	<i>M. javanica</i>	Okra & mungbean (<i>Vigna radiata</i> (L.) R. Wilczek)	Pakistan	Inhibition of J2 hatching and larval death. Production of nematicidal compounds	Siddiqui <i>et al.</i> (2001)
<i>Trichoderma</i> spp.	<i>M. incognita</i>	Pea (<i>Pisum sativum</i> , L.)	Egypt	Reduction in number of galls, egg masses and J2 per root system	El-Nagdi <i>et al.</i> (2019)
<i>Trichoderma</i> spp.	<i>M. incognita</i>	Tomato	Egypt	Decrease root galling, egg masses, number of females and J2 while improving host growth parameters	Abdel-lateif & Bakr (2018)
<i>Trichoderma</i> spp.	<i>M. incognita</i>	Tomato	Leuven, Belgium	Lower J2-densities, egg production and galling damage	Affokpon <i>et al.</i> (2011)
<i>Trichoderma</i> spp.	<i>M. incognita</i>	Tomato	India	Reduced galling, egg mass production, fecundity and population density	Khan <i>et al.</i> (2018)
<i>Trichoderma</i> spp.	<i>M. incognita</i> & <i>M. javanica</i>	<i>In vitro</i>	Russia	Metabolite excretion to paralyze/kill J2	Migunova <i>et al.</i> (2018)
<i>Trichoderma</i> spp.	<i>M. javanica</i>	Soybean & tomato	South Africa	J2 mortality; reduced number of galls and egg masses	Chinheya (2015)

Table 2-3 cont: Studies showing different *Trichoderma* spp. that were used as biocontrol agents of *Meloidogyne* spp. for a range of crops and across different countries.

<i>Trichoderma</i> spp.	<i>M. incognita</i>	Tomato	Italy	Nemato-toxic action	Leonetti <i>et al.</i> (2014)
<i>Trichoderma</i> spp.	<i>M. incognita</i>	Sunflower	Egypt	Production of metabolites	Mohammed <i>et al.</i> (2014)
<i>Trichoderma</i> spp.	<i>M. javanica</i>	Eggplant	Saudi Arabia	Toxic metabolites and inhibit nematode penetration and development	Bokhari (2009)

2.6 *Clonostachys rosea* as a biological control agent of *Meloidogyne*

According to De Andrade Carvalho *et al.* (2018), *Clonostachys* serves as a biological control agent by acting as a hyperparasite, competing for resources against plant pathogens, and inducing host plant resistance. The genus *Clonostachys* has shown to be harmless to humans and is at most pathogenic to plants (Sutton *et al.*, 1997; Pinotti & Santos, 2013). *Clonostachys rosea* also acts as an entomopathogenic fungus on *Bemisia tabaci* (whitefly) (Anwar *et al.*, 2018) and interacts with other microorganisms by producing a variety of metabolites that are toxic to fungi, bacteria, and insects; thus, being useful as a biocontrol agent of various pests and pathogens (Yu & Sutton, 1997; Zhang *et al.*, 2008; Anwar *et al.*, 2018).

Just like *Trichoderma*, *C. rosea* possess PGP traits that for example improved leaf area, bole-girth diameter, and root dry weight in palm oil (*Elaeis guineensis*) (Goh *et al.*, 2020). A PGP metabolite IK726 is produced by *C. rosea* and associated with increasing plant growth and altering shoot nutrient content (Ravnskov *et al.*, 2006; Bennett & Whipps, 2008).

In terms of its nematicidal effect, Migunova *et al.* (2018) found that *C. rosea* KAV1 & *C. rosea* KAV2 was effective in causing 100% mortality of *M. incognita* J2 during *in vitro* tests. Another study conducted in Southern Iraq found a maximum reduction of 59.73% in egg hatching of *Meloidogyne* spp. while using *C. rosea* culture filtrates in petri dishes (Lafta & Kasim, 2019). It is therefore reasonable to derive from these studies that *C. rosea* can be considered as an efficient biological control agent (Ravnskov *et al.*, 2006; Bennett & Whipps, 2008; Hussain *et al.*, 2017). Table 2-4 contributes to more evidence of the successful biocontrol of plant-parasitic nematodes utilizing *C. rosea*.

Table 2-4: Studies in which *Clonostachys rosea* has been used as a biocontrol agent of *Meloidogyne* spp. with information given on the target nematode species, host crop, the area where the research was done and the mode of action of the fungus.

<i>Clonostachys</i> spp.	Target plant-parasitic nematode species	Crop	Country	Effect on nematode life stage	Reference
<i>C. rosea</i>	Plant-parasitic nematodes	Variety of crops	Sweden	Toxicity and immobilization towards motile nematode life stages	Iqbal (2019)
<i>C. rosea</i>	<i>Meloidogyne</i> spp.	<i>In vitro</i>	Iraq	Reduction of second-stage juvenile (J2) hatching	Lafta & Kasim (2019)
<i>C. rosea</i>	<i>Meloidogyne</i> spp.	Soybean	Japan	Penetrates nematode cuticle and cause destruction of body content	Toju & Tanaka (2019)
<i>C. rosea</i>	<i>M. incognita</i>	<i>In vitro</i>	Czech Republic	Attraction or repulsion of RKN's to nematophagous fungi irrespective of trap formation	Hussain <i>et al.</i> (2018)
<i>C. rosea</i>	<i>M. incognita</i> & <i>M. javanica</i>	<i>In vitro</i>	Russia	Toxic metabolite production against nematodes	Migunova <i>et al.</i> (2018)
<i>C. rosea</i>	<i>M. hapla</i>	Carrot	Czech Republic	Parasitism of eggs and J2	Hussain <i>et al.</i> (2017)

2.7 Commercial products, containing only fungal or fungal and bacterial strains, available for use against plant-parasitic nematodes

A limited number of products containing fungi and bacteria as the active ingredients, for the biological control of nematodes, have been registered in SA (Van Biljon, 2017; Table 2-5). A range of commercial products have, however, been released worldwide as described in Table 2-5 (Tranier *et al.*, 2014). The commercial availability of these products is proof that fungal agents are a valuable management strategy to reduce densities of plant-parasitic nematodes and mitigate the damage they cause.

Table 2-5: Commercial fungal products released worldwide for the use against plant-parasitic and specifically root-knot nematodes (Tranier *et al.*, 2014).

Name	Organism	Target nematode genus and/or species	Company
BIO-ACT®/MELOCON WG	<i>Paecilomyces lilacinus</i>	<i>Globodera</i> spp., <i>Heterodera avenae</i> (Khan <i>et al.</i> , 2006), <i>Meloidogyne</i> spp. (Brand <i>et al.</i> , 2010) <i>M. incognita</i> & <i>M. hapla</i> (Kiewnick & Sikora, 2004) <i>M. javanica</i> (Khan <i>et al.</i> 2006; Holland <i>et al.</i> , 1999) <i>M. incognita</i> (Mukhtar <i>et al.</i> , 2013; Kiewnick & Sikora, 2006), <i>Pratylenchus</i> spp. & <i>Radopholus similis</i> (Mendoza <i>et al.</i> , 2007; Khan <i>et al.</i> , 2006)	Bayer CropScience Biologics GmbH, Germany
BOTANIGARD®/MYCONTROL®	<i>Beauveria bassiana</i>	<i>Meloidogyne incognita</i> (Ekanayake & Jayasundara, 1994), <i>M. hapla</i> (Liu <i>et al.</i> , 2008)	ARLA (The Pest Management Regulatory Agency), Canada
BIOSTAT®	<i>Purpureocillium lilacinum</i>	<i>Ditylenchus</i> spp., <i>Helicotylenchus</i> spp., <i>Meloidogyne</i> spp., <i>Pratylenchus</i> spp., <i>Radopholus</i> spp., <i>Rotylenchulus</i> spp. & <i>Tylenchus</i> spp. (Laverlam, 2023)	EPA (Environmental Protection Agency), LAM International, Montana, USA
MYCOTAL®	<i>Verticillium lecanii</i>	<i>Meloidogyne incognita</i> (Meyer 1999)	KOPPERT, Netherlands
TRIANUM®	<i>Trichoderma harzianum</i> , <i>T. virens</i> & <i>T. viride</i>	<i>Meloidogyne hapla</i> (Masadeh <i>et al.</i> , 2004), <i>Meloidogyne</i> spp. (González <i>et al.</i> , 2012)	KOPPERT, Netherlands
NEMAXXION BIOL®	<i>Bacillus subtilis</i> , <i>Paecilomyces</i> spp. & <i>Trichoderma</i> spp.	<i>Meloidogyne</i> spp.	GreenCorp, Mexico
REM G®	<i>Arthrobotrys</i> spp., <i>Dactyllela</i> spp., <i>Glomus</i> spp. & <i>Paecilomyces</i> spp.	Plant-parasitic nematodes	Green Solutions, Italy

2.8 Conclusion

Since literature demonstrates the use of fungal biological control agents as having the potential to manage root-knot nematodes, and other plant-parasitic genera, in grain and leguminous crops (Ashoub, *et al.*, 2009; Izuogo & Abiri, 2015), further research should be conducted to identify superior strains that can be used. Whether these biocontrol agents are used alone or in conjunction, they generally have a positive effect in controlling plant-parasitic nematodes, especially root-knot nematodes, although such an effect can be variable ranging from low to high efficacy (Tables 2-3, 2-4 & 2-5). The control of different *Meloidogyne* spp. is also effective on a

variety of crops other than grains and legumes (Jones *et al.*, 2013; Tables 2-3 & 2-4) demonstrating the value of fungal biocontrol in crop rotation systems. It is important to consider IPM when using fungal biological control agents, since the effect of these agents can often not replace the effect that chemical nematicides has (Kerry, 1997; Chintehya, 2015), but they represent a valuable contribution towards sustainable crop production and preservation of our most valuable and irreplaceable natural resources namely soil, water, and the atmosphere.

CHAPTER 3 MATERIALS AND METHODS

In this chapter the *in vitro* methodology is explained that was used to evaluate the effect of i) viable spores of five fungal isolates (*Clonostachys rosea*, *Trichoderma asperellum*, *Trichoderma asperellum* kd, *Trichoderma harzianum*, and *Trichoderma virens*) that were reared on nutrient substrates and ii) spores of two isolates formulated as products (Eco-T® and CR; Andermatt-PHP Pty Ltd) on the motility of second-stage juveniles (J2) of the root-knot nematode species *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949.

In addition, the methodology (of the *in vivo* experiments) used to evaluate the effect of the fungal isolates' spores on the reproduction of *M. incognita* females in roots of soybean (*Glycine max* L. Merr.) are provided. Finally, information on the techniques applied to verify the identity of the *M. incognita* population and the five fungal isolates used are also given.

3.1 *Meloidogyne* nematode population

3.1.1 Origin, identification, and *in vivo* rearing

Eggs and second-stage juveniles (J2) of a *Meloidogyne* sp. population were obtained from roots and tubers of potato plants (*Solanum tuberosum*, L.) grown in the Sandveld (Western Cape Province, South Africa). The species was identified as *M. incognita* using perineal-pattern morphology of mature females (Marais *et al.*, 2017; Kleynhans, 1991) as well as the molecular sequence characterised amplified region – polymerase chain reaction (SCAR-PCR) (Rashidifard *et al.*, 2019) approach. This species was used for a life-stage and life-cycle study of MSc student Raymond Collett, with a detailed description given of the techniques used to identify it (Collett, 2020).

The *M. incognita* population used for this study was reared from single-egg masses *in vivo* in roots of tomato (*Solanum lycopersicum*, L.) seedlings (cv. Moneymaker) in a glasshouse of the Nematology Unit (Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa) at a mean temperature range of ± 25 °C as described by Collett (2020). When the population was 30 – 40 days old, the roots were harvested to obtain eggs and J2 for inoculation purposes. The 20-ℓ plastic pots, in which tomato seedlings were grown, were filled with the same volume of a sandy soil, purchased from Witpoort Sand & Stone (Ventersdorp, North-West Province, South Africa). The soil contained 97,6% sand, 1,2% silt, 1,3% clay, and 0,06% organic matter (Collet, 2020). The pH of the soil was 5.73 (H₂O). To ensure all soil-borne

pathogens were eliminated, the soil was fumigated with Telone® II (97,5% active ingredient/a.i.) 1,3-dichloropropene) at a dosage rate of 150 l/ha four weeks before the onset of the *M. incognita* rearing process.

3.1.2 Preparation of *Meloidogyne* sp. nematode inoculum for experimental purposes

Thirty to forty days after *in vivo* rearing of the *M. incognita* population commenced, the roots of infected tomato plants were uprooted, washed with tap/borehole water [pH (H₂O) of 7.3] to remove excess soil, and cut into 2-5 mm pieces. An adapted NaOCl method (Riekert, 1995) was used that consisted of a 1% sodium hypochlorite (NaOCl) solution used to extract *M. incognita* nematode eggs from roots. The solution was shaken for 4 min on an orbital shaker to aid the release and suspension of the eggs from the gelatinous matrix surrounding it. Then, the solution with the eggs were then decanted onto a series of sieves, that was adapted from the methods of Riekert (1995) and Marais *et al.*, 2017), stacked from top to bottom: 250-µm, 75-µm, 63-µm, and 20-µm apertures. During the sieving process the roots on the top sieve were gently rinsed with tap water to allow the eggs to settle on the mesh of the bottom 20-µm aperture sieve. The eggs on the 20-µm aperture sieve were rinsed to remove any remaining NaOCl and then poured onto a 25-µm aperture sieve that was submerged in a 2-l capacity plastic container filled with approximately 750 ml of tap water.

The container with the sieve (containing the *M. incognita* nematode eggs) were incubated in a temperature-regulated growth chamber at 28 °C allowing the first-stage juveniles (J1) to develop and hatch into J2 that migrated through the mesh of the sieve into the surrounding water. At 24 hr intervals, the hatched J2 were isolated by decanting them onto a 20-µm mesh sieve and washing them into a 100-ml capacity glass beaker. The J2 obtained were counted using a De Grisse dish (De Grisse, 1963) and a stereomicroscope (60× magnification) according to the following process: 5 droplets of 10 µl of the J2 suspension that was continuously stirred (using a magnetic stirrer at a low-speed setting) were pipetted into a de Grisse dish and counted. The process was repeated six times, representing six replicates, totalling 30 droplets in which the J2 were counted to estimate their density. Finally, the total number of hatched J2 obtained were calculated to allow further experimentation.

3.2 Fungal isolates

3.2.1 Identification and cultivation of fungal isolates

Viable spores of five pure culture and potential fungal isolates, viz. *C. rosea*, *T. asperellum*, *T. asperellum* kd, *T. harzianum*, and *T. virens* were obtained from Andermatt Plant Health Products (Pty) Ltd. for experimental purposes. The fungal isolates were identified to species level by

Inqaba Biotechnical Industries (Pty) Ltd, trading as 'inqaba biotec' in sub-Saharan Africa (<https://inqababiotec.co.za>), with the use of a Sanger sequencing [ABI 3500xl Genetic Analyzer]. The Internal Transcribed Spacer (ITS) region and the ITS 1 and 4 primer sets (forward and reverse, respectively) were used for species identification followed by BLAST analysis of deposited sequences of the isolates that are available on the NCBI database (<https://www.ncbi.nlm.nih.gov>). This general information was supplied by Andermatt-PHP Ltd Pty since their personnel did the analysis.

For initial *in vitro* experiments, potato-dextrose-agar (PDA) (Merck®) were plated in 90-cm diameter Petri dishes on which the five fungal isolates were reared individually at 25 °C in a temperature-regulated growth cabinet. The plates for each isolate were sub-cultured and the spores extracted 7-14 days after the onset of incubation (depending on the sporulation of each strain). Spores for each subculture and strain were obtained by flooding the PDA layer with 10 ml of a 0.25% Ringer's solution after which the mycelia/spores were scraped of the agar using a sterilized scalpel (Figure 3-1). The resulting suspension for each isolate was then filtered through a cheesecloth into sterile McCartney bottles using a pipette (Figure 3-1). Serial dilutions of 10^{-1} and 10^{-2} were made from the original concentration of each strain and spores were counted using a haemocytometer to determine the spore density for each isolate (Avin, 2019).



Figure 3-1: Extraction and filtration of fungal mycelia from a potato-dextrose-agar plate using a sterilized scalpel to obtain fungal spores for experimental purpose (Marné Pretorius, NWU).

However, the fungal growth on PDA was not as effective in terms of obtaining sufficient numbers of spores as was needed for experimental purposes and therefore an alternative method of cultivation was required to enable that enough spores could be obtained. The first *in vitro* experiments (initial and repeat) were, therefore, performed using only 10^8 spores/ml of each fungal isolate as the highest concentration.

To enhance the growth of fungal isolates, a bran-based substrate as provided by Andermatt-PHP (Pty) Ltd. was used as food source to rear the fungal strains. The method used to prepare this substrate is intellectual property (IP) of Andermatt-PHP (Pty) Ltd. and is therefore not described. The bran substrate was transferred to 250 ml capacity glass jars that were autoclaved for 20 min at 121 °C. A few small blocks of PDA containing fungal isolate of each strain was transferred, from the Petri dishes in which they were reared, to the sterile jars and incubated at 25 °C for one to two weeks depending on the fungal growth (Figure 3-2).



Figure 3-2: Constructed jam jars containing growth medium and fungal isolates, incubated at $\pm 25^{\circ}\text{C}$ before extraction of fungal spores for experimental purposes (Marné Pretorius, NWU).

After incubation, the content was suspended with distilled water and filtered through a cheesecloth to collect fungal spores (Figure 3-3). The solution was then diluted and counted using a haemocytometer (Neubauer, Merck S.A.) (Aberkane *et al.*, 2002; <https://agristudentbikash.wordpress.com/2015/06/18/counting-fungal-spores-using-a-haemocytometer>), and suspension concentrations of 10^9 spores/ml as the highest were obtained. The original solutions were used for both *in vitro* and *in vivo* analyses, with the following concentrations being prepared/diluted: 10^7 , 10^8 , and 10^9 spores/ml.



Figure 3-3: A visual representation of the extraction of *Clonostachys rosea* from bran-soy flour using distilled water (Marné Pretorius, NWU).

A germination test was also performed on all of these fungal isolates as well as the two formulated products to determine the viability of the fungal spores showed in Figure 3-4 a & b (Paul *et al.*, 1993).

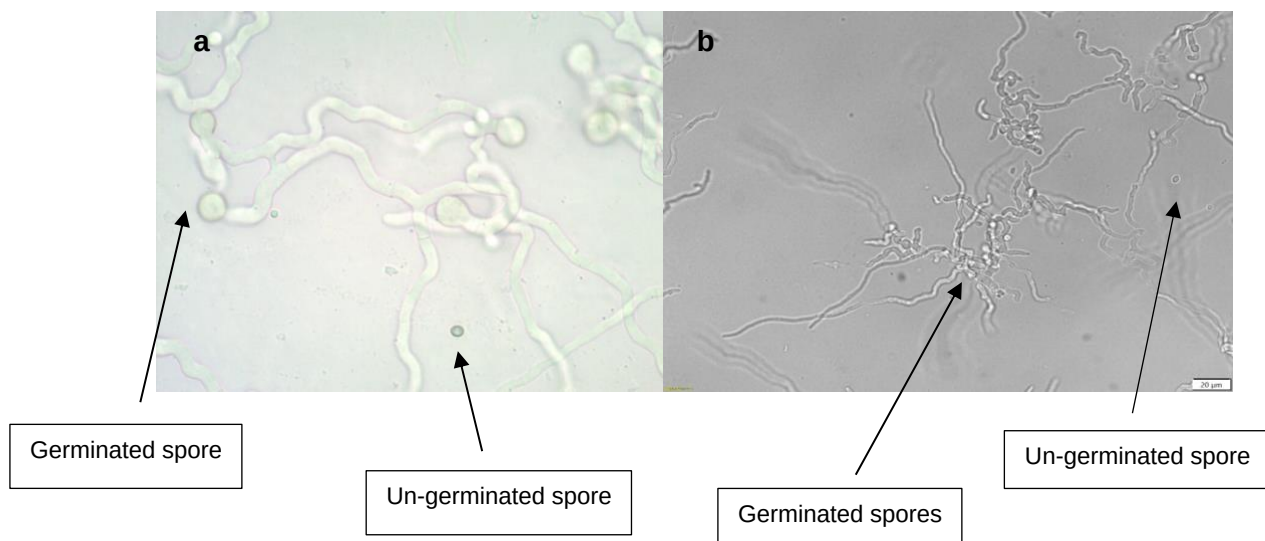


Figure 3-4: A visual representation of the germination of a) *Trichoderma asperellum* strain kd (Eco-T®) and b) *Clonostachys rosea* strain PHP 1701 (CR) when extracted from bran-substrate (Gilmore Pambuka, Andermatt Plant Health Products (Pty) Ltd.).

3.3. Screening of fungal isolates for their effect on motility of *M. incognita* second-stage juveniles (*in vitro* analysis)

3.3.1. *In vitro* screening utilising viable fungal spores from reared colonies

In vitro screening experiments were conducted individually for the five fungal isolates. Sterilised 24-well, flat bottom, clear polystyrene plates with lids (Greiner Bio-one) were used (Engelbrecht, 2018) for the *in vitro* experiments with one well plate being used for each fungal isolate. For each fungal isolate the following concentrations were used: 10^7 , 10^8 , and 10^9 spores/ml, while an ethanol (96%) upper control, and a water control (dH_2O) were also included as treatments (Figure 3-6) to test against J2 motility. One millilitre of spores of each of the fungal isolates and for each concentration were added to each well of the plate. Thereafter, 100 μl of nematode J2 inoculum (containing 100 J2) were added to each well, after removing excess water from the J2 suspension to apply the correct concentration. These well plates containing the J2 suspended in the fungal spores of each isolate were incubated in a growth chamber at 25 °C. The number of immotile J2 was counted at 24, 48, and 72 hrs after incubation using an inverted microscope (Nikon Eclipse TS100-F; 10x magnification) as illustrated in Figure 3-5. With each count, photos were taken of

the motile and immotile/paralysed J2. The experiment was repeated for each fungal isolate and data were statistically analysed.



Figure 3-5: Method of counting immotile *Meloidogyne incognita* juveniles using an inverted microscope (Marné Pretorius, NWU).

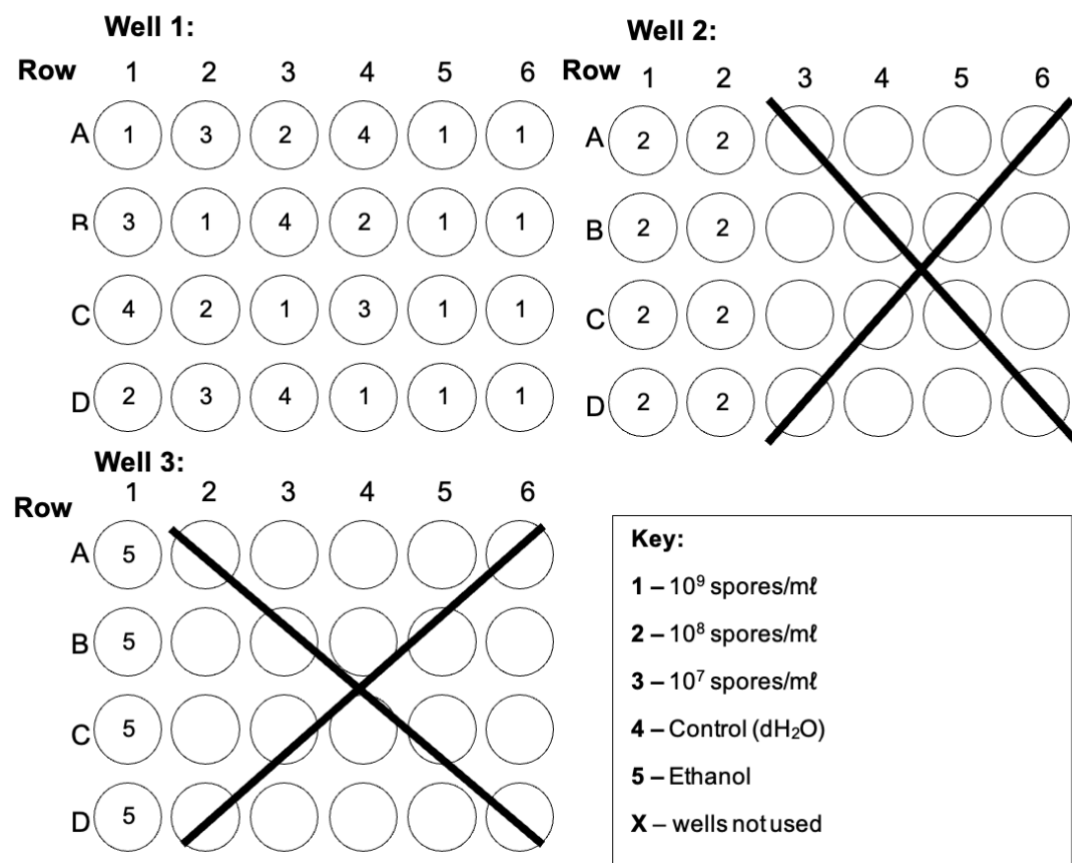


Figure 3-6: *In vitro* well-plate experimental layout (randomized complete block design) used for screening each of the fungal isolates' living spores against *Meloidogyne incognita* to determine its effect on second-stage juvenile motility (Marné Pretorius, NWU).

The incubation of ethanol was done in a separate plate to prevent the fumes from spreading and confounding results obtained for the fungal isolates. The highest concentrations of fungal isolates were incubated in repetition for counting purposes because the fungal isolates were very dense at 10^8 and 10^9 spores/ml, and J2 were not visible through the spore suspension. Therefore, using a 1 ml pipette, the solutions were transferred to a Peters counting slide (Marais *et al.*, 2017) in 200 μ l drops after which 1 ml of distilled water was added to each drop to dilute the solution and make *M. incognita* J2 visible for counting. After addition of the water was performed, J2 were quickly counted and immotility determined. Each replicate, for each time interval, was discarded after counting and recording the motility of the J2.

Apart from the experiments performed with the fungal isolates, a treatment containing bran extract (extract from the bran-based substrate only) was tested against the J2 under the same experimental conditions to determine if the substrate itself had any effect on the motility of the J2.

3.3.2. *In vitro* screening using formulated fungal products:

Similarly, as was done for the viable fungal spores of the five isolates tested (see paragraph 3.3.1) for its effects on *M. incognita* J2 motility, *in vitro* screening was conducted for the two formulated products, Eco-T® (a.i. *T. asperellum* strain kd) and CR (a.i. *C. rosea* strain PHP 1701). These products are owned and manufactured by Andermatt Plant Health Products (Pty) Ltd. Eco-T® is commercially registered in South Africa on a variety of vegetables, crops, ornamentals, and *Eucalyptus* species as bio-fungicide to suppress root diseases and enhance plant growth (Andermatt-PHP, 2021), while CR is not commercially available at present. The following concentrations of each of the two products were used as treatments: 0,4 g/l dH₂O; 0,6 g/l dH₂O; 0,8 g/l dH₂O; 1 g/l dH₂O; ethanol (96%) as the upper, and dH₂O as the untreated control (Figure 3-7).

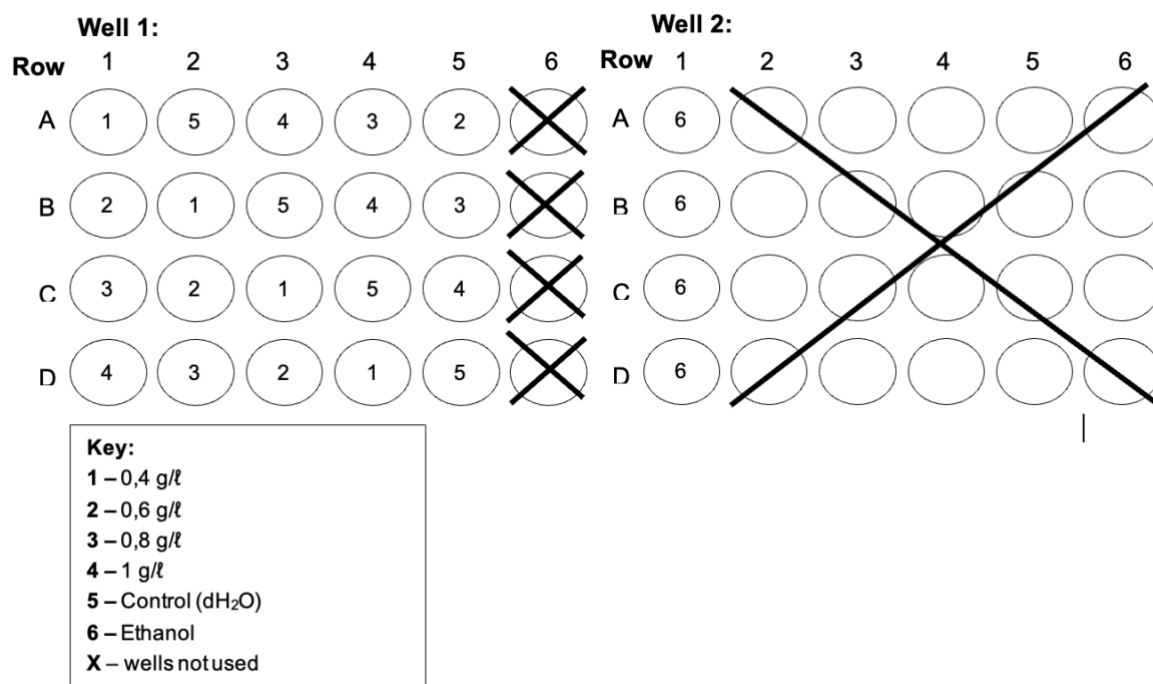


Figure 3-7: *In vitro* well-plate experimental layout, randomized complete block design, used for screening of each formulated fungal product against second-stage juveniles of *Meloidogyne incognita* to record its effect on motility (Marné Pretorius, NWU).

3.4. Screening of fungal isolates for their effect on *M. incognita* reproduction (*in vivo* analysis)

3.4.1. Preparation of soil and pots for *in vivo* experiments

Telone II-fumigated soil (from the same source as used for *M. incognita in vivo* rearing; see paragraph 3.1.1) was mixed in a 1:4 ratio with potting soil (Culterra Professional Potting Mix, <https://culterra.co.za/category/products/soil/>) that is characterised by providing good drainage and aeration; is bark and peat based with added nutrients to ensure vigorous and sustained plant growth; is pest and disease free which was sterilized in an autoclave at 121 °C for 1 hr. The soil-compost mixture was added to 1-l capacity, plastic pots and marked to represent each treatment and replicate. The *in vivo* experiments were conducted in a glasshouse at an ambient temperature range of 19±2 °C (min) and 25±2 °C (max). Soil-compost substrate in pots were irrigated with borehole water [pH 7.3 (H₂O)] before planting followed by watering every 2-3 days depending on demand.

Using a marking pen, 10 holes were made in the soil-compost substrate of each pot and one soybean seed added to each hole. Then, 300 µl of *Bradyrhizobium japonicum* (Soygro microbial inoculant; <http://www.soygro.co.za/soybeans/>) were applied for nitrogen fixation to soybean seed per hole with a concentration of 10 g *B. japonicum* per 100 ml of dH₂O. Nematode inoculum

representing ± 1000 J2 and eggs per pot were next applied to each seed per hole as 1-ml aliquots. The egg and J2 inoculum were prepared as explained in paragraph 3.1.2. Thereafter, either the seed-treatment or direct, drench soil application was applied.

3.4.2. Preparation of fungal isolates: seed treatments (ST) and direct, drench soil (DA) applications

Living fungal spores of the five isolates and the two formulated fungal products (Eco-T® and CR) were applied as seed-treatments (ST) and direct soil drench applications (DA). The Round-up Ready® soybean *M. incognita* susceptible cultivar DM 6.8i (Mbatyoti *et al.*, 2021) was used in this study and was obtained from the Agricultural Research Council (ARC-Grain Crops) in Potchefstroom, North-West Province, South Africa. Surface sterilization of the seeds took place by rinsing it with a 3% NaOCl solution for 2.5 min. The seeds were then rinsed with sterile dH₂O five times before planting.

3.4.2.1. Seed-treatment (ST):

To optimise the efficacy of the fungal spore seed treatments, a spore sticker solution was prepared as by Pambuka (2014), by dissolving 1 g of carboxymethyl cellulose (CMC) in 30 ml of dH₂O in a glass beaker. Thirty millilitre of the spore sticker solution was added to each of the conidial suspensions 10^7 and 10^8 spores/ml of each fungal isolate, suspended in separate glass beakers, and shaken on an orbital shaker at 150 rpm for 1 hr. Surface sterilized soybean seeds were added to each of the conidial-sticker-spore concentration solutions, and shaken on an orbital shaker at 150 rpm for 20 min. The conidial-sticker-spore concentration solutions were then decanted, and the seeds dried overnight (16-18 hr) in a laminar flow cabinet before being planted (four seeds per pot for living fungal isolate, and ten seeds per pot for formulated fungal product experiments) in the soil-compost substrate (see paragraph 3.4.1).

The soybean seeds used for the untreated control were directly put under laminar flow to dry, 16-18 hrs, after surface sterilization with the 3% NaOCl solution. No sticker solution or conidia were thus coated on the seed of the untreated control.

3.4.2.2. Direct, drench soil application (DA):

During the direct, soil drench application of the fungal isolates to the soil, a 1 ml conidial suspension of 10^7 and 10^8 spores/ml were prepared and applied into the holes made in the soil, next to the surface sterilised soybean seeds directly after nematode inoculum and *Bradyrhizobium* were applied.

3.4.3. *In vivo* screening using living fungal spores

The two treatments, ST and DA, with the different fungal strains at different concentrations (10^8 and 10^7 spores/ml), together with an untreated control (surface sterilized seeds only), was used for the *in vivo* experiments (Figure 3-8).

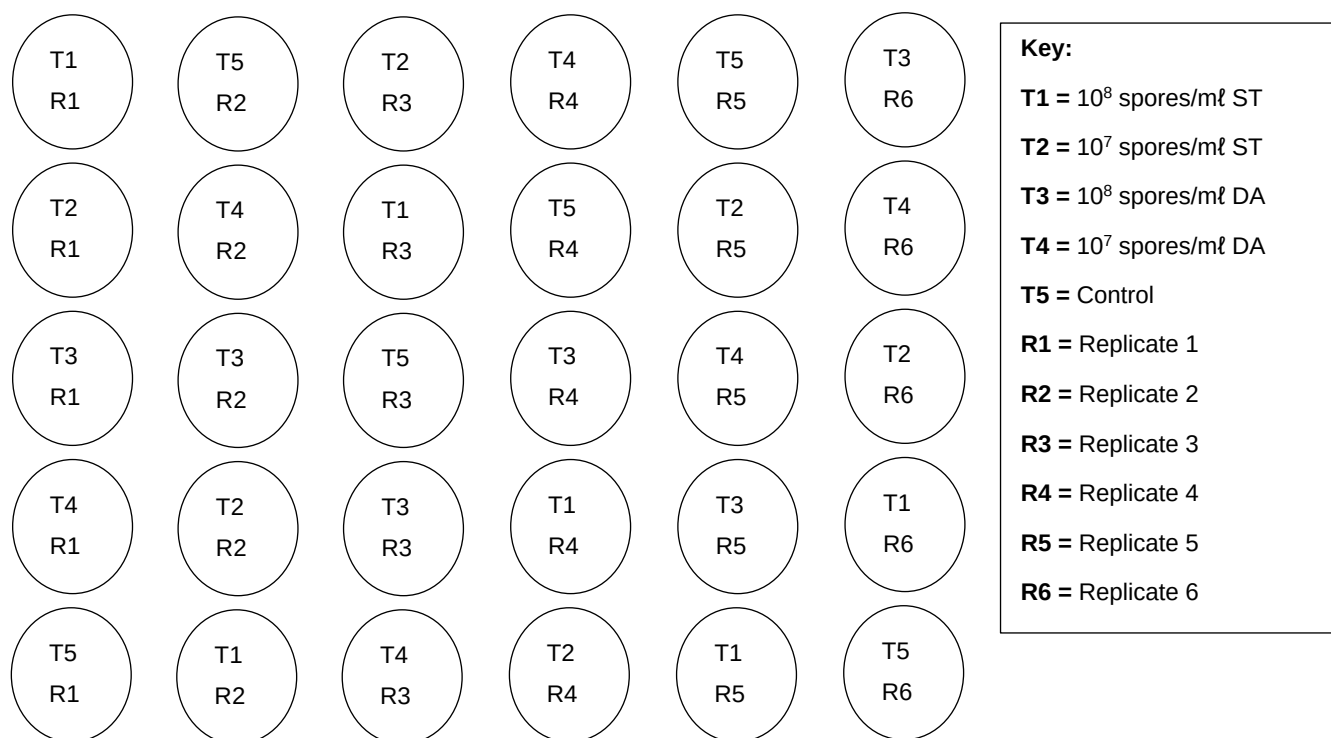


Figure 3-8: *In vivo* randomized split-plot design trial plan used for screening living fungal spores against *Meloidogyne incognita* reproduction (Marné Pretorius, NWU).

Twenty-eight days after planting (DAP) (representing the first sampling interval) two of the four soybean plants sown were removed from pots for nematode analyses. The aerial plant parts were excised, and the root mass determined, followed by recording the *M. incognita* gall rating for each plant's root system using the following galling index: 0 = no galling; 1 = trace infection with a few small galls; 2 = 25% roots galled; 3 = 26 to 50%; 4 = 51 to 75%; and 5 > 75% roots galled (Hussey & Janssen, 2002). Thereafter, *M. incognita* life-stages were extracted from 20 g roots using a modified NaOCl method followed by the sugar centrifugal flotation method (Marais *et al.*, 2017). In addition, 200 g of rhizosphere soil was also removed from each pot and extracted using the decanting and sieving method followed by the sugar centrifugal flotation method (Marais *et al.*, 2017). *Meloidogyne incognita* life stages were counted, as indicated in the data tables in Chapter 5, for each treatment and replicated for both root and soil samples using a De Grisse counting dish (Marais *et al.*, 2017) as explained in paragraph 3.1.2. A second sampling interval took place 56 DAP when the remaining two soybean plants per

pot were removed, and the same procedures followed (as during 28 DAP) to determine the root mass, *M. incognita* nematode galling, and number of *M. incognita* life stages in root and soil samples.

3.4.4. Formulated fungal products

The two application methods (ST and DA) with the two formulated fungal products at different concentrations (0,4 g/l; 0,6 g/l; 0,8 g/l; 1 g/l), the untreated control (surface sterilized seeds only) and a negative control representing a Velum® 10G treatment (a.i. fluopyram/pyridinylethylbenzamide @ 500 g/l used at a dosage rate of 0,34 g per 1-l pot) were included for the *in vivo* experiments (Figure 3-9). Ten soybean seeds were planted per pot and treated with *B. japonicum* as explained in paragraph 3.4.1. for the live fungal spore experiment.

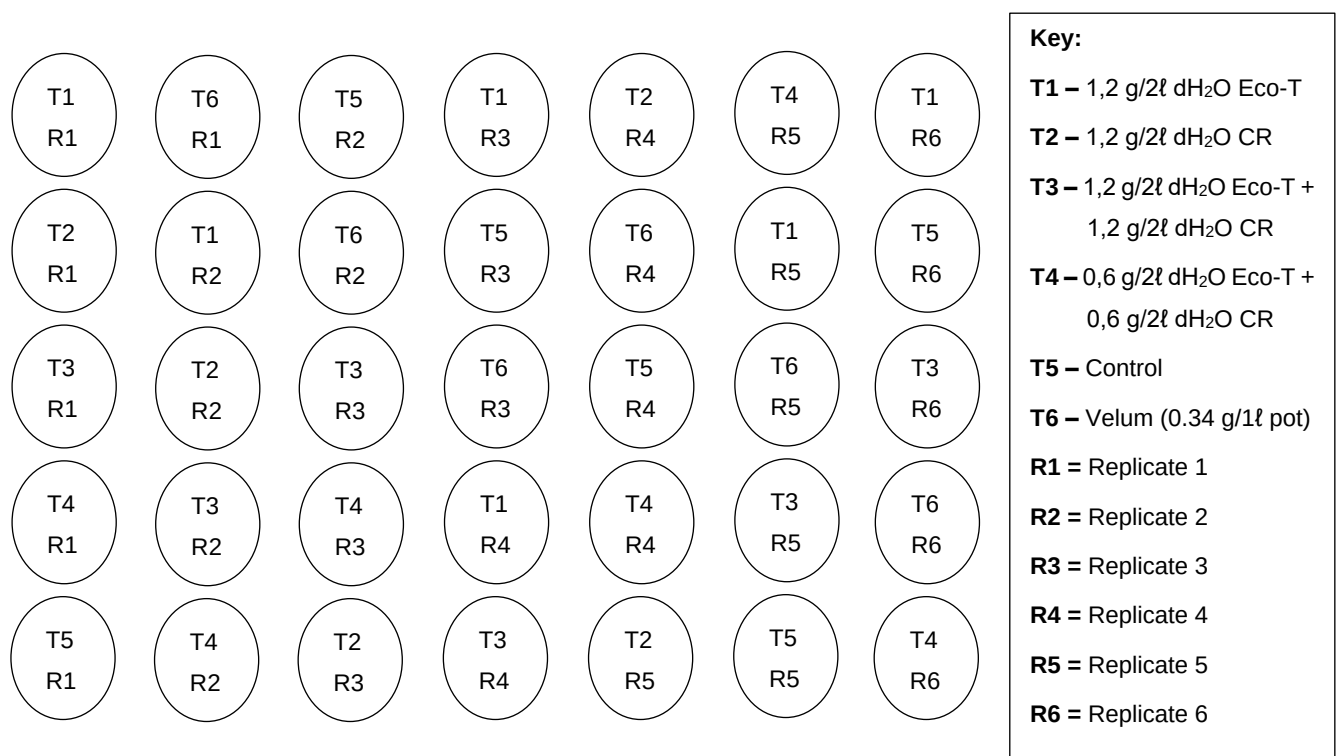


Figure 3-9: *In vivo* randomized split-plot design trial plan used for evaluating the effects of two formulated fungal products on the reproduction of *Meloidogyne incognita* (Marné Pretorius, NWU).

Two weeks after the experiment commenced, a follow-up direct soil drench application was performed for each of the fungal treatments, ST and DA, using the same rates applied at the beginning of the experiment (0,4 g/l; 0,6 g/l; 0,8 g/l; 1 g/l). The follow-up DA application was repeated every two weeks for the full term of the experiment to optimize application of the products.

At 48 DAP the soybean plants were removed from the pots. Aerial height and mass, as well as root mass, were measured for each plant. The *M. incognita* gall rating (see paragraph 3.4.3.) was also done for each plant. The root system of each plant was then stained with phloxine B (0,15 mg per 1 l of tap water) (Holbrook *et al.*, 1983) (Figure 3-10), and the red-stained *M. incognita* egg masses counted. This was done by immersing and suspending the infected roots in the phloxine-B solution in a square, plastic 1- l capacity container for 20 min. The egg masses were then counted with a commercial magnifying glass. The next step entailed that *M. incognita* life-stages were extracted from the roots of the whole root system of each plant (after it was cut into 2-5 mm pieces) using a modified NaOCl and sugar flotation method (Marais *et al.*, 2017). *Meloidogyne incognita* J2 were extracted from the soil samples using the decanting and sieving methods followed by the sugar-centrifugal flotation method (Marais *et al.*, 2017). *Meloidogyne incognita* life stages in root and soil samples were counted using a De Grisse counting dish (De Grisse, 1963; Van den Berg *et al.*, 2017), and statistically analysed.



Figure 3-10: Staining and counting of *Meloidogyne incognita* egg masses on infected soybean roots from an *in vivo* trial (Marné Pretorius, NWU).

3.5. Statistical analysis

Parameters measured for the *in vitro* experiments included: J2 motility at 24 h, 48 h, and 72 h after the experiments commenced. For the *in vivo* experiments the following parameters were recorded: aerial plant mass, aerial plant height, root mass, *M. incognita* galling, *M. incognita* egg mass counts, *M. incognita* counts in the root system of each plant and the corresponding soil (200 g). Each experiment was repeated once to compare and verify the data obtained.

Data obtained from *in vitro* and *in vivo* experiments were analysed making use of Statistica 13.3 Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>). Raw data as well as $\log(x+1)$ transformed data for the initial and repeat experiments (*in vivo* and *in vivo*) for each fungal isolate and/or product were subjected to Repeated Measures ANOVA and the means of treatments compared with the Tukey HSD Test ($P < 0.05$). The data was also subjected to Levene's test for homogeneity of variance.

CHAPTER 4 RESULTS AND DISCUSSION – *IN VITRO* EXPERIMENTS

4.1 Introduction

The testing of potential biological nematicides is important since this is the direction in which the global market is moving (Kumar & Purohit, 2012; Toju & Tenaka, 2019). Producers need access to alternative strategies to protect their crops and mitigate the adverse impact of plant-parasitic nematodes (Kumar & Dara, 2021).

With respect to *Trichoderma* spp., fungi play a large role in the effective control of a wide range of species of root-knot nematodes (*Meloidogyne* Goeldi, 1892) (Goettel *et al.*, 2001; Woo *et al.*, 2014). Research has shown that numerous fungal genera and strains have been used as biocontrol agents of species of *Meloidogyne* (Harman, 2000; Verma *et al.*, 2007; Vinale *et al.*, 2008; Al-Hazmi & TariqJaveed, 2016; Cristóbal-Alejo *et al.*, 2021; Mahaptra *et al.*, 2021). *Trichoderma harzianum* is for example amongst one of the most effective fungal strains used that negatively impact the motility of second-stage juveniles (J2) and reproduction of *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949 (Singh *et al.*, 2021). Although limited information in this regard exists for *Clonostachys rosea*, this fungal strain has been found to effectively induce *M. incognita* J2 mortality by 100% and hatching in eggs of J2 of *Meloidogyne* spp. by 59,73% during *in vitro* experiments (Migunova *et al.*, 2018; Lafta & Kasim, 2019).

It is also important to indicate why *M. incognita* was used as the target *Meloidogyne* spp. for biological control utilizing fungi. This specific plant-parasitic nematode is known for its destruction in grain production areas globally (Fourie *et al.*, 2010; Jones *et al.*, 2013). Moreover, there are only a limited number of nematicides available in areas with root-knot nematode problems, especially when considering products registered on specific crops such as soybean (Briedenhann, 2018; Engelbrecht *et al.*, 2021).

The main mode of action utilized by *Trichoderma* spp. is the production of extracellular degrading enzymes (including chitinolytic) which have a nemato-toxic potential and parasitizes plant-parasitic nematode life stages or damage their eggs (Szabó *et al.*, 2012; Fan *et al.*, 2020; Lakmini & Amarasinghe, 2021). Another mechanism is through nematode-trapping using specialized structures to trap nematodes (Lafta & Kasim, 2019). *Clonostachys rosea* on the other hand utilizes antibiosis, direct parasitism, and enzyme secretion to reduce *M. incognita* population densities (Yang *et al.*, 2010; Iqbal, 2019; Cristóbal-Alejo *et al.*, 2021).

The aim of this study was therefore to analyse the *in vitro* effect of spores of five fungal strains of which stock cultures were obtained from Andermatt-PHP (Pty) Ltd., South Africa (<http://ander-matt->

php.co.za), on the motility of J2 *M. incognita* South African population during three time intervals (24, 48 and 72 h after the experiments commenced) (see Paragraph 3.3.1., Chapter 3 Materials and Methods). The two fungal genera (*Trichoderma* and *Clonostachys*) represented by five fungal strains used for this study were *Trichoderma asperellum*, *Trichoderma asperellum* kd, *T. harzianum*, *Trichoderma virens* and *C. rosea*. In addition, two formulated products namely CR (active ingredient/a.i. *C. rosea* strain PHP 1701) and Eco-T® (a.i. *T. asperellum* strain kd) were also evaluated for their effect on J2 motility in this study.

The data are reflected as the percentage J2 immotilized by the fungal spore concentrations and were subjected to either ANOVA (formulated products), Repeated Measures ANOVA (for the fungal strains for which J2 immotility data were obtained during the three respective time intervals) and Factorial ANOVA (to compare the two trials of each fungal strain) using TIBCO Statistica™ (<https://www.statistica.com>), Version 14.0.0.15. Means for each treatment were separated by the Tukey HSD Test (P=0.05).

4.2 Results: *In vitro* screening utilizing viable fungal spores from reared colonies

Two bran substrate experiments were performed as an upper control before commencing with the different *in vitro* experiments to determine whether this substrate, used for rearing the strains, had a negative effect on *Meloidogyne* J2. Since J2 immotility was zero (data not shown) for all concentrations of all the fungal strains tested, this indicated that the bran substrate did not adversely affect J2 motility and was safe to use as the rearing medium.

4.2.1 *Clonostachys rosea*

A significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments reacted different for the two *in vitro* experiments, and/or for the different time intervals (24, 48 and 72 h) (Figure 4-1; Table 4-1).

For Experiment 1 the highest *C. rosea* concentration, 10^9 spores/mL, for all respective time intervals (24 h, 48 h and 72 h) and the intermediate concentration of 10^8 spores/mL (only for the 72 h time interval) showed the highest J2 immotility of 100% (Figure 4-1; Table 4-1). The J2 immotility for these treatments were not significantly higher than the 10^8 spores/mL for the 24 h and 48 h intervals but did differ significantly from the lowest concentration of 10^7 and the Untreated Control for all respective time intervals.

For Experiment 2, the highest concentration of 10^9 spores/mL (all time intervals) had the highest J2 immotility (ranging from 82% to 93%), differing significantly from all respective time intervals of

the intermediate 10^8 spores/mL, lowest 10^7 spores/mL and Untreated Control (Figure 4-1; Table 4-1).

The Untreated Control treatments for both experiments had the lowest J2 immotility rates, zero for Experiment 1 and 0,5% - 1,7% for Experiment 2 (Figure 4-1; Table 4-1). The J2 immotility of these treatments were not significantly different from the lowest 10^7 spores/mL treatments (24 h and 72 h) of Experiment 1 and intermediate 10^8 spores/mL (24 h and 48 h) of Experiment 2. In contrast to this, the Untreated Control treatments differed significantly from the 10^7 spores/mL (48 h) of Experiment 1 and 10^8 spores/mL (72 h) of Experiment 2.

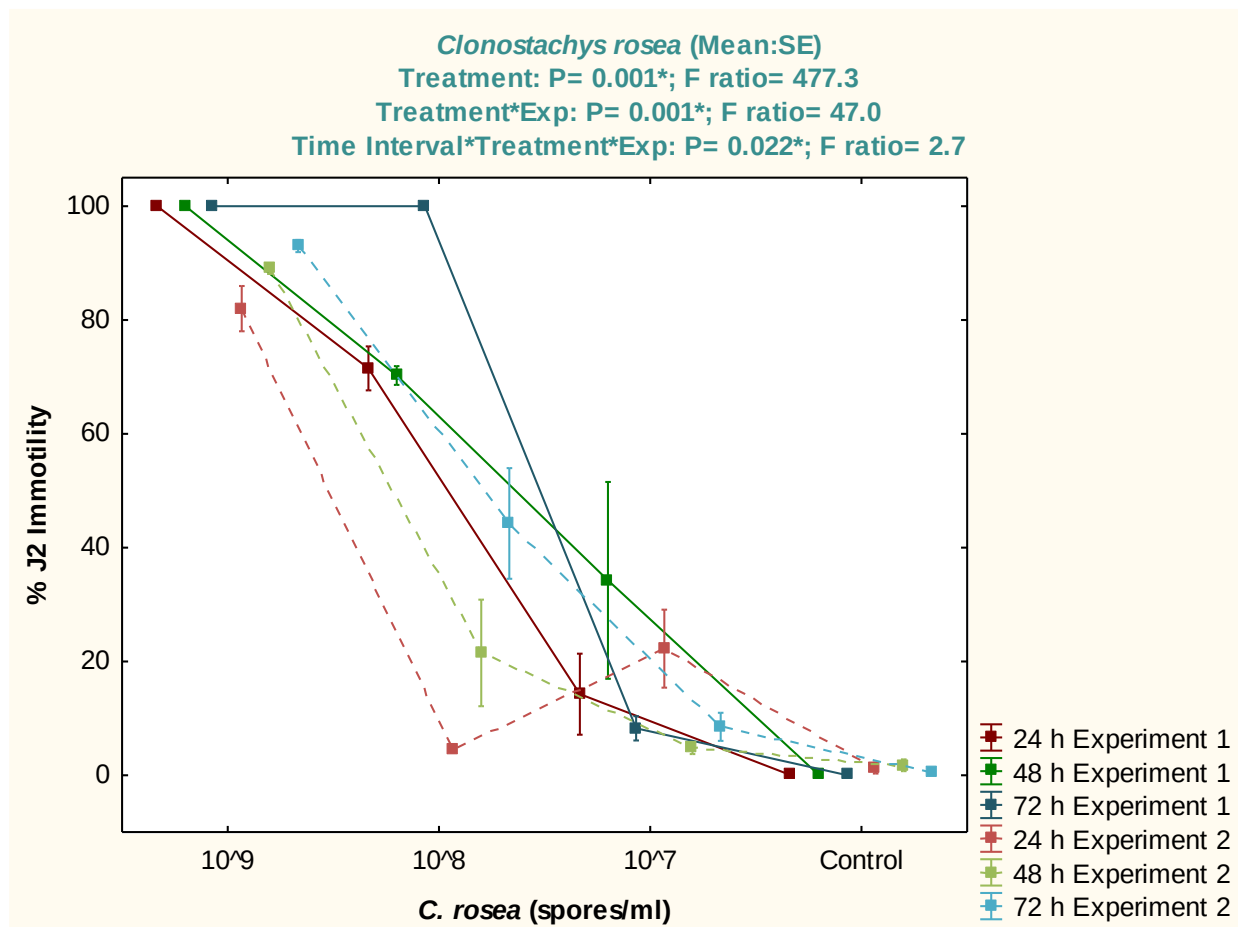


Figure 4-1: Effect of different concentrations (spores per mL) of *Clonostachys rosea* on the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (*in vitro* analysis).

Table 4-1: Effect of different concentrations of *Clonostachys rosea* on second-stage (J2) juvenile motility of *Meloidogyne incognita* (*in vitro* study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (dH ₂ O)	24 h	0,0 aA	1,2 aA
Untreated Control (dH ₂ O)	48 h	0,0 aA	1,7 aA
Untreated Control (dH ₂ O)	72 h	0,0 aA	0,5 aA
10 ⁷	24 h	14,2 acAB	22,2 acABC
10 ⁷	48 h	34,2 cBC	4,7 aA
10 ⁷	72 h	8,2 acAB	8,5 aAB
10 ⁸	24 h	71,5 dDEG	4,5 aA
10 ⁸	48 h	70,2 dDE	21,5 aABC
10 ⁸	72 h	100,0 bdFG	44,2 cCD
10 ⁹	24 h	100,0 bF	82,0 bEFG
10 ⁹	48 h	100,0 bF	89,0 bEFG
10 ⁹	72 h	100,0 bF	93,0 bEFG
P value		0.001*	0.001*
F ratio		420.1	167.1
Interaction data: Treatment x Experiment			
P value		0.001*	
F ratio		47.0	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.022*	
F ratio		2.7	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.2.2 *Trichoderma asperellum*

A significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments reacted differently for the two *in vitro* experiments, and/or for the different time intervals (24, 48 and 72 h) (Figure 4-2; Table 4-2).

For Experiment 1, the highest concentrations of 10⁹ spores/ml 48 h interval (99,5%) and 10⁹ spores/ml 72 h interval (99%), and the intermediate concentrations of 10⁸ spores/ml 24 h interval (94,5%) and 10⁸ spores/ml 72 h interval (98%) showed the highest J2 immotility. These results were significantly higher than the 10⁹ spores/ml (24 h time interval) and 10⁸ spores/ml (48 h time

interval), as well as for all respective time intervals of the 10^7 spores/ml and the Untreated Control (Figure 4-2; Table 4-2).

For Experiment 2, the highest concentration of 10^9 spores/ml for the 48 and 72 h and intermediate concentrations of 10^8 spores/ml (for all respective time intervals) had the highest J2 immotility ranging from 89,5% to 98,2% (Figure 4-2; Table 4-2). These treatments had significantly higher J2 immotility than the 10^9 spores/ml for the 24 h time interval, and for the respective time intervals of the 10^7 spores/ml and the Untreated Control.

The J2 immotility rates of the Untreated Control did not differ significantly from the lowest concentration of 10^7 spores/ml (all respective time intervals) for the first experiment but was significantly lower for the 72 h time interval of the 10^7 spores/ml for the second experiment (Figure 4-2; Table 4-2).

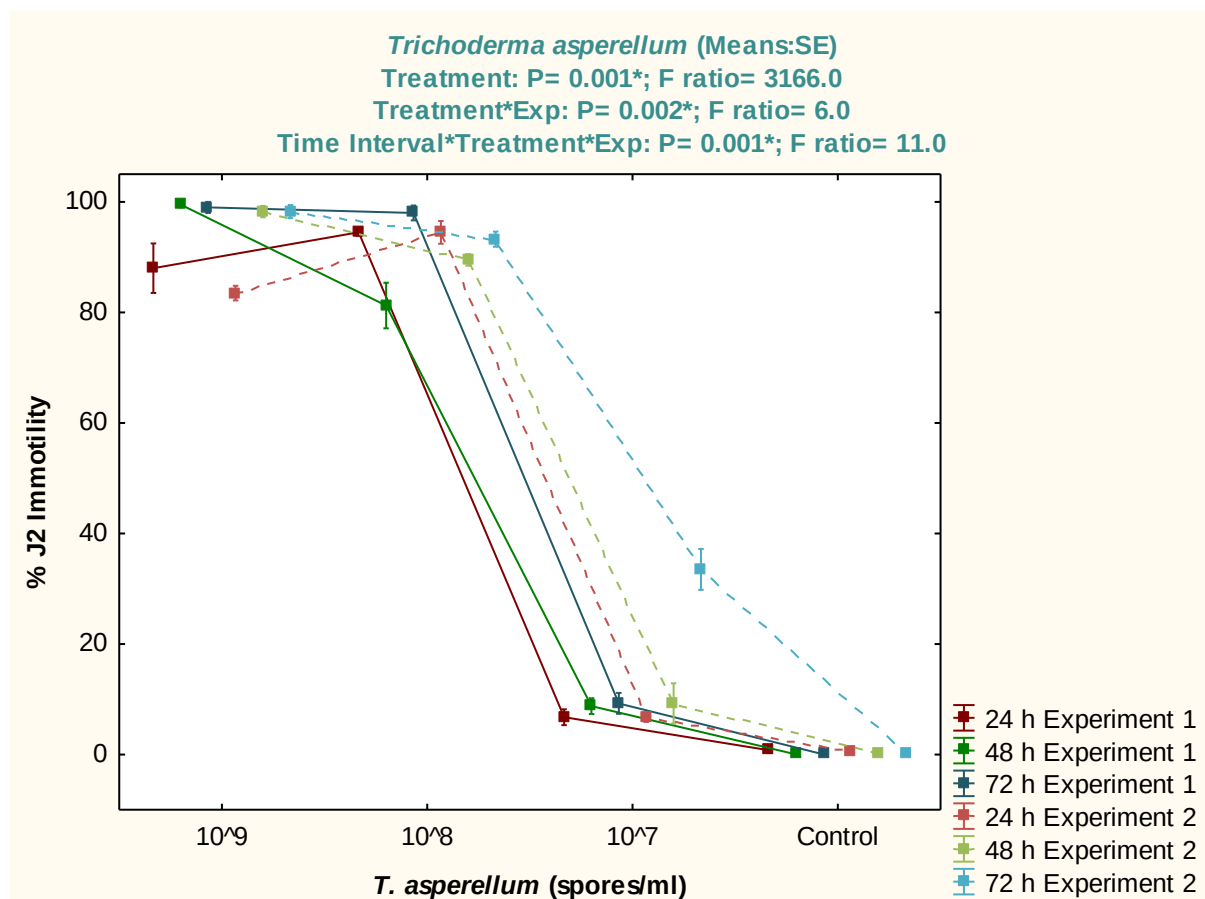


Figure 4-2: Effect of different concentrations (spores per ml) of *Trichoderma asperellum* on the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (*in vitro* analysis).

Table 4-2: Effect of different concentrations of *Trichoderma asperellum* on second-stage (J2) juvenile motility of *Meloidogyne incognita* (*in vitro* study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (dH ₂ O)	24 h	0,7 aA	0,5 bcA
Untreated Control (dH ₂ O)	48 h	0,0 aA	0,0 bA
Untreated Control (dH ₂ O)	72 h	0,0 aA	0,0 bA
10 ⁷	24 h	6,7 aA	6,7 bcA
10 ⁷	48 h	8,7 aA	9,2 cA
10 ⁷	72 h	9,2 aA	33,5 eB
10 ⁸	24 h	94,5 bdEF	94,5 aEF
10 ⁸	48 h	81,2 cC	89,5 adCDEF
10 ⁸	72 h	98,0 bEF	93,2 aDEF
10 ⁹	24 h	88,0 cdCDE	83,5 dCD
10 ⁹	48 h	99,5 bF	98,2 aEF
10 ⁹	72 h	99,0 bF	98,2 aEF
P value		0.001*	0.001*
F ratio		1693.0	1484.0
Interaction data: Treatment x Experiment			
P value		0.002*	
F ratio		6.0	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.001*	
F ratio		11.0	

Means were separated with the Tukey HSD Test where $P \leq 0.05$, with significance indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.2.3 *Trichoderma asperellum* kd

No significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments did not react differently for the two *in vitro* experiments, and/or for the different time intervals (24, 48 and 72 h) (Figure 4-3; Table 4-3).

For Experiment 1, the highest *T. asperellum* kd concentration of 10⁹ spores/ml (for all of the respective time intervals) and the intermediate concentration of 10⁸ spores/ml (after 24 and 48 h) showed the highest J2 immotility, ranging from 75.2% to 87.2% (Figure 4-3; Table 4-3). These were significantly higher than those of the time intervals of the lowest 10⁷ spores/ml treatments

and the Untreated Control, but not significantly different from the 10^8 spores/ml treatment of the 72 h time interval.

For Experiment 2, the same trend was evident for the time intervals of the highest (10^9 spores/ml) and those of the intermediate (10^8 spores/ml) treatments as explained above for Experiment 1 (Figure 4-3; Table 4-3).

The J2 immotility rates of the Untreated Control were significantly lower than that of the lowest concentration (10^7 spores/ml) for the three respective time intervals of Experiment 1, but not from that of the 48 h and 72 h time intervals of the 10^7 spores/ml treatments of Experiment 2 (Figure 4-3; Table 4-3).

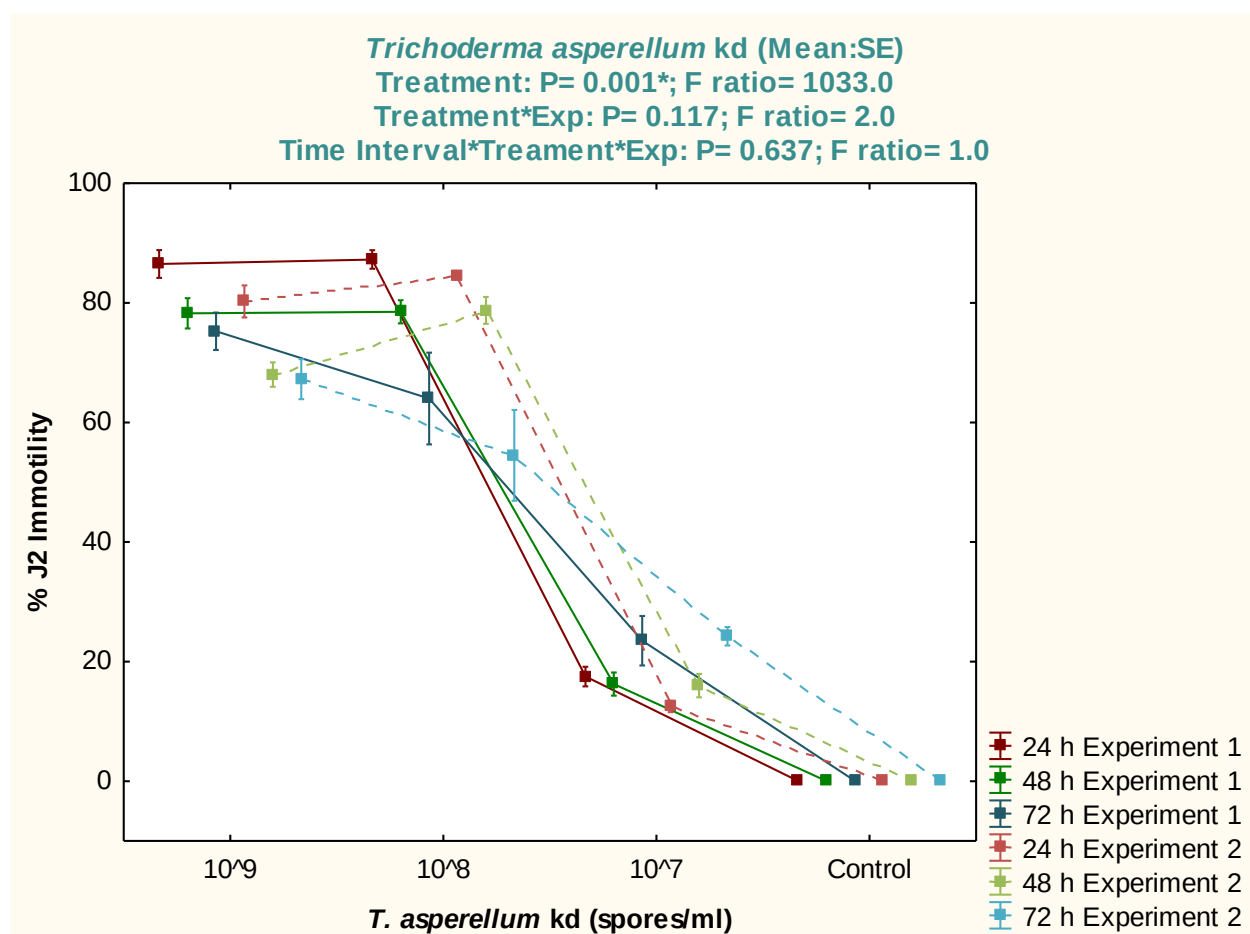


Figure 4-3: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* kd in the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (in vitro analysis).

Table 4-3: Data (%) of the effect of different concentrations of *Trichoderma asperellum* kd on second-stage (J2) juvenile motility of *Meloidogyne incognita* (*in vitro* study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (dH ₂ O)	24 h	0,0 cA	0,0 aA
Untreated Control (dH ₂ O)	48 h	0,0 cA	0,0 aA
Untreated Control (dH ₂ O)	72 h	0,0 cA	0,0 aA
10 ⁷	24 h	17,5 dB	12,5 acAB
10 ⁷	48 h	16,2 dB	16,0 cB
10 ⁷	72 h	23,5 dB	24,2 cB
10 ⁸	24 h	87,2 aF	84,5 eF
10 ⁸	48 h	78,5 abDEF	78,7 beDEF
10 ⁸	72 h	64,0 bCD	54,5 dC
10 ⁹	24 h	86,5 aF	80,2 beEF
10 ⁹	48 h	78,2 abDEF	68,0 bdCDE
10 ⁹	72 h	75,2 abDEF	67,2 bdCDE
P value		0.001*	0.001*
F ratio		658.0	413.5
Interaction data: Treatment x Experiment			
P value		0.117	
F ratio		2.0	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.637	
F ratio		1.0	

Means were separated with the Tukey HSD Test where $P \leq 0.05$, with significance indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.2.4 *Trichoderma harzianum*

A significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments reacted differently for the two *in vitro* experiments, and/or for the different time intervals (24, 48 and 72 h) (Figure 4-4; Table 4-4).

For Experiment 1, the highest *T. harzianum* concentration of 10⁹ spores/mL, at the three respective time intervals and the intermediate concentration of 10⁸ spores/mL (24 h only) had the highest J2 immotility, ranging from 80,7% to 91,7% (Figure 4-4; Table 4-4). The J2 immotility rates of these treatments were significantly higher than those of all the time intervals of the lowest 10⁷ spores/mL

treatment and the Untreated Control. The J2 immotility rates of the three 10^8 spores/ml for the respective time intervals did, however, not differ from each other.

For Experiment 2, the 72 h time interval of the highest 10^9 spores/ml had the highest J2 immotility rate of 91,5%, not differing significantly from that of the 48 h time interval (80,2%). In contrast, these results were significantly higher than that of 24 h time interval of the 10^9 spores/ml treatment as well as those of all time intervals of the 10^8 spores/ml treatment, the 10^7 spores/ml treatment and Untreated Control (Figure 4-4; Table 4-4)

The J2 immotility rates of the Untreated Control were zero for Experiment 1 (all time intervals) differing significantly from all time intervals of the lowest (10^7 spores/ml), intermediate (10^8 spores/ml) and highest (10^9 spores/ml) treatments (Figure 4-4; Table 4-4). For Experiment 2, the J2 immotility rates of the Untreated Control were the lowest (ranging from 0,7% to 2,7%), not differing significantly from the respective time intervals of the lowest 10^7 spores/ml treatment, but significantly lower than that of the intermediate (10^8 spores/ml) and highest (10^9 spores/ml) treatments.

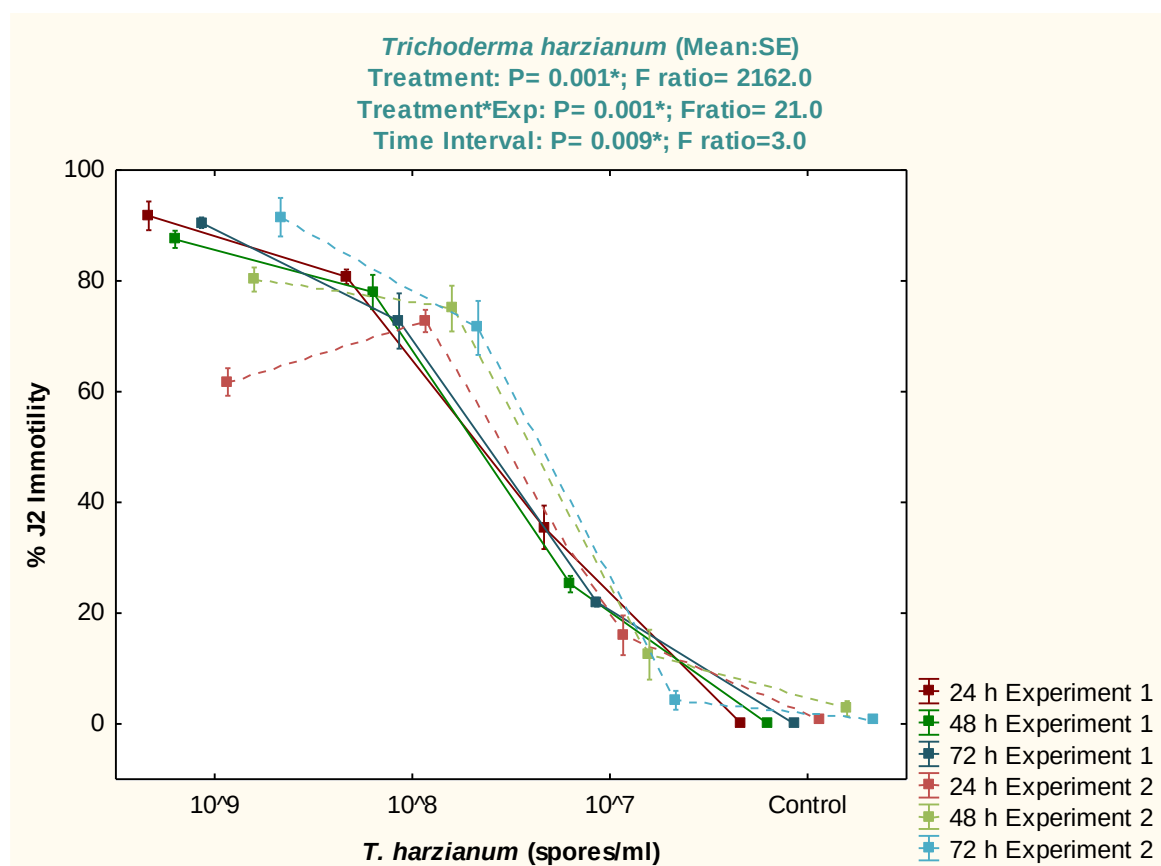


Figure 4-4: The effect of different concentrations (spores per ml) of *Trichoderma harzianum* on the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (*in vitro* analysis).

Table 4-4: Data (%) of the effect of different concentrations of *Trichoderma harzianum* in second-stage (J2) juvenile motility of *Meloidogyne incognita* (*in vitro* study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (dH ₂ O)	24 h	0,0 bA	0,7 aA
Untreated Control (dH ₂ O)	48 h	0,0 bA	2,7 abAB
Untreated Control (dH ₂ O)	72 h	0,0 bA	0,7 aA
10 ⁷	24 h	35,5 fD	16,0 bBC
10 ⁷	48 h	25,2 efCD	12,5 abABC
10 ⁷	72 h	22,0 eCD	4,2 abAB
10 ⁸	24 h	80,7 acdFGH	72,7 cdEF
10 ⁸	48 h	78,0 cdFGH	75,0 cdEFG
10 ⁸	72 h	72,7 cEF	71,5 cdEF
10 ⁹	24 h	91,7 aH	61,7 cE
10 ⁹	48 h	87,5 adGH	80,2 deFGH
10 ⁹	72 h	90,5 aH	91,5 eH
P value		0.001*	0.001*
F ratio		1022.0	1179.0
Interaction data: Treatment x Experiment			
P value		0.001*	
F ratio		21.0	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.009*	
F ratio		3.0	

Means were separated with the Tukey HSD Test where $P \leq 0.05$, with significance indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.2.5 *Trichoderma virens*

No significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments did not react differently for the two *in vitro* experiments, and/or for the different time intervals (24, 48 and 72 h) (Figure 4-5; Table 4-5).

For Experiment 1, the 48 h and 72 h time intervals of the highest concentration of 10⁹ spores/ml and the three respective time intervals of the intermediate 10⁸ spores/ml treatment had the highest J2 immotility, ranging from 85,2% to 100% (Figure 4-5; Table 4-5). The J2 immotility of these treatments were significantly higher than those of the 24 h time interval of the 10⁹ spores/ml

treatment, and those of the three time intervals for the 10^7 spores/ml treatment and the Untreated Control.

For Experiment 2, the highest *T. virens* concentration of 10^9 spores/ml (three respective time intervals) and the intermediate concentration of 10^8 spores/ml (all time intervals) had the highest J2 immotility, ranging from 85,2% to 97,7% (Figure 4-5; Table 4-5). The J2 immotility rates of these treatments were significantly higher than those of all the time intervals of the lowest 10^7 spores/ml treatment and the Untreated Control.

The J2 immotility rates of the Untreated Control were the lowest of all the treatments equalling zero and were significantly lower than that of the lowest concentration of 10^7 spores/ml, intermediate concentration (10^8 spores/ml) and highest concentration (10^9 spores/ml) for the three respective time intervals for both experiments (Figure 4-5; Table 4-5).

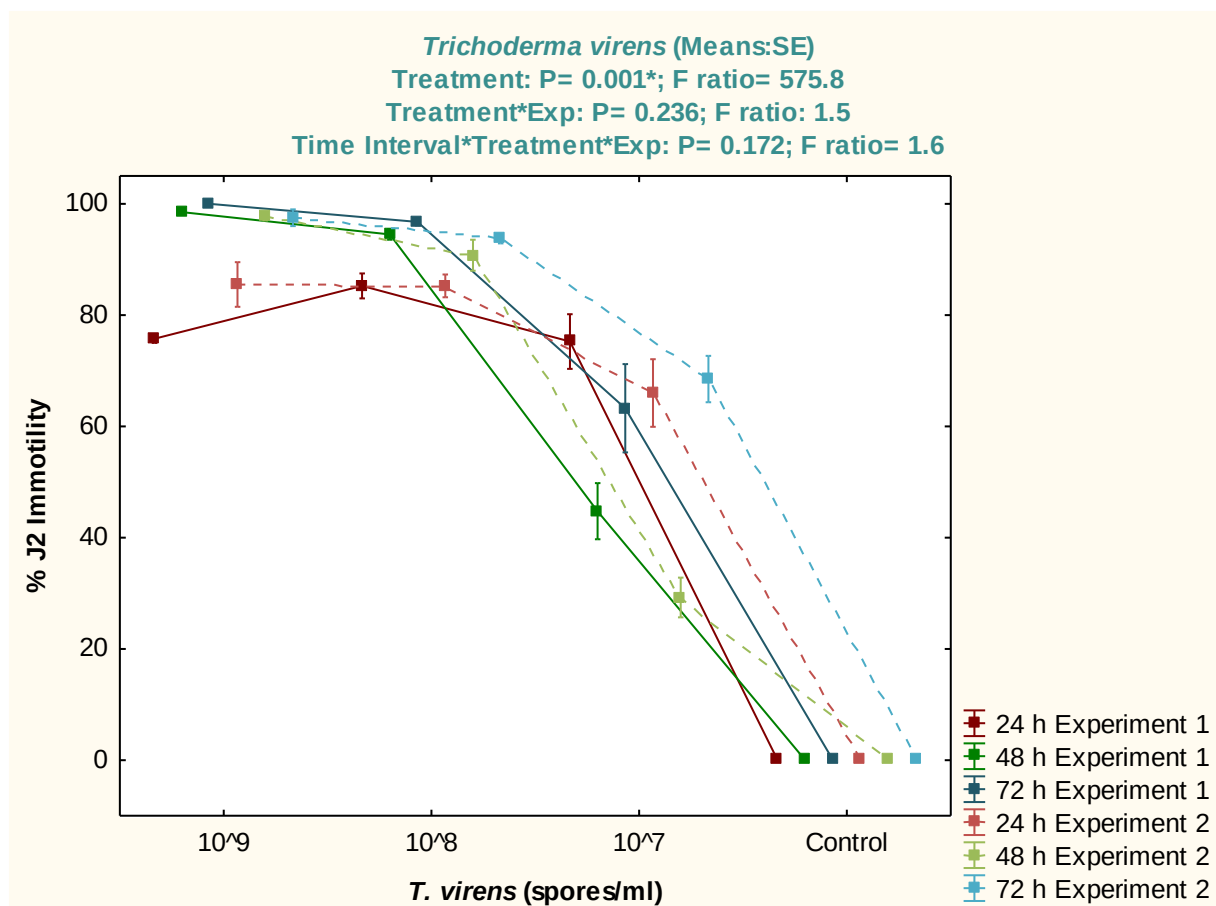


Figure 4-5: The effect of different concentrations (spores per ml) of *Trichoderma virens* on the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (in vitro analysis).

Table 4-5: Data (%) of the effect of different concentrations of *Trichoderma virens* on second-stage (J2) juvenile motility of *Meloidogyne incognita* (*in vitro* study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (dH ₂ O)	24 h	0,0 bA	0,0 bA
Untreated Control (dH ₂ O)	48 h	0,0 bA	0,0 bA
Untreated Control (dH ₂ O)	72 h	0,0 bA	0,0 bA
10 ⁷	24 h	75,2 cdCD	66,0 cC
10 ⁷	48 h	44,7 eB	29,2 dB
10 ⁷	72 h	63,2 cC	68,5 cC
10 ⁸	24 h	85,2 adDE	85,2 aDE
10 ⁸	48 h	94,5 aE	90,7 aDE
10 ⁸	72 h	96,7 aE	93,7 aE
10 ⁹	24 h	75,7 cdCD	85,5 aDE
10 ⁹	48 h	98,5 aE	97,7 aE
10 ⁹	72 h	100,0 aE	97,5 aE
P value		0.001*	0.001*
F ratio		1058.0	242.6
Interaction data: Treatment x Experiment			
P value		0.29*	
F ratio		4.0	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.088	
F ratio		2.0	

Means were separated with the Tukey HSD Test where $P \leq 0.05$, with significance indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.3 Results: *In vitro* screening utilizing formulated fungal-based products

4.3.1 Product CR (a.i. *Clonostachys rosea* strain PHP 1701)

No significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments did not react differently for the two *in vitro* experiments, and/or for the different time intervals (24, 48 and 72 h) (Figure 4-6; Table 4-6).

For Experiment 1, the highest CR concentrations, 0.8 g product/L H₂O and 1 g product/L H₂O showed the highest J2 immotility rates of 17% (for the 72 h of the 1 g product/L H₂O), followed by the 72 h of the 0.4 g product/L H₂O treatment with 16,2%, the 72 h for the 0.8 g product/L H₂O treatment with 13,7%, the 48 h for the 0.8 g product/L H₂O treatment with 12%, the 48 h of the

0.4 g product/L H₂O treatment with 9%, the 72 h of the 0.6 g product/L H₂O treatment with 8,2% and the 48 h of the 0.6 g product/L H₂O treatment with 7,7% (Figure 4-6; Table 4-6). Although the J2 immotility rates of these treatments were the highest, it did not differ significantly from the other treatments accentuating that the J2 immotility rates were relatively low with data showing high variation among the six replicates for this experiment.

For Experiment 2, a similar trend was evident as for Experiment 1 with the 72 h of the 0.8 g product/L H₂O treatment having the highest J2 immotility rate of 13,5%, followed by the 72 h of the 1 g product/L H₂O treatment with 12,7%, the 48 h of the 0.4 g product/L H₂O treatment with 9,5%, the 72 h of the 0.6 g product/L H₂O treatment with 9%, the 48 h of the 0.8 g product/L H₂O treatment with 8,5%, the 48 h of the 1 g product/L H₂O treatment with 5,2%, the 48 h of the 0.6 g product/L H₂O treatment with 4,5%, and the 24 h of the 0.4 g product/L H₂O treatment with 3% (Figure 4-6; Table 4-6). Although these treatments had the highest J2 immotility rates, they were not significantly different from the J2 immotility rates of the other treatments, agreeing with results of the first experiment that the J2 immotility rates were relatively low with data showing high variation among the six replicates.

The J2 immotility rates of the Untreated Control were the lowest of all the treatments, 0% - 1% for both experiments (Figure 4-6; Table 4-6). These results were significantly lower than the 1 g product/L H₂O and 0.4 g product/L H₂O (72 h only) treatments but did not differ significantly from the 0.4 g product/L H₂O (24 h and 48 h) treatment, the 0.6 g product/L H₂O (all time intervals) treatment, the 0.8 g product/L (24 h time interval) treatment or the 1 g product/L (24 h and 48 h) treatment for Experiment 1. For Experiment 2 the Untreated Control (all time intervals) were significantly lower than the 1 g product/L H₂O, 0.8 g product/L H₂O and 0.4 g product/L H₂O (72 h only) treatments, but not significantly lower than the 0.4 g product/L H₂O (24 h), the 0.6 g product/L H₂O (all time intervals), the 0.8 g product/L H₂O (24 h and 48 h) or the 1 g product/L H₂O (24 h and 48 h) treatments.

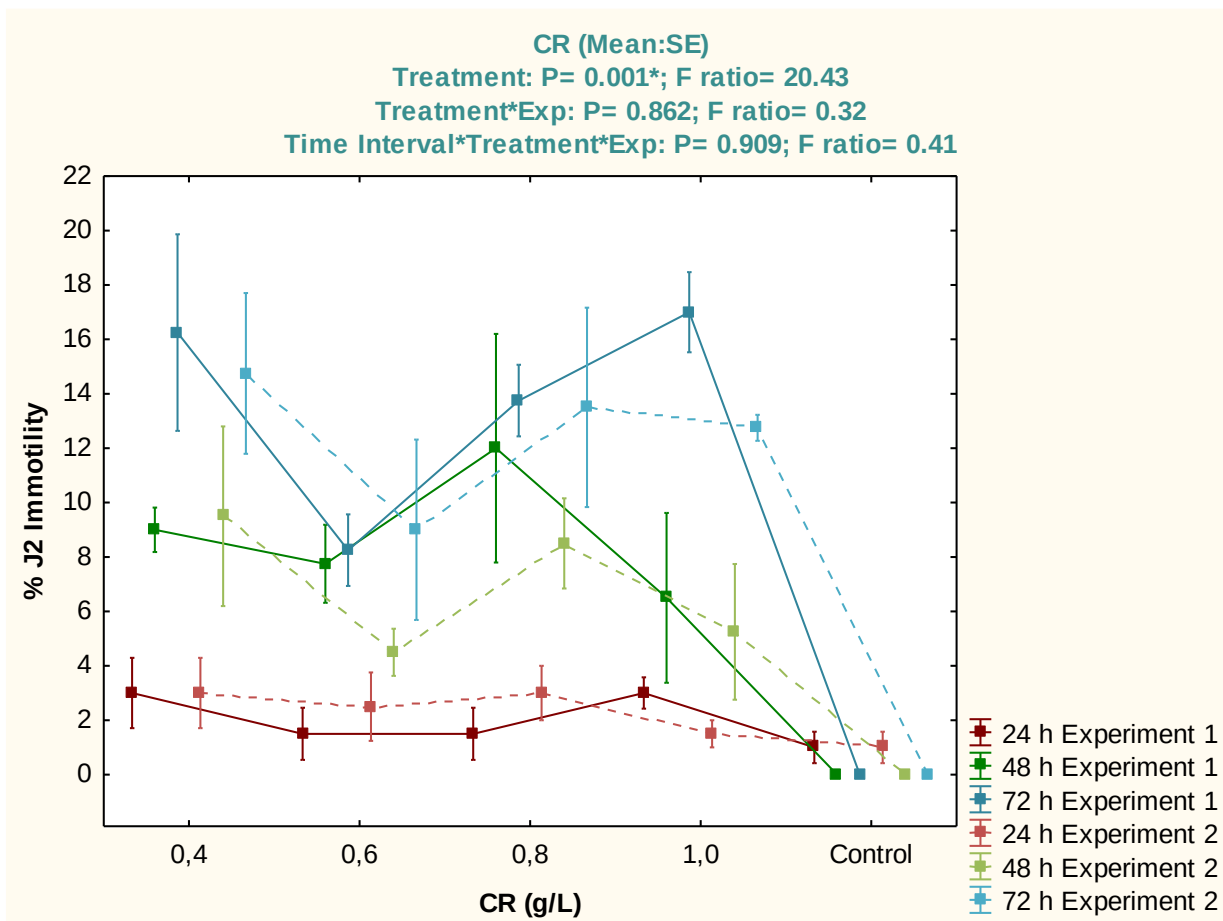


Figure 4-6: The effect of different concentrations (g/L) of CR (a.i. *Clonostachys rosea* strain PHP 1701) on the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (*in vitro* analysis).

Table 4-6: Data (%) of the effect of different concentrations of CR (a.i. *Clonostachys rosea* strain PHP 1701) on second-stage (J2) juvenile motility of *Meloidogyne incognita* (*in vitro* study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (dH ₂ O)	24 h	1,0 abA	1,0 abA
Untreated Control (dH ₂ O)	48 h	0,0 aA	0,0 aA
Untreated Control (dH ₂ O)	72 h	0,0 aA	0,0 aA
0.4 g/L dH ₂ O	24 h	3,0 abABC	3,0 abABC
0.4 g/L dH ₂ O	48 h	9,0 bcdeABCDEFGF	9,5 bcdABCDEFGF
0.4 g/L dH ₂ O	72 h	16,2 deFG	14,7 dEFG
0.6 g/L dH ₂ O	24 h	1,5 abA	2,5 abAB
0.6 g/L dH ₂ O	48 h	7,7 abcdABCDEFGF	4,5 abcABCD
0.6 g/L dH ₂ O	72 h	8,2 abcdeABCDEFGF	9,0 abcdABCDEFGF
0.8 g/L dH ₂ O	24 h	1,5 abA	3,0 abABC
0.8 g/L dH ₂ O	48 h	12,0 cdeBCDEFG	8,5 abcdABCDEFGF
0.8 g/L dH ₂ O	72 h	13,7 cdeDEFG	13,5 cdDEFG
1 g/L dH ₂ O	24 h	3,0 abABC	1,5 abABC
1 g/L dH ₂ O	48 h	6,5 abcABCDEF	5,2 abcABCDE
1 g/L dH ₂ O	72 h	17,0 eG	12,7 cdCDEFG
P value		0.001*	0.001*
F ratio		17.74	14.57
Interaction data: Treatment x Experiment			
P value		0.862	
F ratio		0.32	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.909	
F ratio		0.41	

Means were separated with the Tukey HSD Test where $P \leq 0.05$, with significance indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.3.2 Product Eco-T® (a.i. *Trichoderma asperellum* strain kd)

No significant interaction was evident for Treatment x Time Interval x Experiment indicating that the treatments did not react differently for the two *in vitro* experiments and the different time intervals (24, 48 and 72 h) (Figure 4-7; Table 4-7).

For Experiment 1, the highest J2 immotility rate was recorded for the 24 h time interval of the 0.6 g product/L H₂O treatment (27,7%), followed by 20% J2 immotility for the 24 h and 72 h time intervals of the 1 g product/L H₂O treatment (Figure 4-7; Table 4-7). Although the J2 immotility rates of these treatments were the highest, it did not differ significantly from the other treatment concentrations accentuating that the J2 immotility rates were relatively low for this experiment with data showing high variation among the six replicates.

For Experiment 2, the highest J2 immotility rate was 29,7% for the 24 h time interval of the 0.8 g product/L H₂O treatment, followed by 26,5% and 19,5% for the 24 h and 72 h time intervals of the 0.6 g product/L treatment, respectively (Figure 4-7; Table 4-7). Although the J2 immotility rates of these treatments were the highest, it did not differ significantly from the other treatments agreeing with low J2 immotility rates with data showing high variation among the six replicates, reported for Experiment 1.

The J2 immotility rates of the Untreated Control were the lowest of all the treatments, 0% - 0,7% for Experiment 1 and 0% - 0,5% for Experiment 2 (Figure 4-7; Table 4-7). These results were significantly lower than the lowest 0.4 g product/L H₂O (all time intervals) for both experiments and the 0.8 g product/L H₂O (24 h) of Experiment 2 but did not differ significantly from the other treatment concentrations for all time intervals.

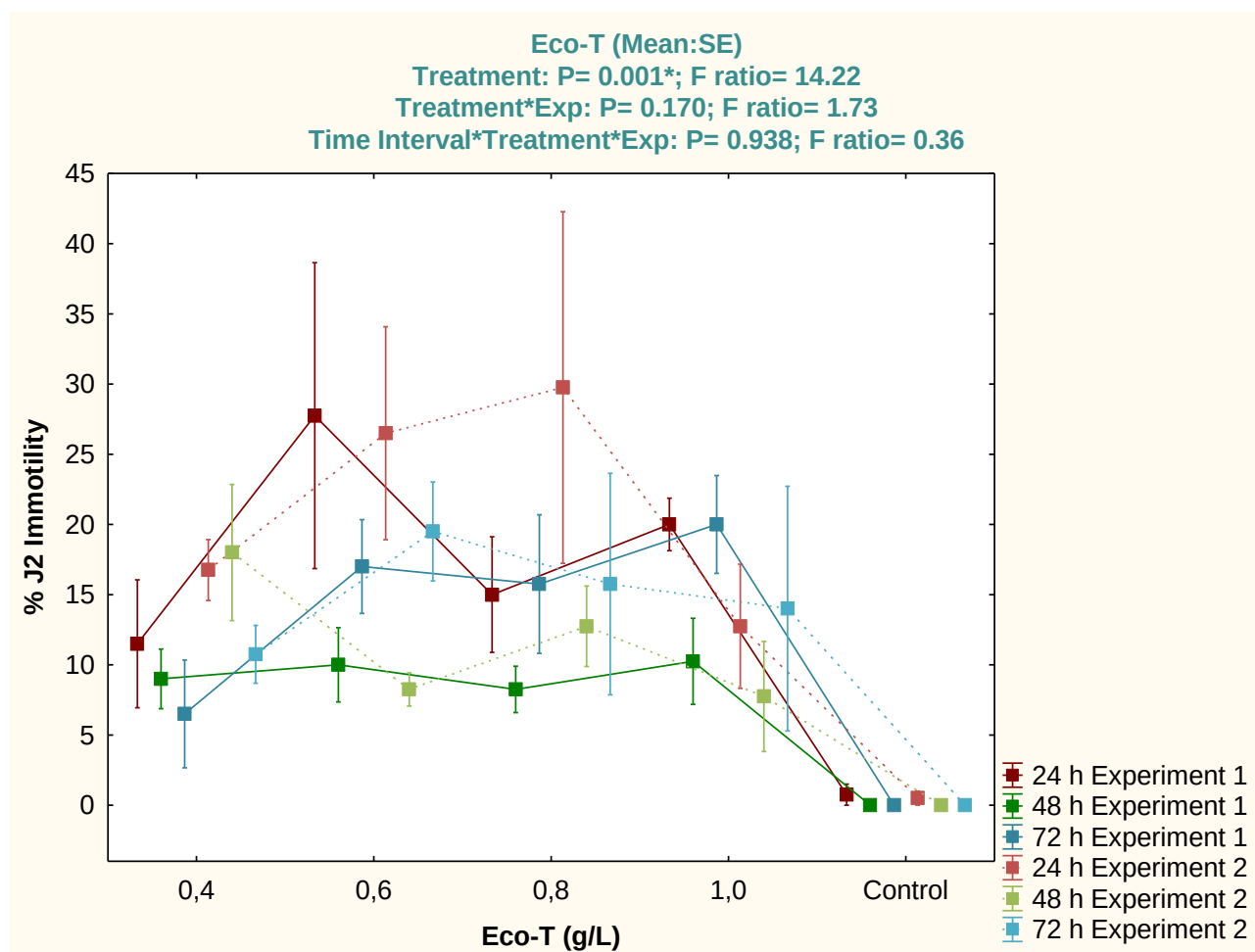


Figure 4-7: The effect of different concentrations (g/L) of Eco-T® (a.i. *Trichoderma asperellum* strain kd) on the motility of second-stage (J2) juveniles of *Meloidogyne incognita* (*in vitro* analysis).

Table 4-7: Data (%) of the effect of different concentrations of Eco-T® (a.i. *Trichoderma asperellum* strain kd) on second-stage (J2) juvenile motility of *Meloidogyne incognita* (in vitro study).

Treatment	Time interval	Experiment 1	Experiment 2
		% J2 Immotility	
Untreated Control (H ₂ O)	24 h	0,7 aA	0,5 aA
Untreated Control (H ₂ O)	48 h	0,0 aA	0,0 aA
Untreated Control (H ₂ O)	72 h	0,0 aA	0,0 aA
0.4 g/L dH ₂ O	24 h	11,5 abcAB	16,7 abAB
0.4 g/L dH ₂ O	48 h	9,0 abcAB	18,0 abAB
0.4 g/L dH ₂ O	72 h	6,5 abAB	10,7 abAB
0.6 g/L dH ₂ O	24 h	27,7 cB	26,5 bB
0.6 g/L dH ₂ O	48 h	10,0 abcAB	8,2 abAB
0.6 g/L dH ₂ O	72 h	17,0 abcAB	19,5 abAB
0.8 g/L dH ₂ O	24 h	15,0 abcAB	29,7 bB
0.8 g/L dH ₂ O	48 h	8,2 abAB	12,7 abAB
0.8 g/L dH ₂ O	72 h	15,7 abcAB	15,7 abAB
1 g/L dH ₂ O	24 h	20,0 bcAB	12,7 abAB
1 g/L dH ₂ O	48 h	10,2 abcAB	7,7 abAB
1 g/L dH ₂ O	72 h	20,0 bcAB	14,0 abAB
P value		0.001*	0.001*
F ratio		11.72	12.04
Interaction data: Treatment x Experiment			
P value		0.170	
F ratio		1.73	
Interaction data: Treatment x Time Interval x Experiment			
P value		0.938	
F ratio		0.36	

Means were separated with the Tukey HSD Test where $P \leq 0.05$, with significance indicated by *; different small letters for treatments and time intervals indicate significance for each experiment, while different capital letters for treatments and time intervals indicate significance among the two experiments.

4.4 Discussion

During this study the parameter used to evaluate the effect of the fungal strains and formulated products on the biology of *M. incognita* J2 was immotility, since it gave an indication of the effect of the active substances present in the spore concentrations used against this motile, vermiform life stage. This parameter has been used successfully for various studies of this nature using spores of fungal strains (Dababat & Sikora, 2007; Mascarín *et al.*, 2012; Hussain *et al.*, 2017;

Hemeda & El Deeb 2019) and bacteria (Kamran *et al.*, 2014; Jansen-Girgan *et al.*, 2016). It is also easy to see during microscopic analyses when a J2 is not moving (immotile) in the substrate concentrate. Mortality is another parameter that is used by authors, but although J2s are generally considered dead when they are not moving for a certain time, this is problematic since they might only be paralyzed. Since most of the authors reporting on such studies did not use a dedicated stain (Sharon *et al.*, 2007; Hussain *et al.*, 2017) to elucidate whether J2s were dead, it represents a challenge since data may not reflect the biological state of the J2s suspended in such products. Another parameter used is parasitism of J2 by the fungal spores, e.g., *Meloidogyne javanica* J2 as recorded by Sharon *et al.* (2007).

The *in vitro* reared fungal strains tested, generally showed high efficacy in terms of the ability of their spores to immotilize J2 of the South African *M. incognita* population used in this *in vitro*, well-plate study. Generally, the highest (10^9 spores/ml) and intermediate (10^8 spores/ml) concentrations of all the fungal strains showed >70% J2 immotility rates, with *C. rosea* generally being superior (100% J2 immotilized by the 10^9 spores/ml concentration), followed by *T. virens*, *T. harzianum*, *T. asperellum* and *T. asperellum* kd.

The data generated during this study is mostly in agreement with those reported from across the globe for the *in vitro* efficacy of fungal spores in terms of their adverse effect on J2 biology of *Meloidogyne* spp. (Woo *et al.*, 2014; Hussain *et al.*, 2017) and specifically for *M. incognita* (Mascarin *et al.*, 2012; Migunova *et al.*, 2018). The opposite was, however, observed for the *in vitro* efficacy of the two fungal-based products, namely CR and Eco-T® which generally resulted in J2 immotilization of <30%. In contrast, both Chinheya (2015) and Pambuka (2014) reported that the formulated products had an efficacy substantially higher than 30% (from 64% to 79%) when evaluated against *M. javanica*.

The efficacy of the *C. rosea* 10^9 spores/ml concentration in reducing *M. incognita* J2 by 100% in this study mirrors results by Migunova *et al.* (2018), that a 7×10^6 concentration of this fungal species adversely impacted on the J2 motility of a Russian (Moscow) *M. incognita* population, with an immotilization rate of 100%. Furthermore, Lafta & Kasim (2019) showed that hatching of J2 of *Meloidogyne* spp. was reduced by 59,73% during *in vitro* experiments, while Hussain *et al.* (2017) found values of 86,4% and 86% for egg parasitism and J2 mortality of *Meloidogyne hapla* Chitwood, 1949, respectively. These are the only three published sources that could be found indicating the efficacy of *C. rosea* in terms of its adverse effects on the biology of *Meloidogyne* spp. J2. Although J2 hatching and mortality were not investigated during the South African study, the adverse effect on J2 biology (measured as motility) was confirmed by these results implying that this fungal species could be further explored for its nematicide effects and potential

bionematicidal activity to this highly damaging plant-parasitic genus causing extensive damage to crops (Jones *et al.*, 2013).

Results obtained for the *T. asperellum* 10^9 spores/ml in immotilizing *M. incognita* J2s by 83,5% to 99,5% demonstrated the superior effect of the spore concentrate to adversely impact the biology of this nematode specie. Although no other reports could be found on the effect of *T. asperellum* on *M. incognita* J2 motility, data of this South African study substantiated those by Hemeda & El Deeb (2019) showing the adverse effect of this fungal strain, namely a reduction in J2 hatching of 88% for *M. incognita* and an average of 71,96% J2 mortality of *M. javanica* Treub, 1885 after 48 h exposure to an unknown concentration. Sharon *et al.* (2007) further demonstrated that 95,5% of eggs were parasitized *M. javanica* after 24 h exposure to a 10^5 fungal conidia/ml concentration of *T. asperellum* spores, while a 50,5% J2 mortality rate was recorded. No other published sources could be found regarding the efficacy of *T. asperellum* on *Meloidogyne* spp. J2. As in the case with *C. rosea*, this study confirms that this fungal species is worthy of further research for its nematocidal effect on *Meloidogyne* spp. and possible future bionematicide development.

The J2 immotility data obtained during this study for *T. asperellum* kd showed a slightly lower J2 immotility rate than *T. asperellum* towards *M. incognita*, namely 67,2% to 86,5% for the highest concentration (10^9 spores/ml). No published data is available for *T. asperellum* kd in terms of its effect on the biology of *M. incognita*, but since this strain is closely related to *T. asperellum*, similar conclusions may apply to the impact of *T. asperellum* kd, namely that the fungal species shows potential for being further used as a bionematicide.

By contrast to limited and fragmented reports on the effect of *C. rosea*, *T. asperellum* and *T. asperellum* kd on root-knot nematode J2 biology, ample research exists in this regard for about the *T. harzianum*. Results obtained during the present study showed that for the highest 10^9 spores/ml of *T. harzianum*, J2 immotility ranged between 61,7% to 91,7% agreeing with the adverse effect of this fungal strain on the biology of *M. incognita* when considering reports by various scientists. Migunova *et al.* (2018) showed 100% mortality rates for *M. javanica* and *M. incognita* J2 after 48 h exposure to a 3×10^5 spores/ml concentration; Naserinasab *et al.* (2011) reported that 84% of *M. javanica* eggs were infected 10 days after exposure to a range of concentrations (1:1, 1:10 and 1:100 V/V); Khan *et al.* (2018) demonstrated a 100% *M. incognita* J2 mortality rate and 78,5% J2 hatching inhibition after 4 days of suspension in a 10^5 CFU/ml concentration; Almeida *et al.* (2022) showed a *M. javanica* J2 mortality rate of up to 68,8% 24 h after suspension in a 5×10^7 conidia/ml concentration; Mascarin *et al.* (2012) reported a 64.05% *M. incognita* J2 mortality rate after 1 week suspension in a 1×10^8 conidia/ml concentration; and Jamshidnejad *et al.* (2013) demonstrated *M. javanica* J2 hatching inhibition of 52% and a J2 mortality rate of 63% after 96 h exposure in a 10^6 spores/ml concentration. Taking into

consideration the range of J2 immotility reported for the South African *T. harzianum* strain, results from this study also support results that *M. incognita* J2 hatching was inhibited by 77,8% and *M. javanica* J2 mortality by 58,21% (Hemeda & El Deeb, 2019); 52% J2 mortality and 94% J2 hatching inhibition rates for *M. javanica* (Dawar *et al.*, 2008); *M. javanica* J2 mortality and J2 hatching inhibition rates of 41% and 40%, respectively, and 40% *M. incognita* J2 mortality (Dababat & Sikora, 2007).

Results obtained during this study for *T. virens*, however, differed substantially from those published, namely the highest concentration (10^9 spores/ml) showed a range of 75,7% to 100% *M. incognita* J2 immotility which was higher than reported by Khan *et al.* (2018) for a 75% concentration reducing J2 hatching by 45%, while it showed a J2 mortality level of 39%.

Data obtained for the formulated products, namely CR (a.i. *C. rosea* strain PHP 1701) and Eco-T® (a.i. *T. asperellum* strain kd) respectively, during this study were disappointing in terms of their effects on *M. incognita* J2 immotility. For product Eco-T® J2 immotility ranged from 19,5% to 29,7%, while it was even lower for CR, ranging from 7.7% to 17%. The limited data reported for these two products in terms of their adverse effects on the biology of *Meloidogyne* spp. differ pronouncedly from those of this study. For example, Chinheya (2015) demonstrated a 64% J2 mortality for *M. javanica* (48 h of exposure at a 10^8 spores/g concentration) while Pambuka (2014) reported a J2 mortality rate of 69,3% for *M. javanica* (48 h of exposure at a 10^6 conidia/ml concentration) for the product Eco-T®. Pambuka (2014) also reported that the J2 mortality rate for *M. javanica* was 76% and 68% after 48 h and 24 h of exposure respectively in a 10^6 conidia/ml concentration.

It must be considered that the J2 mortality rates reported by authors for the *Clonostachys* and *Trichoderma* species and strains discussed above, except those results published by Hussain *et al.* (2017) and Sharon *et al.* (2007), are not necessarily supported by staining of individuals of this life stage to confirm that they were dead. Therefore, it is doubtful if these results are accurately reported; the J2 were possibly only immotilized.

4.5 Conclusion

Based on the results generated for the five *in vitro*-reared fungal strains analysed in this project, all of them (and especially the 10^8 and 10^9 spores/ml concentrations) showed potential for further exploitation based on their superior efficacy to immotilize J2s of *M. incognita*. The *in vitro* testing should be seen as the first step in research to test for potential bionematicides and contributes to science in the manner that it gives an initial indication of the mode of action of the fungal spore

suspensions that maybe involved in adversely impacting on J2 biology, rendering such life stages vulnerable to not being able to find and infect the roots of a host plant. Data obtained for the two formulated products were, however, in contrast to those of the *in vitro*-reared fungal strains itself with no explanation to be given at this stage for its poor performance towards immotilizing *M. incognita* J2s.

It is evident that additional ongoing *in vivo*-based research, preferably under prevailing environmental conditions, is necessary to determine the efficacy of these fungal strains. A proposition to improve future research on the fungal strains and products analysed in this project is to combine some of the most effective fungal species to optimize the J2 immotility rate of *Meloidogyne* spp. (specifically *M. incognita* and *M. javanica*). The successful development of these fungal strains and fungal products into biological control agents of *Meloidogyne* spp. are deemed to have a major impact on the agricultural industry, since the market is driven in this direction.

CHAPTER 5 RESULTS AND DISCUSSION – *IN VIVO* EXPERIMENTS

5.1 Introduction

The *in vivo* screening of potential fungal agents as alternative control strategies of plant-parasitic nematodes is important in the sense that it takes place in the soil, e.g., in pots in a glasshouse, in bigger microplots or in a field where prevailing environmental conditions exist. It is known that environmental conditions play a major role in the efficacy of such products (Kerry & Bourne, 2002; Fernandes *et al.*, 2015). Hence, the end goal of research on assessing the biocontrol potential of fungi on target plant-parasitic nematodes, such as *Meloidogyne* Goeldi, 1892, which is the predominant genus parasitizing grain crops in South Africa (Fourie *et al.*, 2015), should be to test it in the soil substrate and ultimately in field trials. *In vivo* testing of fungal agents enables researchers to assess their effect on the reproduction of *Meloidogyne* spp. on all the life stages of the genus (Pocurull *et al.*, 2020) since it affects the life cycle and not only certain life stages such as eggs or second-stage juveniles (J2) that are commonly used in initial *in vitro* experiments.

Despite increased efforts to exploit and test fungal species for their biocontrol effect on plant-parasitic nematodes such as *Meloidogyne* spp., only a limited number of this class of bionematicides are registered globally (Tranier *et al.*, 2014). A few that are registered and available to producers contains spores and secondary metabolites of *Paecilomyces lilacinus* (Mendoza *et al.*, 2007; Mendoza & Sikora, 2009; Crow, 2013), *Pochonia chlamydosporia* (Viggiano *et al.*, 2014; Machado, 2022), *Trichoderma* spp. or *Clonostachys* spp., accentuating the need for the development of more such products (Marrone, 2019; Lahli *et al.*, 2022). On top of this, the application of biological/microbial agents as seed-treatments on commercial scale is a great challenge for researchers, especially the consistency thereof in the field (O'Callaghan, 2016).

The aim of this study was therefore to analyse the *in vivo* effect of spores of five fungal strains, obtained from Andermatt-PHP (Pty) Ltd., South Africa (<http://andermatt-php.co.za>), on the reproduction of *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949 during 48-day glasshouse experiments by means of a seed treatment (ST) and a direct application in the soil (DA). The results presented in this chapter contain nematode (life stage numbers, gall rating data and egg mass numbers) and plant parameter data obtained for both the fungal spores reared from colonies and for the two formulated fungal products namely CR (active ingredient/a.i. *Clonostachys rosea* strain PHP 1701) and Eco-T® (a.i. *Trichoderma asperellum* strain kd) obtained from Andermatt-PHP (Pty) Ltd, South Africa. However, only Eco-T® is registered locally (Agri-Intel, 2021). For screening of the live fungal isolates as well as the formulated fungal

products, $\pm 1\ 000$ J2 were inoculated per 1- ℓ pot prior to application of the fungal products and the environmentally friendly nematicide standard Velum® 1 GR. See paragraph 3.4.1, Chapter 3 for the detailed methodology followed during the execution of these experiments.

5.2 Results: *In vivo* screening utilizing viable fungal spores from reared colonies

5.2.1 Nematode root data: 48 days after planting (DAP)

Graphs are provided only for those fungal strains that significantly reduced all the life stages (eggs, all juvenile stages, females, and males) of *M. incognita* in soybean roots when compared to the Untreated Control (representing sterilized, tap water). Data that showed no significance among treatments for *Meloidogyne* life stages is presented in ANNEXURE I. The data obtained hence implied that the reproduction of *M. incognita* was adversely affected when compared to the Untreated Control.

5.2.1.1 *Clonostachys rosea*

The different concentrations of *C. rosea* did not significantly reduce *M. incognita* life-stage numbers per 20 g roots when compared to the Untreated Control; the mean life-stage numbers for the different *C. rosea* concentrations and Untreated Control were in a relatively narrow numerical range (Table 5-1).

By contrast to the mean life stage numbers per 20 g roots, the 10^7 spores/ml (DA) of *C. rosea* significantly reduced *M. incognita* gall-formation per root system when compared to the Untreated Control, but not from the other concentrations (Fig 5-1; Table 5-1).

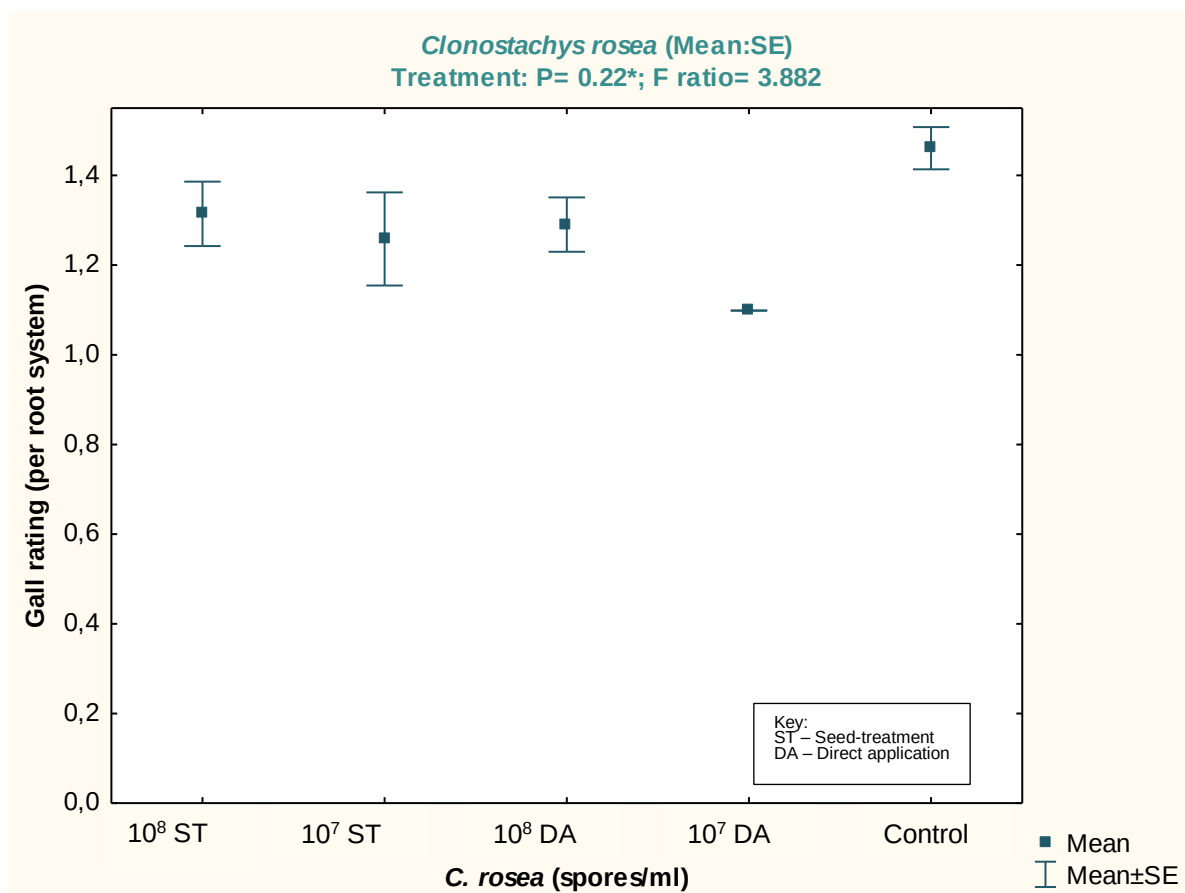


Figure 5-1: The effect of different concentrations (spores per ml) of *Clonostachys rosea* on the gall rating index under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

Table 5-1: The effect of different concentrations (spores per mL) of *Clonostachys rosea* on the reproduction of *Meloidogyne incognita*, referring to the number of all life stages per 20 g roots, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	Numbers of <i>M. incognita</i> life stages per 20 g roots	Gall rating index values / 20 g roots
10⁸ spores / mL (ST)	3,9 (384) a	1,3 (3) ab
10⁷ spores / mL (ST)	5,4 (570) a	1,3 (3) ab
10⁸ spores / mL (DA)	7,0 (1462) a	1,3 (3) ab
10⁷ spores / mL (DA)	5,1 (504) a	1,1 (2) a
Untreated Control	7,1 (1416) a	1,5 (3) b
P value	0.072	0.022
F ratio	2.573	3.882

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.2.1.2 *Trichoderma asperellum*

The 10⁷ spores / mL (DA) treatment for *T. asperellum* showed significantly higher *M. incognita* life stage numbers / 20 g roots compared to the other spore treatments, but not from the Untreated Control (Fig 5-2 a; Table 5-2). The 10⁸ spores / mL (ST) showed significantly lower gall-formation of *M. incognita* per root system when compared to the Untreated Control and the 10⁷ spores / mL (DA) treatment, but not when compared to the 10⁷ spores / mL (ST) and 10⁸ spores / mL (DA) treatments (Fig 5-2 b; Table 5-2).

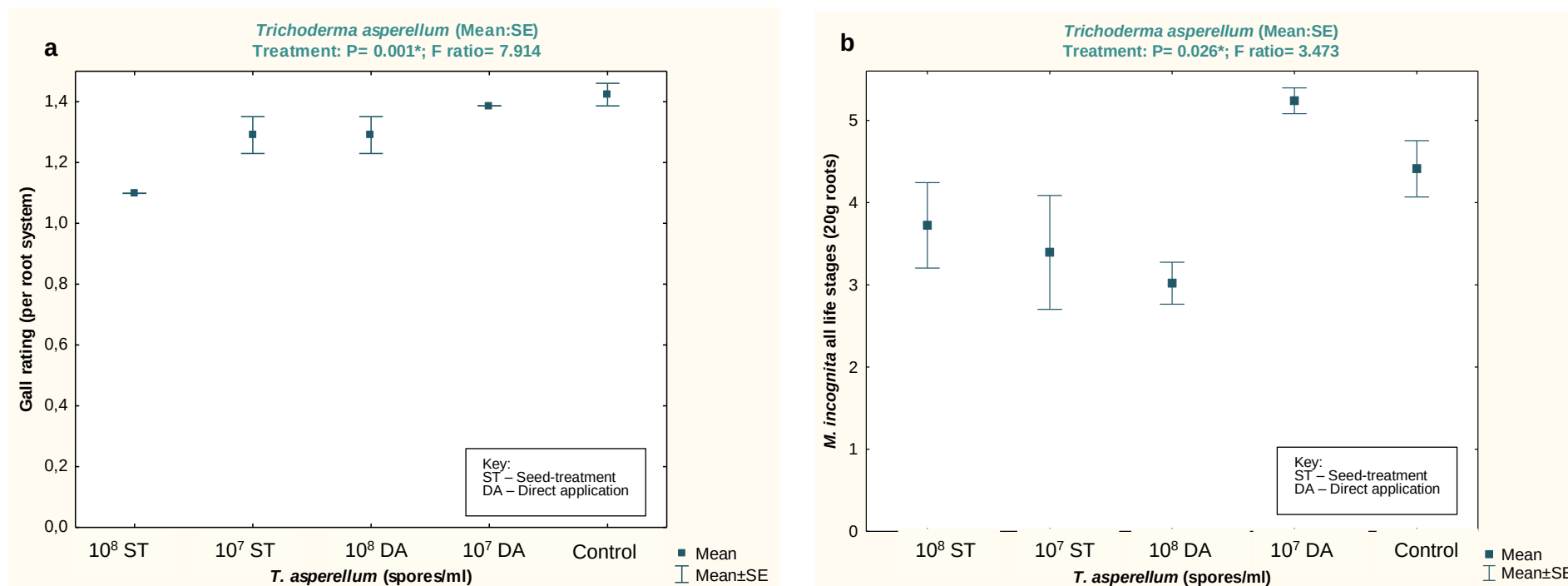


Figure 5-2: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 20 g roots) (a) and data on the gall rating index (b) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

Table 5-2: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* on the reproduction of *Meloidogyne incognita*, referring to the number of all life stages per 20 g roots, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	Numbers of <i>M. incognita</i> life stages per 20 g roots	Gall rating index values / 20 g roots
10⁸ spores / l (ST)	3,7 (75) ab	1,1 (2) b
10⁷ spores / ml (ST)	3,4 (51) ab	1,3 (3) a
10⁸ spores / ml (DA)	3,0 (23) a	1,3 (3) a
10⁷ spores / ml (DA)	5,2 (200) b	1,4 (3) a
Untreated Control	4,4 (103) ab	1,4 (3) a
P value	0.025	0.001*
F ratio	3.533	7.914

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.2.1.3 *Trichoderma asperellum* kd

The different concentrations of *T. asperellum* kd did not significantly reduce *M. incognita* life-stage numbers per 20 g roots when compared to the Untreated Control; the mean life-stage numbers of the different *T. asperellum* kd concentrations and the Untreated Control were in a relatively narrow numerical range (Table 5-3). Also, the different concentrations *T. asperellum* kd did not significantly lower gall-formation of *M. incognita* per root system when compared to the Untreated Control since it was in a relatively narrow numerical range (Table 5-3).

Table 5-3: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* kd on the reproduction of *Meloidogyne incognita*, referring to the number of all life stages per 20 g roots, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	Numbers of <i>M. incognita</i> life stages per 20 g roots	Gall rating index values / 20 g roots
10⁸ spores / ml (ST)	4,0 (112) a	1,5 (4) a
10⁷ spores / ml (ST)	4,6 (295) a	1,4 (3) a
10⁸ spores / ml (DA)	4,7 (165) a	1,5 (3) a
10⁷ spores / ml (DA)	4,7 (479) a	1,5 (4) a
Untreated Control	4,7 (132) a	1,5 (4) a
*P value	0.921	0.358
F ratio	0.225	1.167

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.2.1.4 *Trichoderma harzianum*

The 10⁸ spores / ml (ST & DA) treatments of *T. harzianum* showed significantly lower *M. incognita* life stage numbers / 20 g roots compared to the Untreated Control, but not from the other spore treatments (Fig 5-3; Table 5-4).

Also, the different concentrations of *T. harzianum* did not significantly lower gall-formation of *M. incognita* per root system when compared to the Untreated Control, with the values being in a relatively narrow numerical range (Table 5-4).

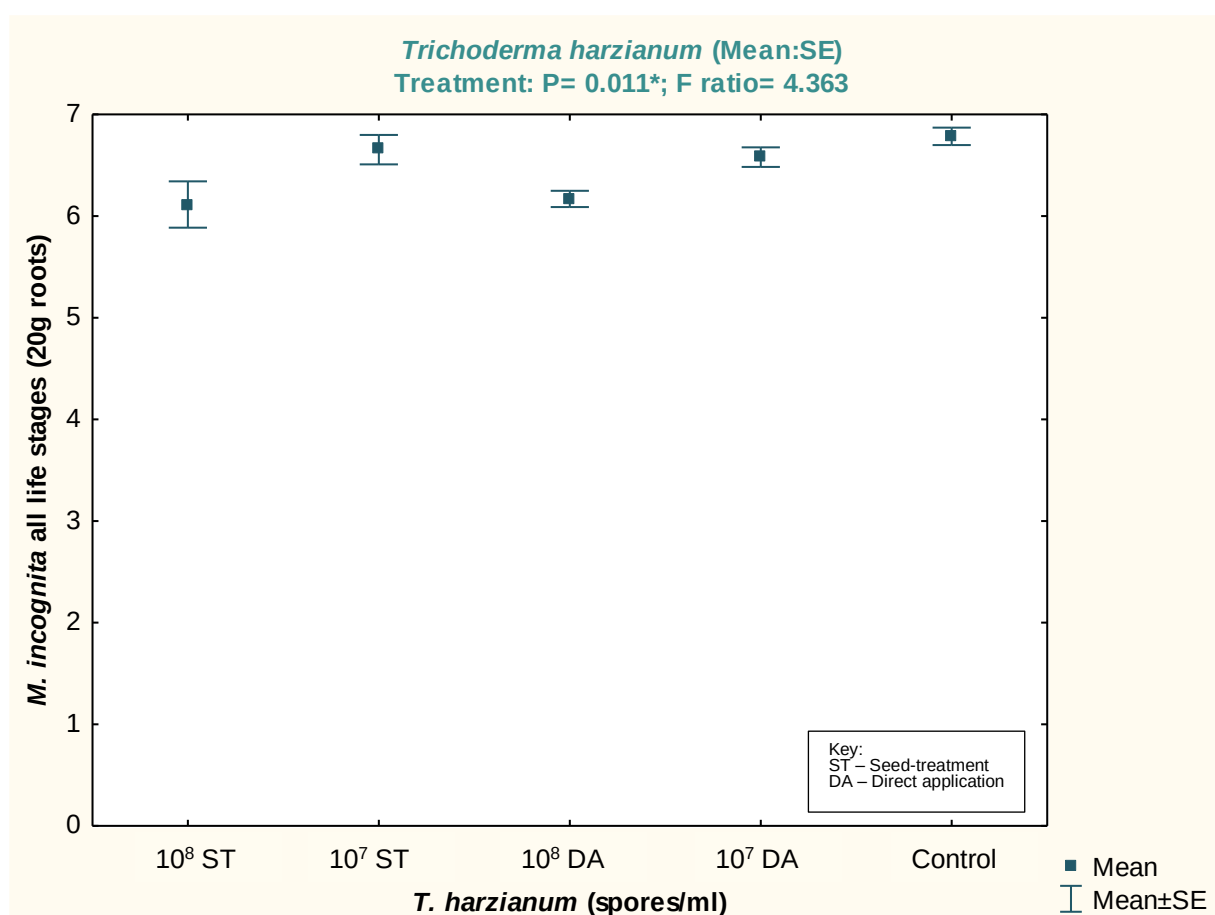


Figure 5-3: The effect of different concentrations (spores per ml) of *Trichoderma harzianum* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 20 g roots) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

Table 5-4: The effect of different concentrations (spores per mL) of *Trichoderma harzianum* on the reproduction of *Meloidogyne incognita*, referring to the number of all life stages per 20 g roots, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	Numbers of <i>M. incognita</i> life stages per 20 g roots	Gall rating index values / 20 g roots
10 ⁸ spores / mL (ST)	6,1 (510) a	1,1 (2) a
10 ⁷ spores / mL (ST)	6,7 (818) ab	1,2 (3) a
10 ⁸ spores / mL (DA)	6,2 (485) a	1,1 (2) a
10 ⁷ spores / mL (DA)	6,6 (737) ab	1,2 (3) a
Untreated Control	6,8 (899) b	1,3 (3) a
P value	0.011	0.192
F ratio	4.363	1.690

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.2.1.5 *Trichoderma virens*

The 10⁸ spores / mL (DA) treatment of *T. virens* showed significantly lower *M. incognita* life stage numbers / 20 g roots compared to the Untreated Control, but not from the other spore treatments (Table 5-5). The 10⁸ spores / mL (ST) and 10⁷ spores / mL (ST) treatments showed significantly lower gall-formation of *M. incognita* per root system when compared to the Untreated Control and the rest of the spore treatments (Fig 5-4; Table 5-5).

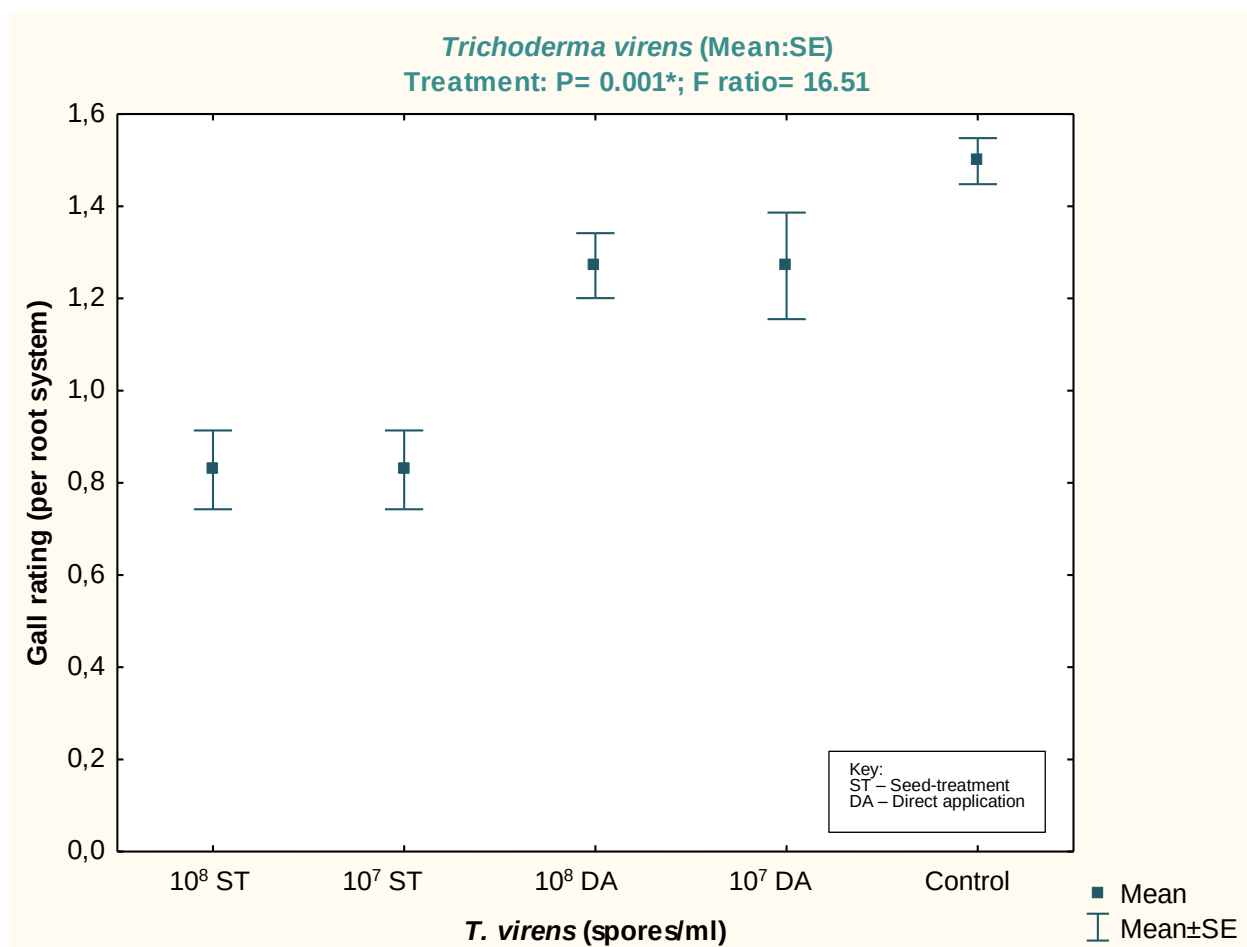


Figure 5-4: The effect of different concentrations (spores per ml) of *Trichoderma virens* on the gall rating index under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

Table 5-5: The effect of different concentrations (spores per mL) of *Trichoderma virens* on the reproduction of *Meloidogyne incognita*, referring to the number of all life stages per 20 g roots, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	Numbers of <i>M. incognita</i> life stages per 20 g roots	Gall rating index values / 20 g roots
10 ⁸ spores / mL (ST)	5,3 (211) ab	0,8 (1) b
10 ⁷ spores / mL (ST)	5,3 (232) ab	0,8 (1) b
10 ⁸ spores / mL (DA)	3,9 (98) a	1,3 (3) a
10 ⁷ spores / mL (DA)	5,7 (313) ab	1,3 (3) a
Untreated Control	6,0 (460) b	1,5 (4) a
P value	0.454	0.001
F ratio	3.779	16.51

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.2.2 Nematode soil data: 48 DAP

5.2.2.1 *Clonostachys rosea*

The different concentrations of *C. rosea* did not significantly reduce *M. incognita* life-stage numbers per 200 g soil when compared to the Untreated Control with the values being in a relatively narrow numerical range (Table 5-6).

5.2.2.2 *Trichoderma asperellum*

The different concentrations of *T. asperellum* did not significantly reduce *M. incognita* life-stage numbers per 200 g soil when compared to the Untreated Control with the values being in a relatively narrow numerical range (Table 5-6).

5.2.2.3 *Trichoderma asperellum* kd

The different concentrations of *T. asperellum* kd did not significantly reduce *M. incognita* life-stage numbers per 200 g soil when compared to the Untreated Control with the values being in a relatively narrow numerical range (Table 5-6).

5.2.2.4 *Trichoderma harzianum*

The 10^8 spores / ml (ST) concentration of *T. harzianum* showed significantly higher *M. incognita* life stage numbers / 200 g soil compared to the other spore treatments, but not from the 10^8 spores / ml (DA) treatment (Fig 5-5; Table 5-6).

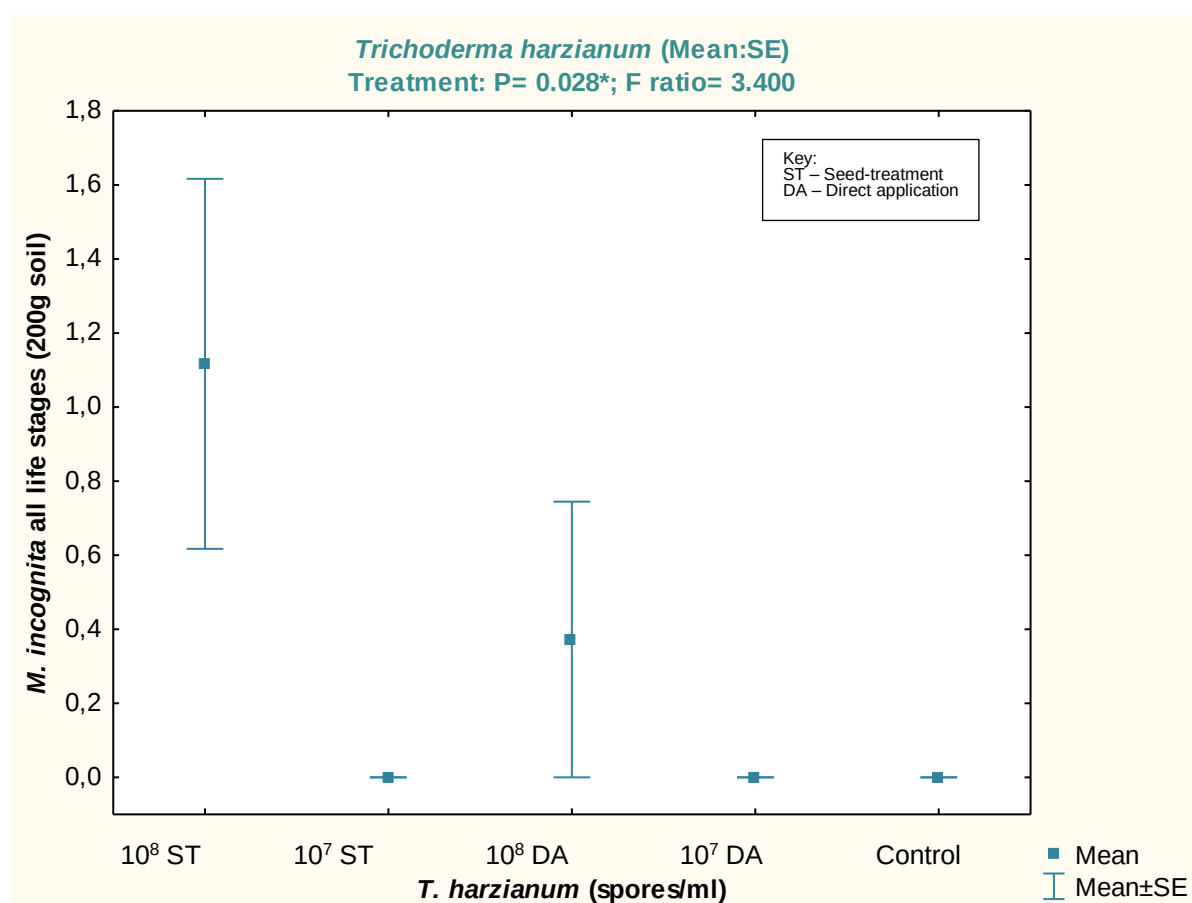


Figure 5-5: The effect of different concentrations (spores per ml) of *Trichoderma harzianum* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 200 g soil) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

5.2.2.5 *Trichoderma virens*

The different concentrations of *T. virens* did not significantly reduce *M. incognita* life-stage numbers per 200 g soil when compared to the Untreated Control with the values being in a relatively narrow numerical range (Table 5-6).

Table 5-6: The effect of different concentrations (spores per mL) of five living fungal isolates on the reproduction of *Meloidogyne incognita*, referring to number of all life stages per 200 g soil, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	All life stages: numbers per 200 g soil				
	<i>C. rosea</i>	<i>T. asperellum</i>	<i>T. asperellum kd</i>	<i>T. harzianum</i>	<i>T. virens</i>
10⁸ spores / mL (ST)	0,0 (0,0) a	1,6 (12,5) a	1,8 (11,1) a	1,1 (4,2) b	1,2 (5,6) a
10⁷ spores / mL (ST)	0,4 (1,4) a	1,8 (9,7) a	2,2 (12,5) a	0,0 (0,0) a	0,7 (2,8) a
10⁸ spores / mL (DA)	0,5 (2,8) a	1,3 (6,9) a	3,3 (33,3) a	0,4 (1,4) ab	0,9 (5,6) a
E10⁷ spores / mL (DA)	0,4 (1,4) a	1,1 (4,2) a	1,7 (15,3) a	0,0 (0,0) a	1,1 (4,2) a
Untreated Control	0,7 (8,3) a	2,4 (15,3) a	3,4 (33,3) a	0,0 (0,0) a	1,7 (11,1) a
P value	0.833	0.573	0.029*	0.028*	0.797
F ratio	0.362	0.745	3.377	3.400	0.413

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.2.3 Plant parameter data: Root mass 48 DAP

5.2.3.1 *Clonostachys rosea*

The different concentrations of *C. rosea* did not significantly reduce the root mass per root system when compared to the Untreated Control, with values being in a relatively narrow numerical range (Table 5-7).

5.2.3.2 *Trichoderma asperellum*

The 10^7 spores / ml (ST) concentration of *T. asperellum* significantly reduced the root mass per root system when compared to the other treatments and the Untreated Control (Fig 5-6; Table 5-7).

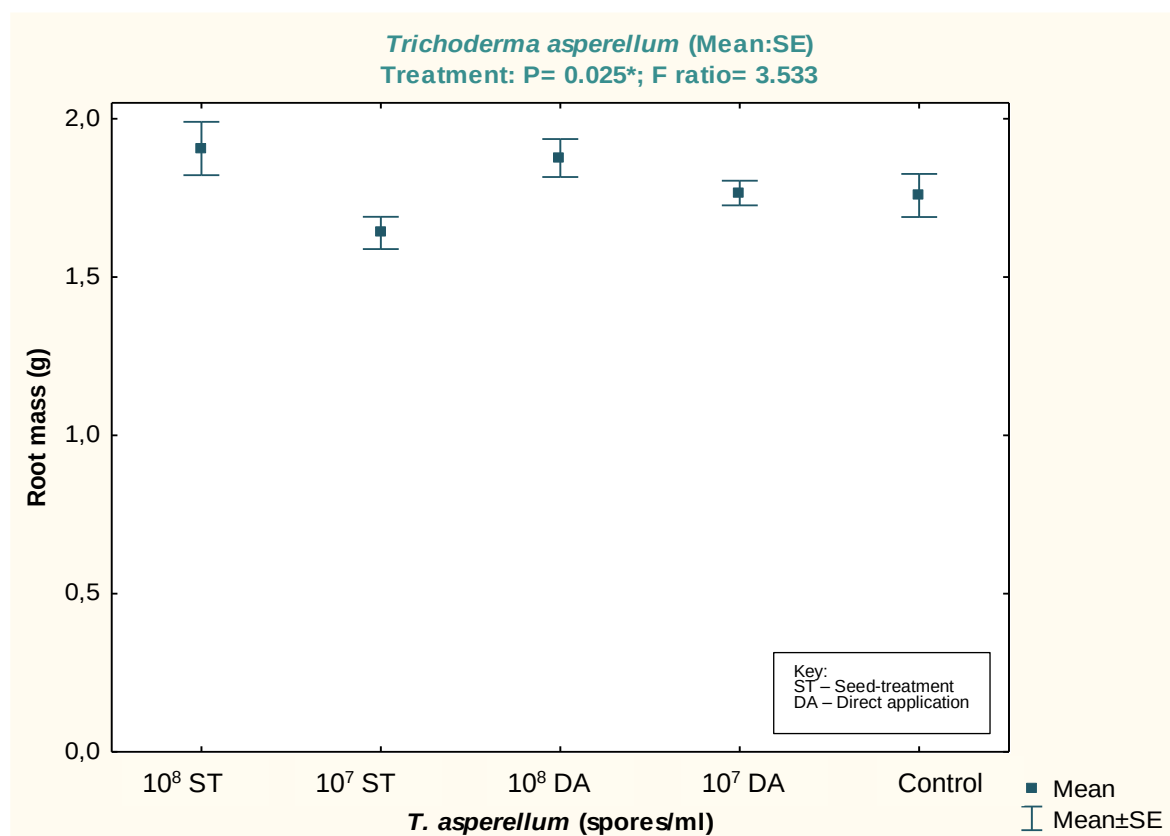


Figure 5-6: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* on the root mass under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

5.2.3.3 *Trichoderma asperellum* kd

The different concentrations of *T. asperellum* kd did not significantly reduce the root mass per root system when compared to the Untreated Control with the values being it was in a relatively narrow numerical range (Table 5-7).

5.2.3.4 *Trichoderma harzianum*

The different concentrations of *T. harzianum* did not significantly reduce the root mass per root system when compared to the Untreated Control with the values being it was in a relatively narrow numerical range (Table 5-7).

5.2.3.5 *Trichoderma virens*

The different concentrations of *T. virens* did not significantly reduce the root mass per root system when compared to the Untreated Control with the values being it was in a relatively narrow numerical range (Table 5-7).

Table 5-7: The effect of different concentrations (spores per mL) of five living fungal isolates on the reproduction of *Meloidogyne incognita*, referring to root mass per root system, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included as a standard.

Treatment name	Root mass (g)				
	<i>C. rosea</i>	<i>T. asperellum</i>	<i>T. asperellum kd</i>	<i>T. harzianum</i>	<i>T. virens</i>
10⁸ spores / mL (ST)	1,9 (6,1) a	1,9 (5,9) b	2,0 (6,1) a	2,0 (6,5) a	2,0 (6,5) a
10⁷ spores / mL (ST)	2,0 (6,8) a	1,6 (4,2) a	1,8 (4,9) a	1,9 (5,8) a	1,9 (5,8) a
10⁸ spores / mL (DA)	1,7 (4,4) a	1,9 (5,6) ab	1,8 (5,4) a	1,9 (5,6) a	1,9 (5,6) a
10⁷ spores / mL (DA)	1,7 (4,8) a	1,8 (4,9) ab	1,8 (5,5) a	1,8 (5,4) a	1,8 (5,4) a
Untreated Control	2,0 (6,3) a	1,8 (4,9) ab	1,9 (6,1) a	1,9 (6,1) a	1,9 (6,1) a
P value	0.284	0.025*	0.921	0.809	0.454
F ratio	1.383	3.533	0.225	0.397	0.956

Means were compared using the Tukey HSD test where $P \leq 0.05$ and indicated by * [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3 Results: *In vivo* screening utilizing formulated fungal-based products: Products CR (a.i. *C. rosea* strain PHP 1701) + Eco-T® (a.i. *T. asperellum* strain kd)

5.3.1 Nematode root data: 48 DAP

5.3.1.1 *Meloidogyne incognita* life stage numbers per 20g roots

A significant interaction was evident for Treatment x Experiment indicating that the treatments reacted differently for the two *in vivo* experiments (Fig 5-7; Table 5-8) namely the 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR and Velum® 1GR treatments for Experiment 1, and the 1,2 g/2 ℓ dH₂O CR treatment for Experiment 2.

For Experiment 1, the Velum® 1GR treatment had the lowest *M. incognita* life stage numbers / 20 g, not differing significantly from the 1,2 g/2 ℓ dH₂O Eco-T® and the Untreated Control but did differ significantly from the 1,2 g/2 ℓ dH₂O CR, 1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR and 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR (Fig 5-7; Table 5-8).

For Experiment 2, no significant differences were evident among the treatments, with their values being in a narrow numerical range (Fig 5-7; Table 5-8).

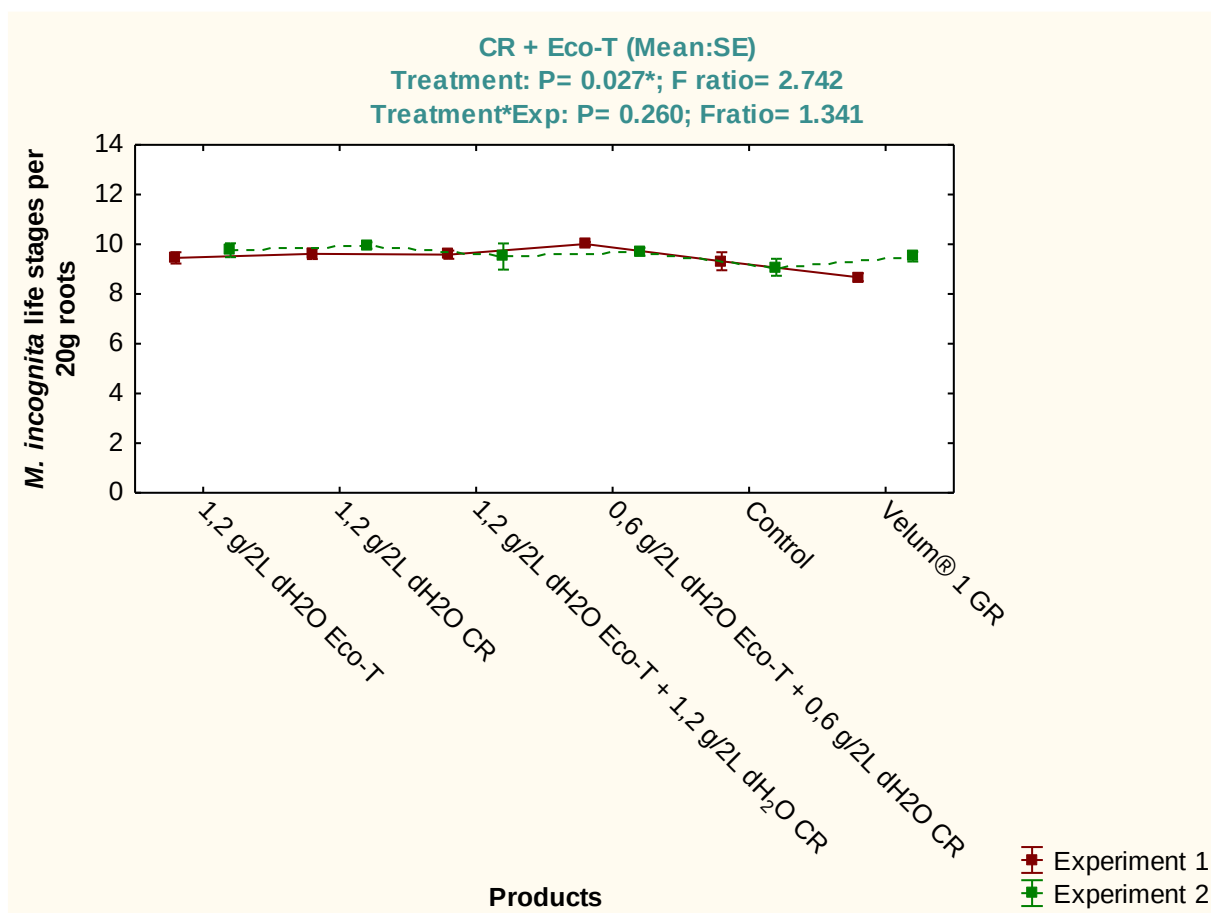


Figure 5-7: The effect of different concentrations (spores per ml) of products CR and Eco-T® on the reproduction of *Meloidogyne incognita* (referring to number of all life stages per 20 g roots) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Table 5-8: Data (20g roots) of the effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to number of life stages per 20 g roots, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Treatment name	All life stages: number per 20 g roots	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH₂O Eco-T®	9,5 (14 565) abAB	6,8 (20 212,8) aAB
1,2 g/2 ℓ dH₂O CR	9,6 (16 762) aAB	10,0 (21 885,4) aA
1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR	9,6 (15 433) aAB	9,5 (21 392,0) aAB
0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR	10,0 (22 715) aA	9,7 (17 316,5) aAB
Untreated Control	9,3 (13 901) abAB	9,1 (11 235,2) aAB
Velum® 1GR	8,7 (6 219) bB	9,5 (14 778,6) aAB
P value	0.002*	0.425
F ratio	5.141	1.024
Interaction data: Treatment x Experiment		
P value	0.031*	
F ratio	2.792	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3.1.2 *Meloidogyne incognita* gall rating / root system

A significant interaction was evident for Treatment x Experiment indicating that the treatments reacted different for the two *in vivo* experiments (Fig 5-8; Table 5-9) namely the 1,2 g/2 ℓ dH₂O CR, 1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR, 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR and the Velum® 1GR treatments for Experiment 1 and 2.

For Experiment 1, the Velum® 1GR and 1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR treatments had the lowest gall ratings which were significantly lower than those of the 1,2 g/2 ℓ dH₂O Eco-T®, 1,2 g/2 ℓ dH₂O CR, 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatments and the Untreated Control (Fig 5-8; Table 5-9).

For Experiment 2, the Velum® 1GR, 1,2 g/2 ℓ dH₂O Eco-T® and 1,2 g/2 ℓ dH₂O CR treatments had the lowest gall rating compared to those of the Untreated Control) which differed significantly from those of the Untreated Control and the 1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR and 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR, but not from the 1,2 g/2 ℓ dH₂O CR (Fig 5-8; Table 5-9).

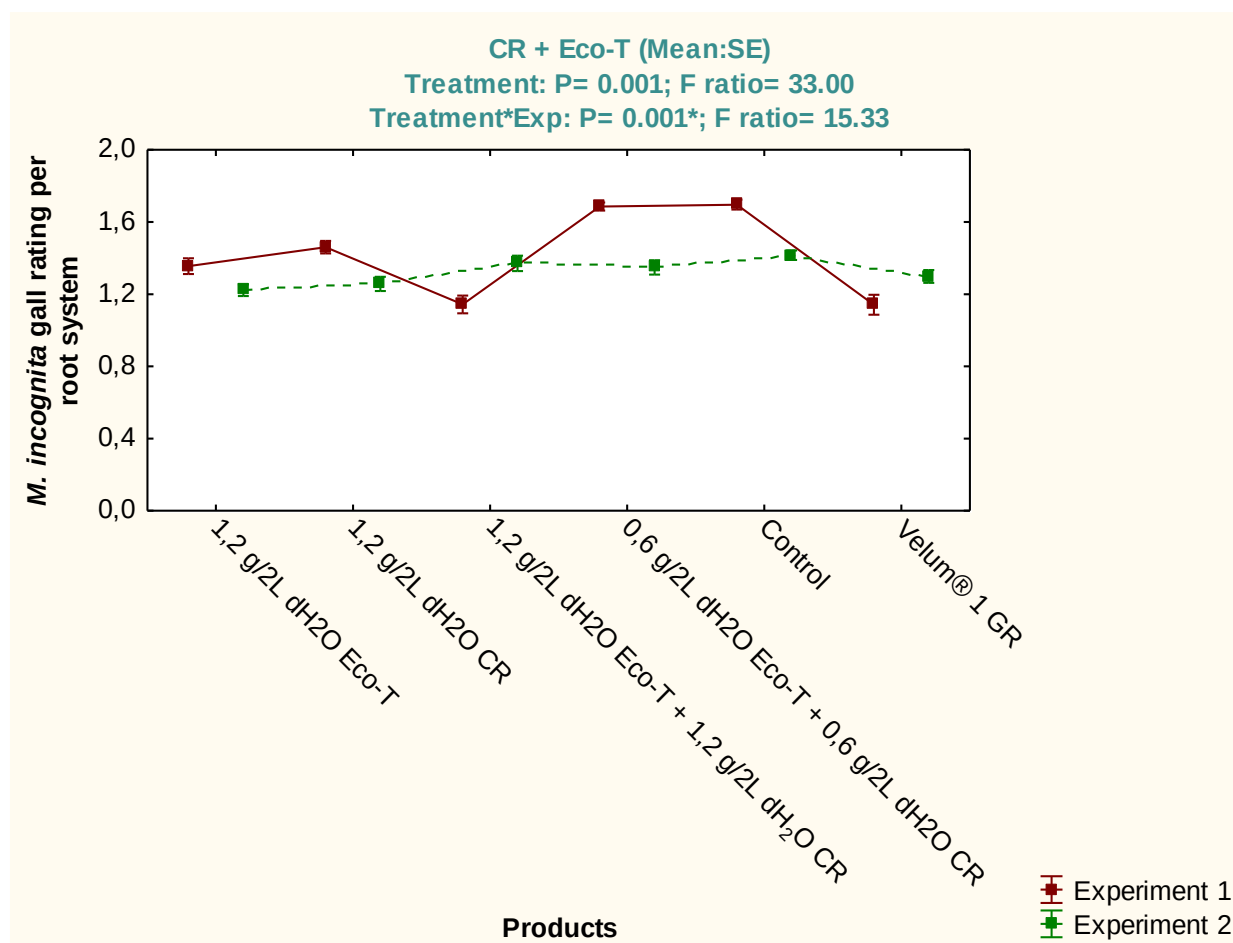


Figure 5-8: The effect of different concentrations (spores per ml) of products CR and Eco-T® on the reproduction of *Meloidogyne incognita* (referring to gall rating per plant root system) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Table 5-9: The effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to gall rating per plant root system, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Treatment name	Gall rating	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH ₂ O Eco-T®	1,4 (3,0) bBCDE	1,2 (2,4) aABC
1,2 g/2 ℓ dH ₂ O CR	1,5 (3,4) bE	1,3 (2,7) abABCD
1,2 g/2 ℓ dH ₂ O Eco-T® + 1,2 g/2 ℓ dH ₂ O CR	1,1 (2,2) aA	1,4 (3,1) bcCDE
0,6 g/2 ℓ dH ₂ O Eco-T® + 0,6 g/2 ℓ dH ₂ O CR	1,7 (4,4) cF	1,4 (2,9) bcBCDE
Untreated Control	1,7 (4,5) cF	1,4 (3,2) cDE
Velum® 1GR	1,1 (2,3) aA	1,2 (2,4) aAB
P value	0.001*	0.001*
F ratio	37.49	7.408
Interaction data: Treatment x Experiment		
P value	0.001*	
F ratio	33.00	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3.1.3 *Meloidogyne incognita* egg mass numbers / root system

A significant interaction was evident for Treatment x Experiment indicating that the treatments reacted different for the two *in vivo* experiments (Fig 5-9; Table 5-10) namely the 1,2 g/2 ℓ dH₂O CR and 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatments for Experiment 1 and 2.

For Experiment 1, Velum® 1GR had the lowest number of egg masses being significantly lower than the 1,2 g/2 ℓ dH₂O Eco-T®, 1,2 g/2 ℓ dH₂O CR, 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatments and the Untreated Control (Fig 5-9; Table 5-10).

For Experiment 2, none of the treatments differed significantly from one another and not from the Untreated Control (Fig 5-9; Table 5-10).

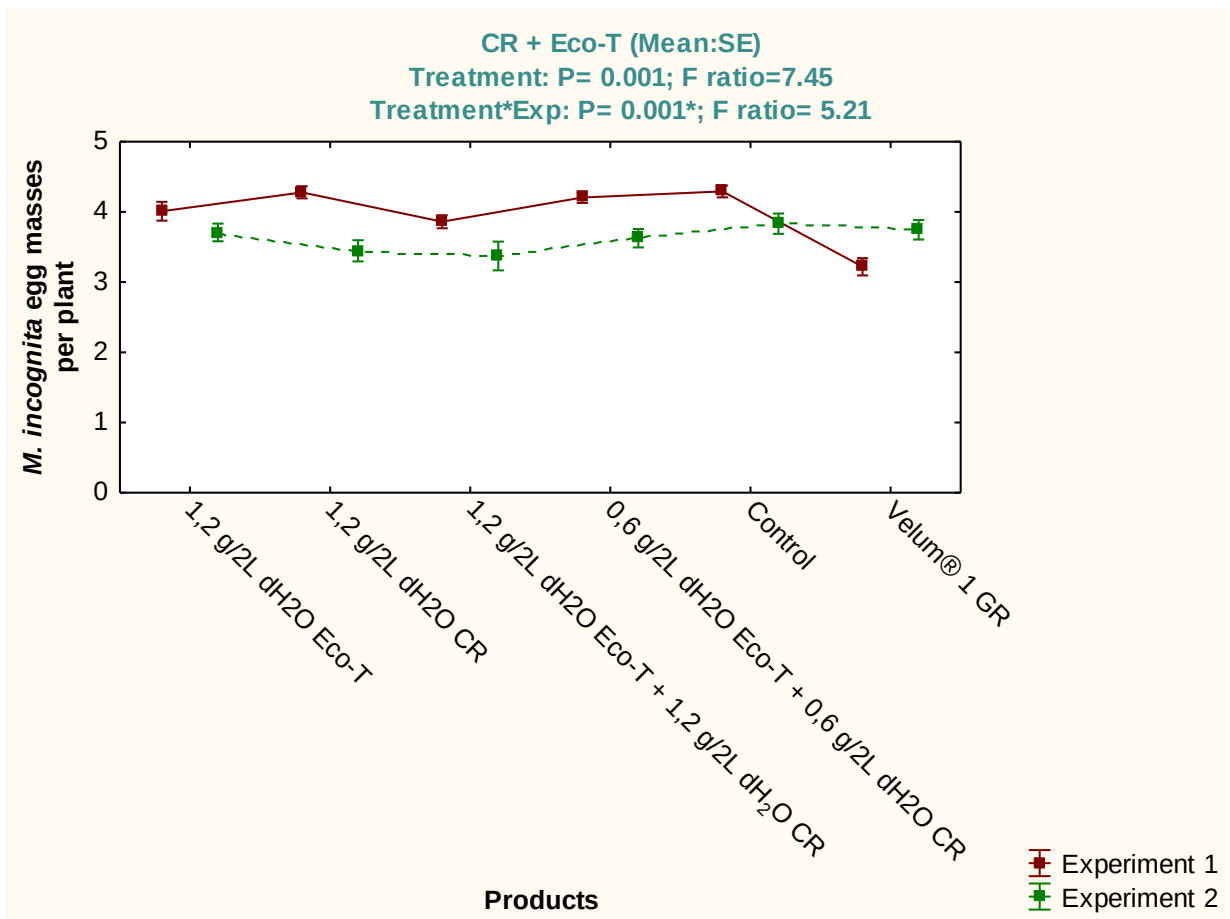


Figure 5-9: The effect of different concentrations (spores per ml) of products CR and Eco-T® on the reproduction of *Meloidogyne incognita* (referring to egg masses per plant root system) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Table 5-20: The effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to egg masses per plant root system, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Treatment name	Number of egg masses: per root system	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH ₂ O Eco-T®	4,0 (63) abBDE	3,5 (39) aABC
1,2 g/2 ℓ dH ₂ O CR	4,3 (78) aE	3,7 (47) aABCD
1,2 g/2 ℓ dH ₂ O Eco-T® + 1,2 g/2 ℓ dH ₂ O CR	3,9 (53) bABDE	3,3 (42) aAC
0,6 g/2 ℓ dH ₂ O Eco-T® + 0,6 g/2 ℓ dH ₂ O CR	4,2 (71) abDE	3,6 (40) aABC
Untreated Control	4,3 (78) aE	3,9 (57) aABDE
Velum® 1GR	3,2 (30) cC	3,6 (49) aABCD
P value	0.001*	0.101
F ratio	18.03	1.878
Interaction data: Treatment x Experiment		
P value	0.001*	
F ratio	7.45	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3.2 Nematode data: soil 48 DAP

No significant interaction was evident for Treatment x Experiment indicating that the treatments did not react differently for the two *in vivo* experiments (Table 5-11). For both Experiment 1 and Experiment 2, none of the treatments differed significantly compared to the Untreated Control.

Table 5-31: The effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to number of life stages per 200 g soil, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Treatment name	All life stages: number per 200 g soil	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH ₂ O Eco-T®	4,6 (138) aA	5,1 (276) aA
1,2 g/2 ℓ dH ₂ O CR	5,3 (268) aA	5,2 (246) aA
1,2 g/2 ℓ dH ₂ O Eco-T® + 1,2 g/2 ℓ dH ₂ O CR	6,0 (444) aA	5,0 (219) aA
0,6 g/2 ℓ dH ₂ O Eco-T® + 0,6 g/2 ℓ dH ₂ O CR	5,4 (275) aA	6,0 (550) aA
Untreated Control	4,8 (272) aA	4,9 (179) aA
Velum® 1GR	5,1 (176) aA	5,1 (189) aA
P value	0.329	0.210
F ratio	1.221	1.552
Interaction data: Treatment x Experiment		
P value	0.512	
F ratio	0.863	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3.3 Plant parameter data

5.3.3.1 Root mass (g) 48 DAP

No significant interaction was evident for Treatment x Experiment 48 DAP indicating that the treatments did not react differently for the two *in vivo* experiments (Table 5-12). For both Experiments 1 & 2, no significant differences were evident among the treatments regarding root mass (g) (Table 5-12).

Table 5-42: Root mass data (g) indicating the effect of different concentrations of products CR and Eco-T® under *in vivo*, glasshouse conditions 48 days after planting and inoculation with fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Treatment name	Root mass (g)	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH₂O Eco-T®	1,5 (4,3) abAB	1,6 (4,4) aAB
1,2 g/2 ℓ dH₂O CR	1,7 (4,9) abAB	1,7 (4,8) aA
1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR	1,5 (4,2) abAB	1,7 (4,8) aA
0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR	1,3 (3,0) aB	1,6 (4,4) aAB
Untreated Control	1,6 (4,6) abAB	1,7 (4,8) aA
Velum® 1GR	1,5 (3,5) abAB	1,6 (4,5) aAB
P value	0.077	0.824
F ratio	2.032	0.434
Interaction data: Treatment x Experiment		
P value	0.094	
F ratio	1.902	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3.3.2 Plant height (cm) 48 DAP

A significant interaction was evident for Treatment x Experiment 48 DAP indicating that the treatments reacted different for the two *in vivo* experiments (Fig 5-10; Table 5-13). This was due to treatments 1,2 g/2 ℓ dH₂O CR, 1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR and 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR for Experiment 1 and 2.

For Experiment 1, the 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatment resulted in plants that were significantly lower compared to those of the Untreated Control, the other product treatments and the standard nematicide Velum® 1GR (Fig 5-10; Table 5-13). Plants that received the Velum® 1GR treatment did, however, not differ significantly in terms of their heights from those of the Untreated Control.

For Experiment 2, the Untreated Control's plants were significantly taller than those of the 1,2 g/2 ℓ dH₂O Eco-T® treatment, but these two treatments did not differ from the other product treatments or the standard nematicide treatment Velum® 1GR (Fig 5-10; Table 5-13).

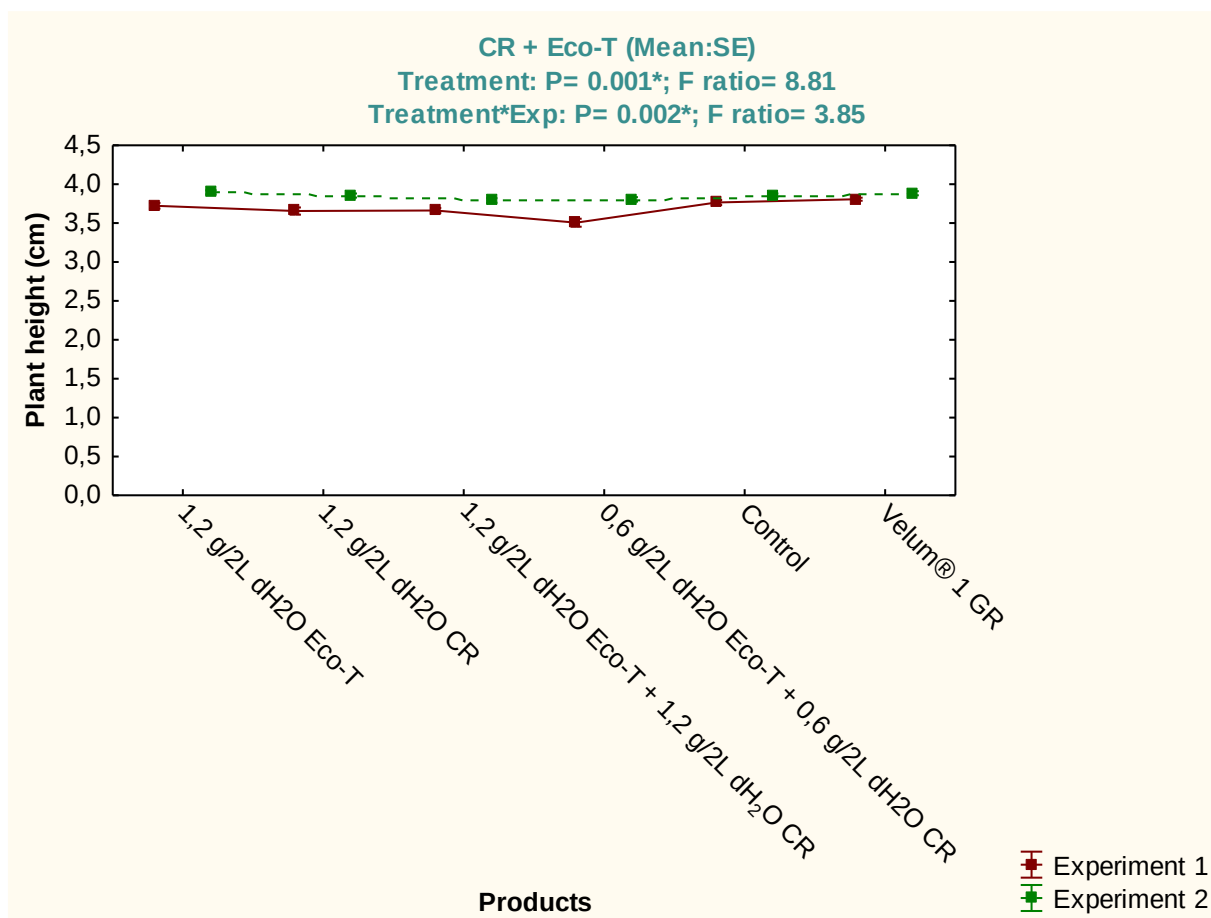


Figure 5-20: The effect of different concentrations (spores per ml) of products CR and Eco-T® on the reproduction of *Meloidogyne incognita* (referring to plant height) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Table 5-53: The effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to plant height, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

	Plant height	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH ₂ O Eco-T®	3,7 (41,0) abACD	3,9 (47,8) abAB
1,2 g/2 ℓ dH ₂ O CR	3,7 (38,7) aC	3,8 (45,6) abAB
1,2 g/2 ℓ dH ₂ O Eco-T® + 1,2 g/2 ℓ dH ₂ O CR	3,7 (38,5) aCD	3,8 (45,0) abAB
0,6 g/2 ℓ dH ₂ O Eco-T® + 0,6 g/2 ℓ dH ₂ O CR	3,5 (33,5) cE	3,8 (43,9) aABCD
Untreated Control	3,8 (42,7) abABCD	3,9 (48,5) bB
Velum® 1GR	3,8 (44,3) bABD	3,8 (45,2) abAB
P value	0.001*	0.025*
F ratio	9.404	2.644
Interaction data: Treatment x Experiment		
P value	0.001*	
F ratio	8.81	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.3.3.3 Aerial fresh plant mass (g) 48 DAP

A significant interaction was evident for Treatment x Experiment 48 DAP indicating that the treatments reacted different for the two *in vivo* experiments (Fig 5-11; Table 5-14) namely the 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatment and Untreated Control for Experiment 1, and the Velum® 1GR treatment for Experiment 2. For Experiment 1, the Untreated Control had a significantly higher plant mass than the 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatment, while it did not differ from the other product treatments and the standard nematicide Velum® 1GR treatment (Fig 5-11; Table 5-14).

For Experiment 2, the Velum® 1GR treatment showed a significantly lower plant mass than the fungal product treatments and the Untreated Control (Figure 5-11; Table 5-14). Plants treated with the 0,6 g/2 ℓ dH₂O Eco-T® + 0,6 g/2 ℓ dH₂O CR treatment had a significantly lower plant mass compared to the 1,2 g/2 ℓ dH₂O Eco-T® + 1,2 g/2 ℓ dH₂O CR treatment but did not differ significantly from the other fungal product treatments and the Untreated Control (Fig 5-11; Table 5-14).

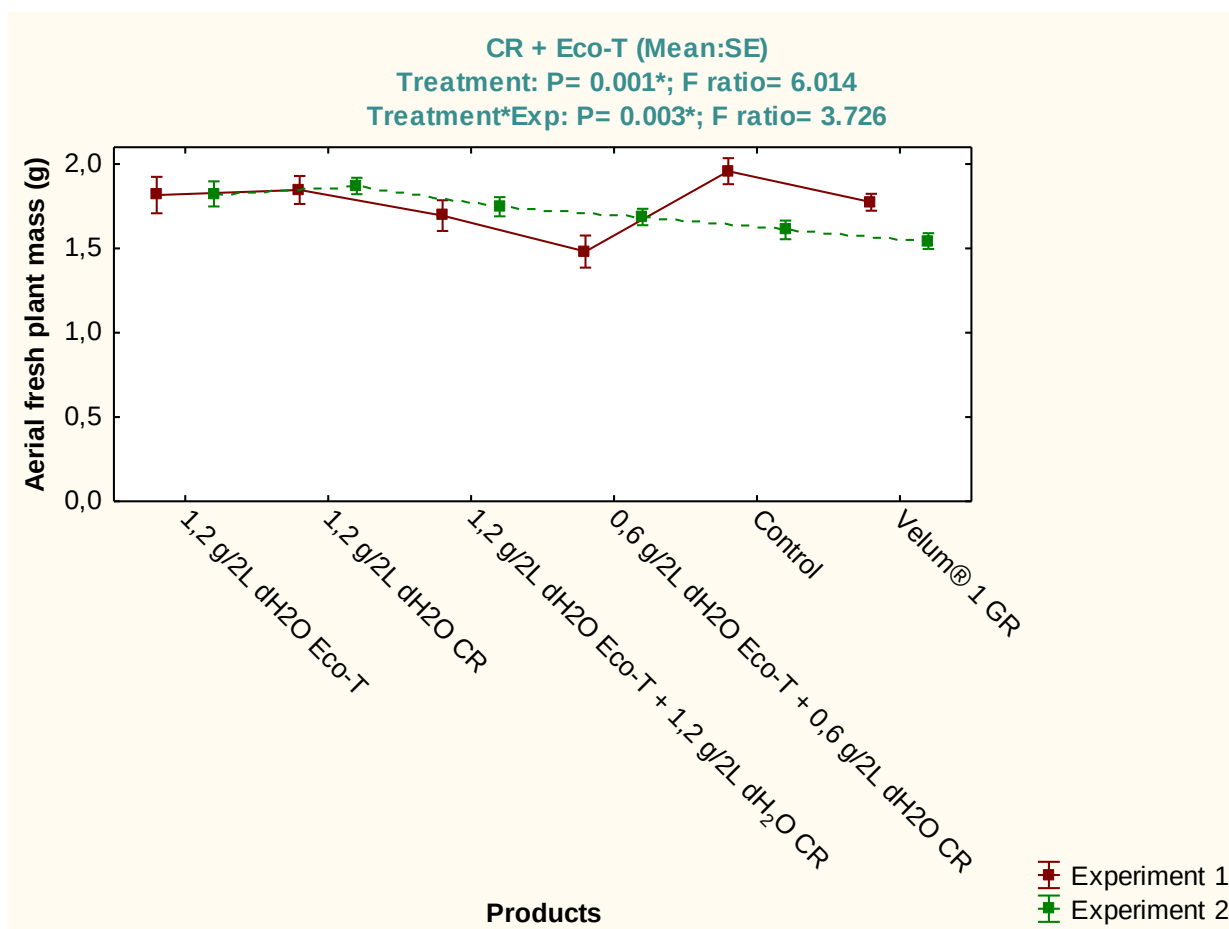


Figure 5-31: The effect of different concentrations (spores per ml) of products CR and Eco-T® on the reproduction of *Meloidogyne incognita* (referring to plant mass) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Table 5-64: The effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to plant mass, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

Treatment name	Plant mass	
	Experiment 1	Experiment 2
1,2 g/2 ℓ dH ₂ O Eco-T®	1,8 (6,0) abABC	1,8 (5,6) abAB
1,2 g/2 ℓ dH ₂ O CR	1,8 (5,9) aAB	1,9 (5,6) abAB
1,2 g/2 ℓ dH ₂ O Eco-T® + 1,2 g/2 ℓ dH ₂ O CR	1,7 (5,1) abABC	1,7 (5,0) aABC
0,6 g/2 ℓ dH ₂ O Eco-T® + 0,6 g/2 ℓ dH ₂ O CR	1,5 (4,1) bC	1,6 (4,2) bAC
Untreated Control	2,0 (6,7) aB	1,7 (4,4) abABC
Velum® 1GR	1,8 (5,1) abABC	1,5 (3,6) cC
P value	0.004*	0.001*
F ratio	3.666	7.517
Interaction data: Treatment x Experiment		
P value	0.001*	
F ratio	6.014	

Means were separated with the Tukey HSD Test significance ($P \leq 0.05$) indicated by *; different small letters for treatments indicate significance among treatments for each experiment (per column), while different capital letters for treatments indicate significance among the treatments of the two experiments (per row). [Factorial Analyses of Variance (ANOVA) using TIBCO Statistica™ Version 14.0.0.15 Software Package (<https://www.statistica.com>)].

5.4 Discussion

During this glasshouse study, various nematode parameters for soil (*M. incognita* eggs and J2 per 200 g soil) and soybean (*Glycine max* L. Merr.) roots (e.g., *M. incognita* life stage numbers per 20 g roots, root gallings per root system, egg mass counts per root system) as well as plant parameters (e.g., root mass, plant height and aerial plant mass) were recorded for *M. incognita* utilising both living fungal isolates and formulated products during a period of 48 days. Most of these parameters have been successfully used in various studies for various crops to determine the efficacy of fungal spores on the reproduction of *Meloidogyne* spp. (Al-Hazmi & TariqJaveed, 2016; El-Nagdi *et al.*, 2019; Cristóbal-Alejo *et al.*, 2021).

The *in vivo* soybean root data showed lower success rates when compared to the *in vitro* results (see paragraph 4.4; Chapter 4) for both the fungal spores reared from communities and the two formulated products in terms of their negative affect on the biology of *M. incognita*. Although reductions in life-stage numbers of *M. incognita* in roots were evident for *in vivo* experiments for some of the strains (*C. rosea*, *T. asperellum*, *T. harzianum* & *T. virens*) reared *in vivo* as well as

for some of the product treatments, it was not necessarily significant. Soil data on the other hand showed no reduction of eggs and J2 of *M. incognita* due to use of the fungal spores of the five strains nor for the two product treatments.

Although spores of the living fungal isolate of *Clonostachys rosea* resulted in a significant reduction in *M. incognita* root-gall formation, it was not evident for life-stage numbers per 20 g root although the 10^8 spores/ml (ST) reduced life-stage numbers by 45% when compared to the Untreated Control. The highest reduction of root galls for *C. rosea* of 26% reported for the 10^7 spores/ml (DA) treatment in this study was substantially lower than the 74% reduction in tomato (*Solanum lycopersicum* L.) root-gall formation of *M. incognita* using $9,5 \times 10^7$ conidia/ml (Cristóbal-Alejo *et al.*, 2021). Similarly, it disagreed with the 82,5% reduction for *Meloidogyne hapla* Chitwood, 1949 root galls (Hussain *et al.*, 2017) on carrot (*Daucus carota* L.). *Meloidogyne incognita* life-stage data per 20 g roots was reduced by 45% and found to be substantially lower than the 73% reduction in *M. incognita* egg numbers in tomato roots reported by Cristóbal-Alejo *et al.* (2021) applying $9,5 \times 10^7$ conidia/ml and that by Hussain *et al.* (2017) demonstrating a reduction of 86,9% for *M. hapla* using 20 ml of a 30% (W/V) solution on carrot. Fresh root mass for this fungal strain was inconclusive opposed to results for *M. hapla* obtained by Hussain *et al.* (2017), showing a 35% increase in carrot root mass using 20 ml of a 30% (W/V). The literature referred to above are the only published sources that could be found indicating the efficacy of *C. rosea* on the reproduction of *Meloidogyne* spp. in crop roots.

Application of spores of the fungal isolate *Trichoderma asperellum* resulted in a significant reduction in both *M. incognita* root gall-formation and life-stage numbers per 20 g roots. The 32% reduction in life-stage numbers is in the same low efficacy range for this strain as reported by Affokpon *et al.* (2011), showing a 13% reduction in juveniles/5 g tomato root using 10^6 spores/ml soil. However, results of this study disagreed with most other reports that showed relatively high efficacy rates for *T. asperellum*: a 98% reduction in *M. incognita* population densities using a $1,9 \times 10^6$ CFU/ml of *T. asperellum* per tomato plant (resistant cultivar) (Pocurull *et al.*, 2020); a 91% reduction in *M. incognita* tomato root galls with 10^8 spores g/soil (Abdel-lateif & Bakr, 2018); 86% and 45% reductions in *M. incognita* J2 and eggs respectively in tomato roots using 10^6 spores/ml soil (Affokpon *et al.*, 2011); an 86% reduction in *M. incognita* female numbers in tomato applying 10^8 spores g/soil (Abdel-lateif & Bakr, 2018); and a 55% reduction in *M. incognita* tomato root gall-formation when applying 2×10^6 CFU/ml concentration (Kumari *et al.*, 2020). Almeida *et al.* (2022), however, showed no reduction in *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 egg and J2 numbers using 5×10^7 conidia/ml on banana (*Musa*, L.). The lack of significant data for the reduction of *M. incognita* life-stages in soil also disagreed with those by: Affokpon *et al.* (2011) that showed a 94% decrease in J2 soil densities using a 10^6 spores/ml concentration;

Abdel-lateif & Bakr (2018) who reported an 86% reduction of *M. incognita* J2 densities using a 10^8 spores/gram soil concentration and Kumari *et al.* (2020) that showed a 46% reduction in J2 densities using a concentration of 2×10^6 CFU/ml. The low 5% increase in soybean root mass in this study were also in conflict with the 27%, 56% and 59% increase in tomato root mass reported by Affokpon *et al.* (2011), Abdel-lateif & Bakr (2018) Kumari *et al.* (2020), respectively. The closely related *Trichoderma asperellum* kd strain showed no significant reduction in neither *M. incognita* root gall-formation nor life-stage numbers per 20 g roots in this study and since no published data is available for this strain in this regard, these results warrant no further discussion. Nonetheless, the data generated during this study for *T. asperillum* kd add valuable information to non-existent data.

Results obtained for the living fungal isolate *Trichoderma harzianum* showed significant, but low reductions of 10% and 9%, respectively, in *M. incognita* life-stage numbers per 20 g roots for the 10^8 spores/ml ST and DA treatments. These results were most probably compromised due to high variation of data among the replicates and disagreed with those by various authors who found the efficacy of *T. harzianum* being substantially higher, not only when tested against *M. incognita*, but also against *Meloidogyne enterolobii* Yang & Eisenback, 1983 and *M. javanica* (Almeida *et al.*, 2022). For *M. incognita*, Affokpon *et al.* (2011) obtained a high efficacy rate (90% reduction in J2 *M. incognita* numbers) for tomato and carrot roots using 10^6 spores/ml soil. A similar high efficacy rate was reported by El-Nagdi *et al.* (2019) equalling a 90% reduction in *M. incognita* densities in pea (*Pisum sativum* L.) using an unspecified concentration. Abdel-lateif & Bakr (2018) too recorded a high reduction rate of up to 84% for *M. incognita* female numbers in tomato roots with a concentration of 10^8 spores/g soil. Intermediate efficacy rates were also recorded: Izuogo & Abiri (2015) demonstrated a 62% reduction in *M. incognita* J2 densities, but low reductions in egg densities (27%) and adult female population densities (21%) after inoculation of soybean plants with a *T. harzianum* T₂₂ solution (30 ml in 15 litres water); Rizvi *et al.* (2018) reported a 67% reduction of *M. incognita* population densities in chickpea (*Cicer arietinum* L.) with a concentration of 10^6 spores/ml. Low efficacy rates, though higher than those found in this study, were recorded by Mascarin *et al.* (2012) with a 49% reduction in *M. incognita* population densities in cucumber (*Cucumis sativus* L.) roots using a 4×10^8 conidia/ml and Pocurull *et al.* (2020) who reported a reduction rate of 48% in *M. incognita* reproduction in tomato roots when inoculated with 10^5 CFU/ml.

Concerning the efficacy of *T. harzianum* against *M. enterolobii*, Jindapunnapat *et al.* (2013) demonstrated a reduction of 60% in a population density in guava (*Psidium guajava* L.) roots with a 10^6 spores/ml concentration. For *M. javanica*, however, from various efficacy rates have been reported: Murslain *et al.* (2014) demonstrated a 83% reduction of J2 densities and 82% reduction

of female numbers for eggplant (*Solanum melongena* L.) using 10^7 spores/ml; Almeida *et al.* (2022) demonstrated a reduction of up to 77% in population densities in banana using a 5×10^7 conidia/ml concentration; Al-Hazmi & TariqJaveed (2016) reported an intermediate reduction of 68% for egg densities in tomato roots using a 10^{10} spores/g soil concentration; Siddiqui *et al.* (2001) showed <50% reductions in J2 densities in okra (*Abelmoschus esculentus* L. Moench) and mungbean (*Vigna radiata* (L.) R. Wilczek) roots using 2.8×10^7 CFU/ml concentration; while Siddiqui & Shaukat (2004) showed a low 28% reduction in population densities in tomato root using 10^5 conidia/ml.

Numerous reports also exist for the effect of *T. harzianum* on root galling caused by various *Meloidogyne* spp. for various crops. The highest reduction in root gall-formation found in this study for the 10^8 spores/ml for both the ST and DA treatments of 15% disagrees with results where high efficacy of the strain were reported as follows: a 75% reduction in *M. incognita* tomato root gall numbers when using a 10^8 g/soil concentration of tomato (Abdel-lateif & Bakr, 2018); a 74% reduction by El-Nagdi *et al.* (2019) for *M. incognita* gall numbers in pea roots using an unspecified concentration; a 69% reduction of *M. incognita* galls on tomato roots by Radwan *et al.* (2012) for a commercial bionatural product (Plant Gard®) with *T. harzianum* as a.i. using 30×10^6 spores/ml; an up to 68% reduction in *M. incognita* galls on carrot roots by Prasad *et al.* (2014) using a 25 g/m² concentration after treatment imposition; a 67% reduction in *M. incognita* chickpea root gall-formation by Rizvi *et al.* (2018) applying a 10^6 spores/plant concentration. Intermediate efficacy was also reported for this strain: Osman *et al.* (2018) showed a 61% reduction in *M. incognita* root galling for eggplant using 10^4 CFU/ml; and a 60% reduction in of *M. incognita* root gall-formation by Izuogo & Abiri (2015) for soybean, using a *T. harzianum* T₂₂ solution (30 ml in 15 l H₂O). Low efficacy rates of <50%, still higher than that reported for this soybean study, were further reported for *T. harzianum* in reducing *M. incognita* reproduction: Affokpon *et al.* (2011) showed a 49% reduction of *M. incognita* in tomato applying 10^6 spores/ml; a 38% decrease in *M. incognita* root gall numbers on tomato roots by Khan *et al.* (2018) using 10^5 CFU/ml; and Dababat & Sikora (2007) recording a 20% reduction of *M. incognita* gall-formation in tomato using a concentration of 5×10^6 spores/ml. For *M. javanica*, other reports demonstrated reductions in root galling with the highest and lowest efficacy of 94% for sunflower (*Helianthus annuus* L.) (Dawar *et al.*, 2008) and 47% for okra (Siddiqui *et al.*, 2001) respectively.

Concerning the effect of spores of the fungal isolate *T. harzianum* in reducing *M. incognita* J2 densities in soil, no significant results were obtained which disagreed with the findings of various authors that showed high reductions of 97% (Radwan *et al.*, 2012), 89% (Abdel-lateif & Bakr, 2018), 71% (Affokpon *et al.*, 2011), 70% (Osman *et al.*, 2018) to intermediate reductions of 65%; Rizvi *et al.* (2018) and 58% (Prasad *et al.*, 2014), and low efficacy of 30% by Khan *et al.* (2018).

For other species such as *M. javanica* life-stages in soil, a few reports showed low efficacy rates of 44% (Al-Hazmi & TariqJaveed, 2016) and 37% (Siddiqui *et al.*, 2001) respectively, in terms of J2 soil density reductions while for *M. enterolobii* J2 soil densities Jindapunnapat *et al.* (2013) reported a high reduction of 94% in soil densities of J2.

Although spores of the fungal isolate *T. harzianum* resulted in a low, but insignificant 5% increase in fresh soybean root mass in this glasshouse study with *M. incognita*, it is in agreement with the report by Khan *et al.* (2018) that showed a 6% increase in fresh root mass and is in the same numerical range 7% (Affokpon *et al.*, 2011) and 12% (Radwan *et al.*, 2012) for tomato. Mascarin *et al.* (2012), however, demonstrated no significant increase in cucumber root mass (13% lower than control) against *M. incognita*. In contrast to the results obtained in this soybean glasshouse study, Abdel-lateif & Bakr (2018) reported a 67% increase in fresh tomato root mass when evaluating it against *M. incognita*, while El-Nagdi *et al.* (2019) demonstrated a low 21% increase in fresh pea root mass and Rizvi *et al.* (2018) recorded a 20% increase in fresh chickpea root mass for *M. incognita*.

For *M. javanica*, high efficacy in terms of okra root mass increase of 73% were reported by Dawar *et al.* (2008), while Chinheya (2015) reported a 61,9% increase in fresh soybean root mass. Siddiqui *et al.* (2001) demonstrated a low, 25% increase in mungbean root mass, while a 7,6% increase in banana root mass was found by Almeida *et al.* (2022), and a non-significant reduction of 53,2% (lower than the control) were reported for *M. javanica* on banana (Murslain *et al.*, 2014). Siddiqui & Shaukat (2004) also reported an insignificant increase in tomato root mass of 6,25% (lower than control) for *M. javanica*. For *M. enterolobii*, Jindapunnapat *et al* (2013), reported a 59,1% increase in guava root mass.

The efficacy of spores of the fungal isolate *Trichoderma virens* tested in this soybean glasshouse study against *M. incognita* resulted in a significant reduction in root-gall formation, but not in life-stage numbers per 20 g roots. In contrast with the 35% reduction of life-stage numbers in soybean roots found in this study, Meyer *et al.* (2001) recorded a 58% reduction egg and J2 densities in roots of gram bell pepper (*Capsicum annuum* L.) using a 10^4 CFU/seed concentration. Compared to this soybean glasshouse study, a low efficacy rate of 12% reduction in *M. incognita* egg numbers have been reported by Khan *et al.* (2018) using a culture suspension of 10^5 CFU/ml. These are the only published sources that could be found indicating the efficacy of *T. virens* on the reproduction of all life-stages of *Meloidogyne* spp. The highest reduction in root gall-formation recorded in this soybean glasshouse study of 47% for both the 10^8 spores/ml (ST) and 10^7 spores/ml (ST) treatments were substantially higher than those reported by Khan *et al* (2018), namely 12%. By contrast, Chinheya (2015) recorded no significant reduction (4% higher

compared to the control) in *M. javanica* soybean root gall numbers using a conidial suspension of 10^8 conidia/ml.

The efficacy of the fungal isolate *T. virens* on the reduction in *M. incognita* life-stage numbers per 200 g soil, showed no significant results in this soybean glasshouse study. This disagrees with a report by Khan *et al.* (2018) showing a 19% reduction in *M. incognita* J2 soil densities using 10^5 CFU/ml. This is the only published source that could be found indicating the efficacy of *T. virens* on the reproduction of all life-stages of *Meloidogyne* spp. in soil. The efficacy of the fungal isolate *T. virens* on the fresh soybean root mass had no significant result (5% higher than the untreated control) and is in agreement with a report by Meyer *et al.* (2001) recording no significant increase in fresh bell pepper root mass (6.6% lower than untreated control), against *M. incognita* with a concentration of 10^4 CFU/seed. By contrast to the results obtained in this glasshouse study the following authors reported higher root mass increases: Chinheya (2015) with a 67% increase for soybean for *M. javanica*; and Khan *et al.* (2018) with a 11% increase for tomato for *M. incognita*.

With regards to the two formulated products, CR and Eco-T®, tested in this soybean glasshouse study against *M. incognita*, only two published sources for these products were available, namely that by Pambuka (2014) and Chinheya (2015). Pambuka (2014) found a reduction in *M. javanica* J2 per gram tomato root of 65% and 67% applying CR as a seed-dressing and a soil drench respectively, when using a concentration of 10^6 spores/g only days after inoculation. This is substantially higher and disagrees with results of this soybean glasshouse study where a combination of CR and Eco-T® (a 0,6 g/2 l H₂O concentration of each) caused an insignificant reduction (7% higher than the untreated control) of *M. incognita* life-stage numbers in soybean roots. Similarly, when compared to this study, Pambuka (2014) found reductions of 52% and 61% in *M. javanica* galling on tomato roots applying CR as a seed dressing and a soil drench, respectively, using a 10^6 spores/g concentration, while *M. javanica* egg mass numbers on tomato roots were reduced by of 38% for both a seed dressing and soil drench. For plant height Pambuka (2014) also reported increases of 33% and 35% using CR as a seed dressing and a soil drench respectively, while for plant mass increases of 42% and 45% were recorded for the seed dressing and soil drench applications respectively. These plant parameter data disagree from those for the soybean glasshouse study in which the effect plant height resulted in a mere 7,9% and 2,6% increase for Experiment 1 & 2 respectively while the plant mass was increased by 25% and 5,9% for Experiment 1 & 2 respectively, which were much lower than the results found by Pambuka (2014).

For Eco-T®, Pambuka (2014) found a reduction in *M. javanica* J2 per gram tomato root of 68,8% and 75,5% as a seed-dressing and a soil drench respectively, with a concentration of 10^6 spores/g, only days after inoculation. This is substantially higher and disagrees with results of this

soybean glasshouse study where a combination of CR and Eco-T® (a 0,6 g/2 l H₂O concentration of each) caused an insignificant reduction (7% higher than the untreated control) of *M. incognita* life-stage numbers in soybean roots. However, the results obtained in this study agree with Chinheya (2015) who found an insignificant reduction in *M. javanica* population of 23,6% (higher than inoculated control) which was inoculated as a root disease complex (with *Rhizoctonia solani*) on soybean, four weeks after inoculation. A root gall reduction of *M. javanica* on tomato of 52,4% and 60,5% as a seed dressing and a soil drench respectively, with a concentration of 10⁶ spores/g 50 days after inoculation was found by Pambuka (2014), while Chinheya (2015) found a 22,4% reduction on soybean inoculated as a root disease complex (with *R. solani*), four weeks after inoculation. *Meloidogyne javanica* egg mass numbers have been reduced by 43,8% and 43,3% for seed dressing and soil drench respectively, with a concentration of 10⁶ spores/g, 50 days after inoculation (Pambuka, 2014). An increase in tomato plant height of 44,3% and 49,6% for seed dressing and soil drench respectively, with a concentration of 10⁶ spores/g, 50 days after inoculation tested against *M. javanica* was found by Pambuka (2014). Lastly, an increase in tomato root mass of 55,2% and 62,8% for seed dressing and soil drench respectively, with a concentration of 10⁶ spores/g, 50 days after inoculation when tested against *M. javanica*, while Chinheya (2015) found a 23,1% increase on soybean inoculated as a root disease complex (with *R. solani*), four weeks after inoculation.

5.5 Conclusion

The five fungal strains analysed in this study for their *in vivo*-based abilities to reduce *M. incognita* numbers in soybean and effect on plant parameters, showed very low to no potential. It is important to look at methods of application as a limitation, since these are living biological agents which tend to be sensitive to the environment (Abd-Elgawad & Askary, 2018). Furthermore, the formulated fungal products tested for its ability to reduce *M. incognita* numbers in soybean and effect on plant parameters, also showed no potential which is in disagreement with the results found by Pambuka (2014). This indicated that the method of application could have influenced the efficacy of the two products, since Pambuka (2014) made use of a soil drench as well as a seed dressing, while this study only made use of the latter.

CHAPTER 6 CONCLUSION

6.1 General conclusion

The aim of this study was to evaluate the effect of fungal strains on *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949 second-stage juvenile (J2) motility (*in vitro* assays) and female reproduction (*in vivo*) utilizing spores of *Trichoderma* spp. strains and *Clonostachys rosea*, as well as those from two formulated products CR (active ingredient/a.i. *C. rosea* strain PHP 1701) and Eco-T® (a.i. *Trichoderma asperellum* strain kd) to determine their potential as biocontrol agents. The successful use of such fungal strains as potential bionematicides will be revolutionary in the sense that it can replace chemical nematicides which are continuously withdrawn from the market (Xiang *et al.*, 2017a; Engelbrecht *et al.*, 2020).

6.2 Testing of the hypotheses

The following hypotheses were tested, while a discussion thereof follows:

- i. Spores of endemic *Trichoderma* spp. and *C. rosea* fungal isolates, as well as those of two formulated products (CR and Eco-T®) will reduce *M. incognita* J2 motility.

Various *in vitro* studies demonstrated the potential nematicidal activity of spores of *C. rosea* due to their significant ability to immotilize *M. incognita* J2 (Migunova *et al.*, 2018; Lafta & Kasim, 2019), with similar results recorded for *Trichoderma asperellum* and *Trichoderma harzianum* (Mascarin *et al.*, 2012; Khan *et al.*, 2018; Migunova *et al.*, 2018; Hemeda & El Deeb, 2019). The strains of these three fungal species, isolated from South African soils, used in this laboratory study against a South African population of *M. incognita* resembled findings in literature about their superior ability to significantly immotilize J2 in *in vitro* experiments. These results indicated J2 immotility rates of 100% for *C. rosea*, >83,5% *T. asperellum* and >61,7% for *T. harzianum*. Moreover, the highest J2 immotility rate of >75,7% recorded for *Trichoderma virens* in this South African study was higher (>36,7%) than reports found in literature (e.g., 39% reported by Khan *et al.* (2018) for *M. incognita*), indicating that this species also has strong nematicidal activity. Unfortunately, although a similar trend was recorded for *Trichoderma asperellum* kd in terms of the superior *in vitro* effect of its spores in causing *M. incognita* J2 immotility (<86,5%), no results could be found in literature for this fungal species in this regard; not for *M. incognita*, nor for any other *Meloidogyne* spp.

Unfortunately, opposed to results recorded for the live spores of the five fungal strains discussed above, data obtained for both the formulated products (CR and Eco-T®) tested in this South African *in vitro* study, yielded insignificant results of <30% reduction in *M. incognita* immotility. Although no data could be found in literature for the efficacy of these two products in terms of J2 immotilization of *M. incognita*, the results disagreed with the only two published sources that exist, namely reports by Pambuka (2014) and Chinheya (2015). These two authors both recorded reductions in J2 immotility ranging from 64% to 79% for *Meloidogyne javanica* when applying these products in *in vitro* experiments. It could be that different batches of these products were used by the latter authors as the one used during this study, but other than that it is challenging to attempt explaining this discrepancy in results.

Results from the *in vitro* experiments done in this South African study showed that all five fungal species evaluated in a first-line screening test showed potential to adversely affect the biology of *M. incognita*, which in this case was J2 motility. The motile J2 is the infective life stage of *Meloidogyne* (Perry & Moens, 2011), and therefore initial testing of a product(s) in a laboratory test (such as the well tests done in this study) that shows potential to interfere with J2 movement or negatively affects its ability to find and infect a host plant, represents the first step in identifying a product with potential bionematicide activity. The first hypothesis is therefore accepted for this *in vitro* study with the spores of the five fungal isolates being superior in its efficacy to immotilize *M. incognita* J2, while although the efficacy of the formulated products was low, they also resulted in reduction of *M. incognita* J2 motility.

- ii. Spores of endemic *Trichoderma* fungal isolates and those of *C. rosea*, as well as the application of formulated products of *C. rosea* and *T. asperellum* strain kd, respectively, will reduce *M. incognita* densities in soybean rhizospheres.

The spores of *Trichoderma* fungal isolates and those of *C. rosea* have been found to reduce *M. incognita* densities in soybean rhizospheres on a global scale (Meyer *et al.*, 2001; Affokpon *et al.*, 2011; El-Nagdi *et al.*, 2019; Cristobal-Alejo *et al.*, 2021). However, low efficacy rates of all *Trichoderma* spp. and *C. rosea* spores in reducing reproduction of *M. incognita* in soybean (*Glycine max*, L. Merr.) roots generally disagreed with published literature. Application of *C. rosea* spores with a 45% reduction in *M. incognita* life-stage densities and a 26% reduction in root galling are, for example, inferior and in disagreement with reductions as high as 87% for these two respective nematode reproduction parameters (Hussain *et al.*, 2017). For *T. virens*, a reduction in *M. incognita* life-stage numbers of 35% for this South African study was lower than the reduction

of 58% reported by Meyer *et al.* (2001), but higher than the 12% reported by Khan *et al.* (2018). Interestingly, a 47% reduction in gall formation found in the present study is substantially higher than the 12% by Khan *et al.* (2018), and no significant reduction by Chinheya (2015). Although low reductions of 32% for *M. incognita* life-stage densities and 21% for root galling for *T. asperellum* were in a similar range as the 13% reported by Affokpon *et al.* (2011), it was considerably lower than reductions of up to 98% (Pocurull *et al.*, 2020) and 91% (Abdel-lateif & Bakr, 2018) for these two respective nematode reproduction parameters. For *T. harzianum* low reductions of 10% for *M. incognita* life-stage densities and 15% in root galling agreed with low efficacy rates of 21% (Izuogo & Abiri, 2015) and 20% (Dababat & Sikora, 2007) respectively for these two nematode reproduction parameters, but disagreed with high reductions of up to 90% (Affokpon *et al.*, 2011; El-Nagdi *et al.*, 2019) and 94%, (Dawar *et al.*, 2008). Interestingly, plant growth parameter data from this South African study generally showed insignificant increases in fresh soybean root mass after application of the five fungal isolates and were homogeneous compared to that existing in literature (Affokpon *et al.*, 2011; Radwan *et al.*, 2012; Murslain *et al.*, 2014; Khan *et al.*, 2018). However, these results disagreed with a 35% increase by Hussain *et al.* (2017) for *C. rosea* and increases of 65% and 59% for *T. asperellum* by Abdel-lateif & Bakr (2018) and Kumari *et al.* (2020), respectively.

The efficacy of the two formulated products CR and Eco-T® applied separately for testing their efficacy in reducing *M. incognita* life-stage numbers also disagreed with intermediate reductions reported for CR and Eco-T® (Pambuka, 2014; Chinheya, 2015), but agreed with low reductions in root gall rating for Eco-T® (Pambuka, 2014). Plant parameter data obtained in this study was inconclusive and also in disagreement with increased plant height of 50% and plant mass of 63% reported previously (Pambuka, 2014).

Application of live spores of the five fungal isolates and those contained in the two formulated products (CR and Eco-T®) resulted in reduction in *M. incognita* reproduction, although to a low degree, for the *in vivo* glasshouse experiment, the second hypothesis was also accepted.

6.3 Future recommendations

- i. Field or microplot studies under prevailing environmental conditions should be conducted to evaluate the potential of the fungal isolates tested in this study, since the initial screenings of such organism using *in vitro* well experiments and glasshouse experiments in which soil has been sterilized, often only represent the first line of investigation. *In vitro* well experiments give an indication of the mode of action of the product which are often considered to be the toxic metabolites (Migunova *et al.*, 2018) and chitinolytic enzymes (Lafta & Kasim, 2019) released by some of these strains. On the other hand, glasshouse testing involved use of soil as the

substrate, but since it was sterilized in this study it did not elucidate the efficacy of these products under conditions in soils which occur in fields of farmers and in which numerous other organisms are present. Studies reported the efficacy of *Trichoderma* spp. and *C. rosea* to reduce *Meloidogyne* spp. densities under field conditions (Kumar *et al.*, 2014; Sasanelli *et al.*, 2016; Fan *et al.*, 2020). But challenges also exist in terms of the application of bioagents in the field since it is considered unstable (Ji *et al.*, 2020).

- ii. It is suspected that the biggest hurdle to overcome is the effective application of these living fungal isolates and formulated products to seed. Further research must be performed to develop more effective methods of application and to supply optimal modes of deliverance. For example, testing different carriers for applying the spores of fungal spp. to seed and directly to soil (Kumar *et al.*, 2014). It could be helpful to increase the exposure time of seed to the fungal strain in its carrier as it was done by Pambuka (2014) for 2 hours instead of 1 hour of exposure as used in this study. Also, the application of powder-based formulations of these fungal spp. should be further explored with inert carriers such as talc or kaolin to enhance efficacy (Prasad & Rangeshwaran, 2000), in contrast with the distilled water used as carrier in this study. From a commercial perspective, such products will also need to be developed to enhance their shelf life, as this is also considered one of the biggest challenges (Kumar *et al.*, 2014), although the formulated products were used well within the range (before the expiry date) of their shelf lifetime.
- iii. The combination of two or more of these fungal spp. must be tested to determine whether the efficacy thereof will be higher than using a single strain alone. Previous results showed higher efficacy when certain fungal strains were used in combination compared to being applied alone (Lafta & Kasim, 2019). Combinability and antagonistic properties should be considered during these tests (Reaves & Crawford, 1994; Szabó *et al.*, 2012), to evaluate the best options for the target *Meloidogyne* spp.

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ANNEXURE I

1. Results: *In vivo* screening utilizing viable fungal spores from reared colonies

1.1. Nematode root data: 48 DAP

1.1.1. *Clonostachys rosea*

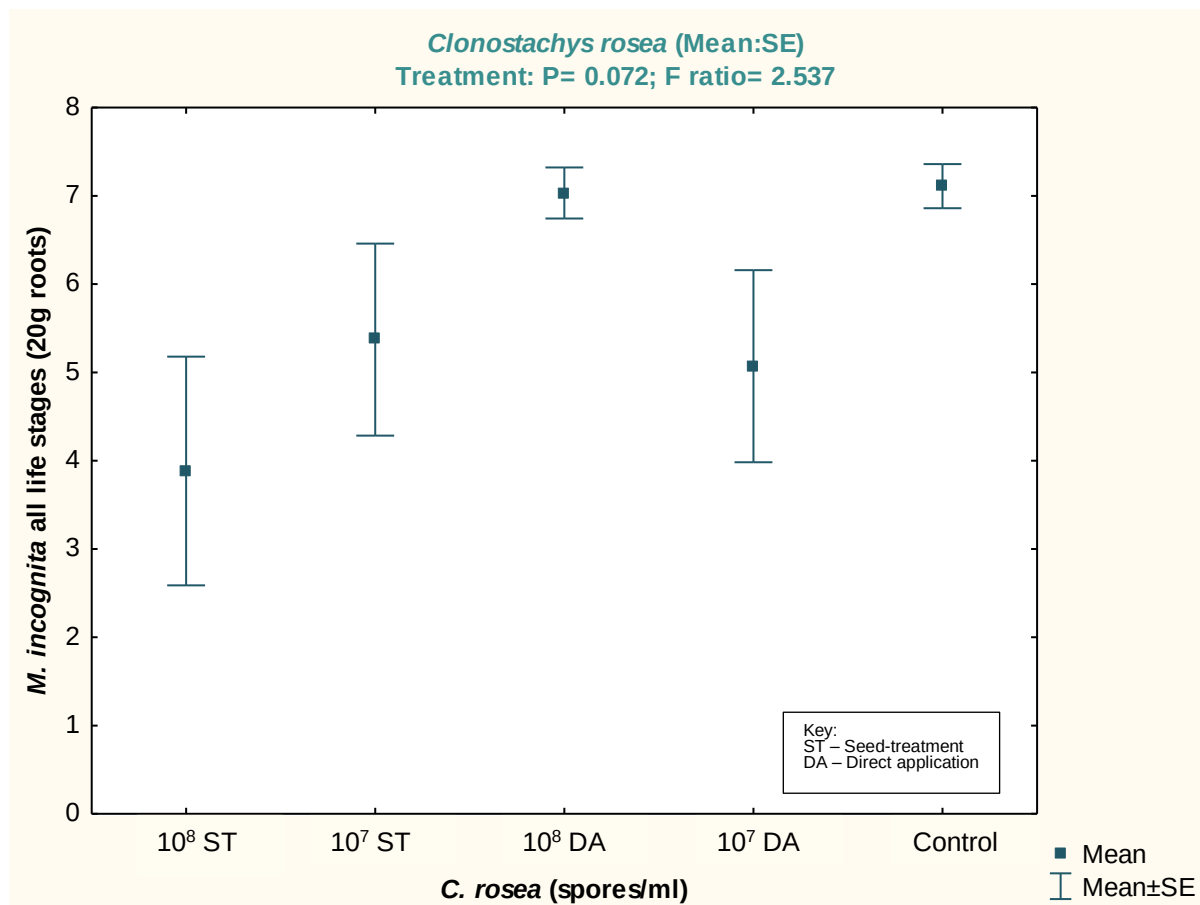


Figure 1: The effect of different concentrations (spores per ml) of *Clonostachys rosea* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 20 g roots) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.1.2. *Trichoderma asperellum* kd

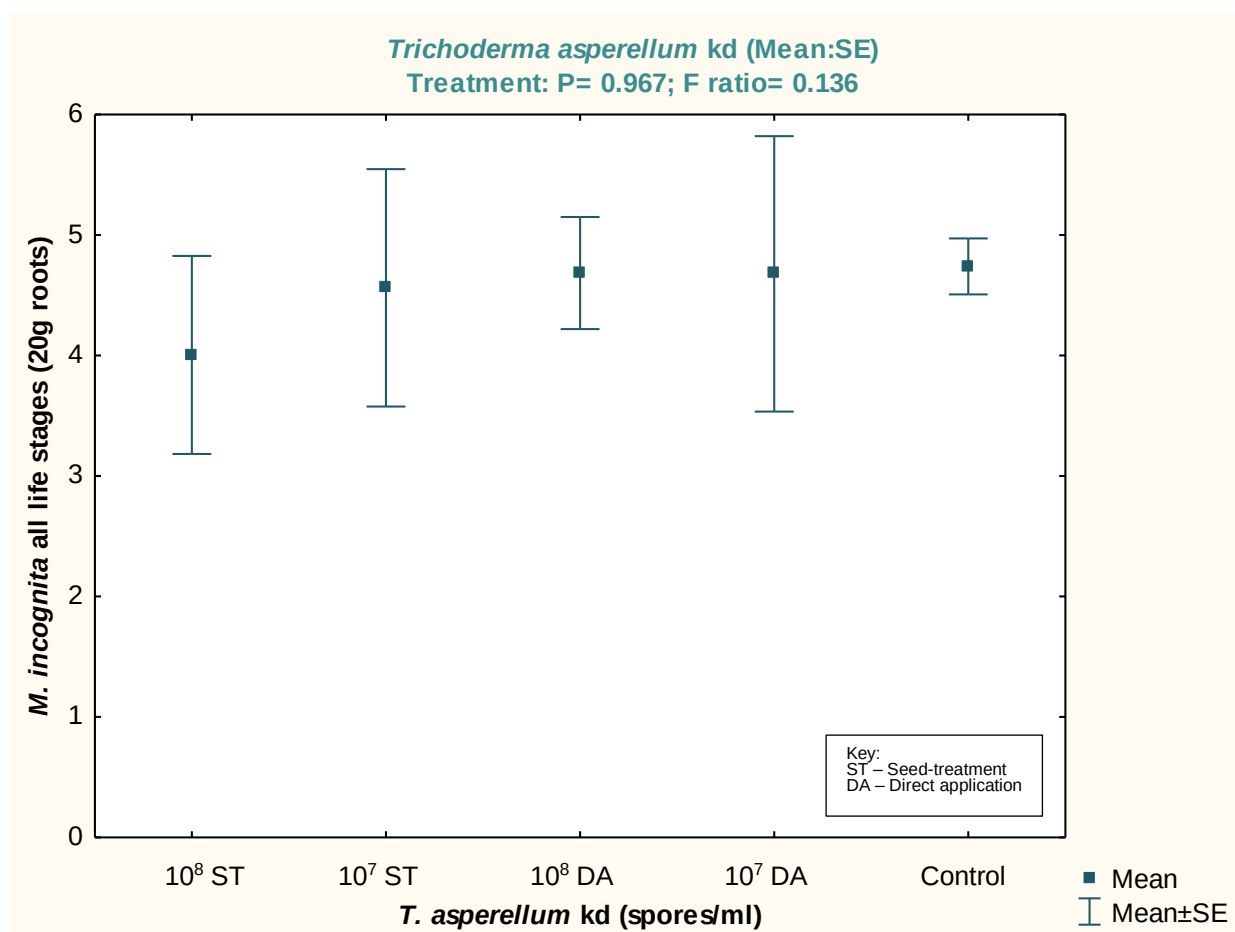


Figure 2: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* kd on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 20 g roots) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

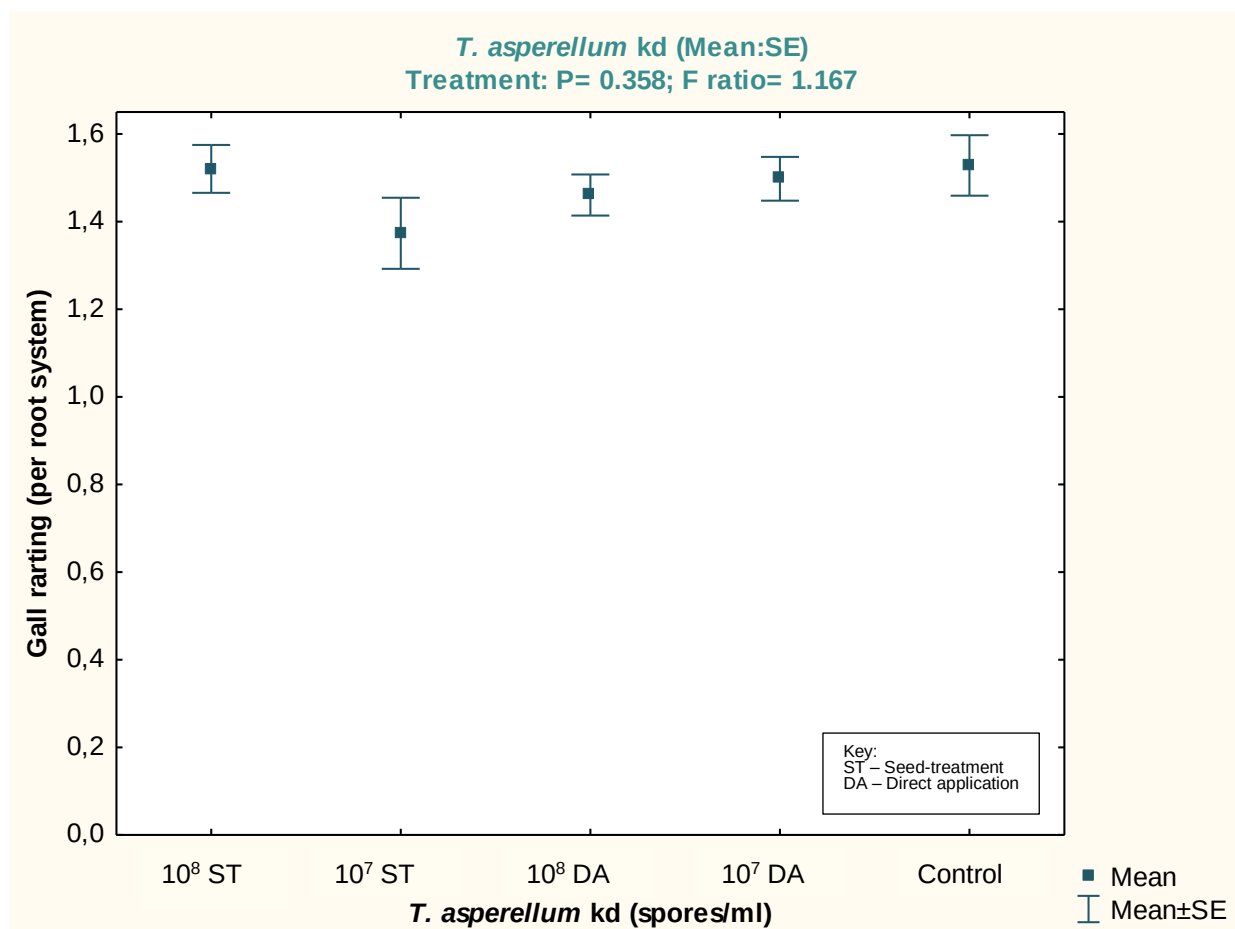


Figure 3: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* kd on the gall rating index under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.1.3. *Trichoderma harzianum*

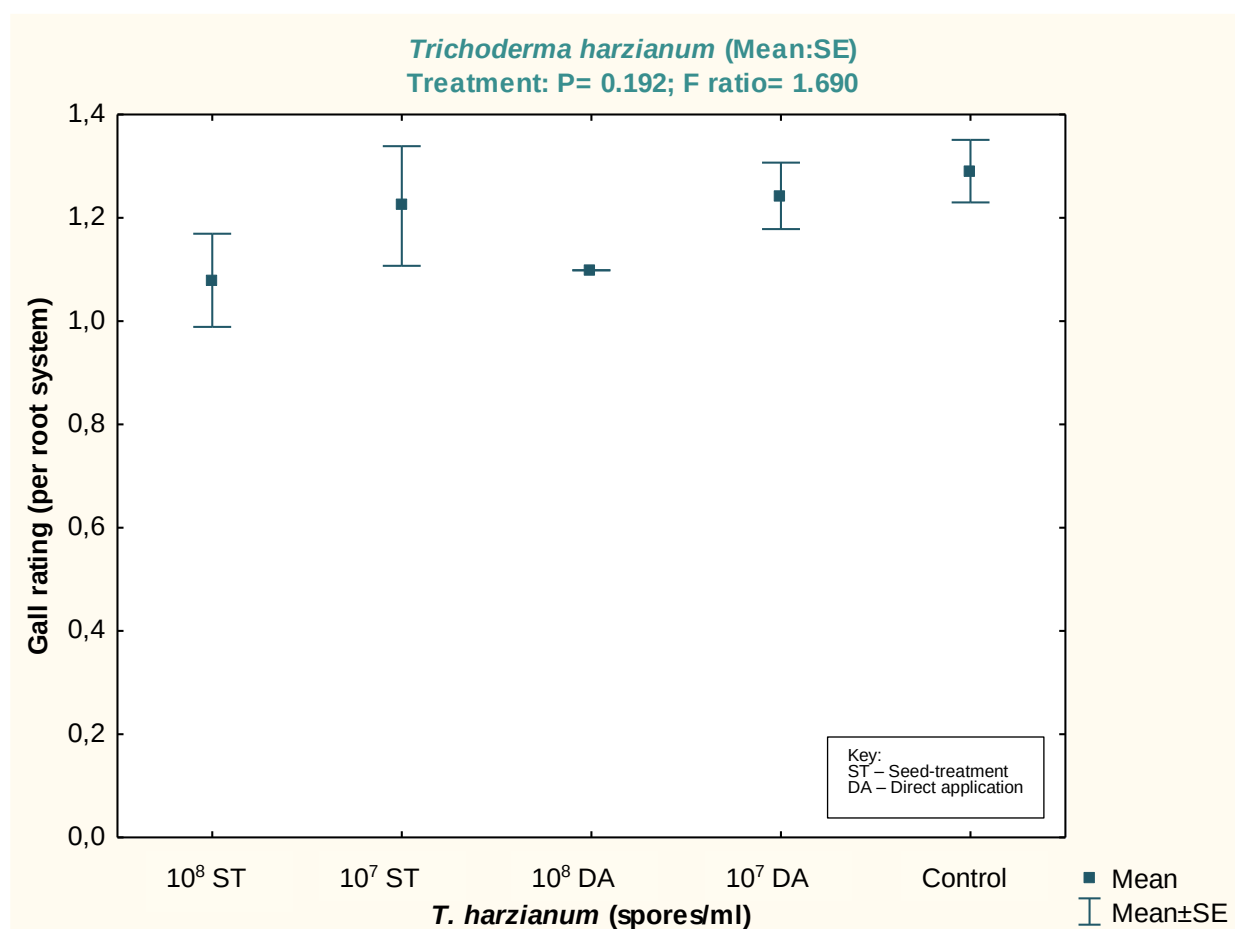


Figure 4: The effect of different concentrations (spores per ml) of *Trichoderma harzianum* on the gall rating index under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.1.4. *Trichoderma virens*

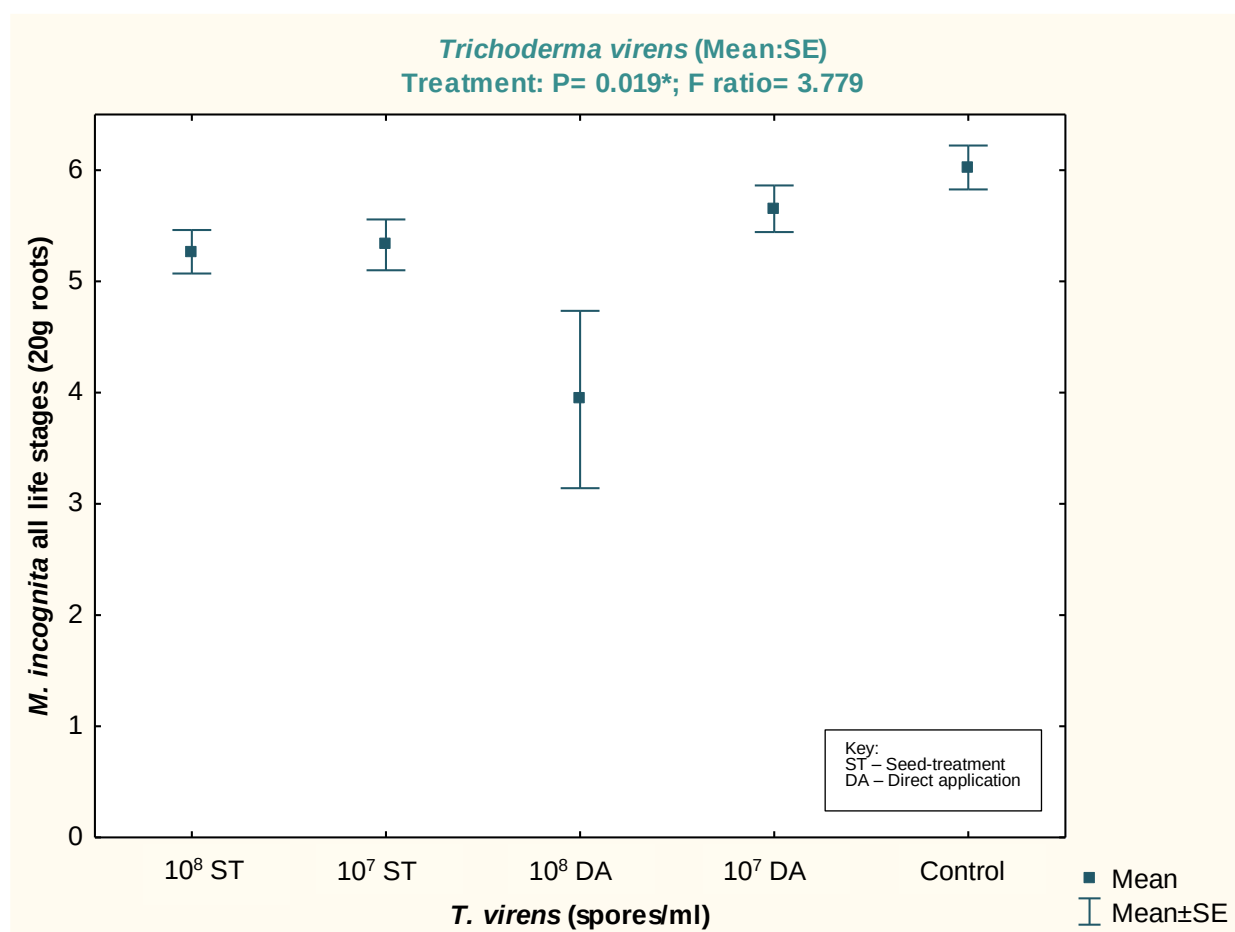


Figure 5: The effect of different concentrations (spores per ml) of *Trichoderma virens* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 20 g roots) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.2. Nematode soil data: 48 DAP

1.2.1. *Clonostachys rosea*

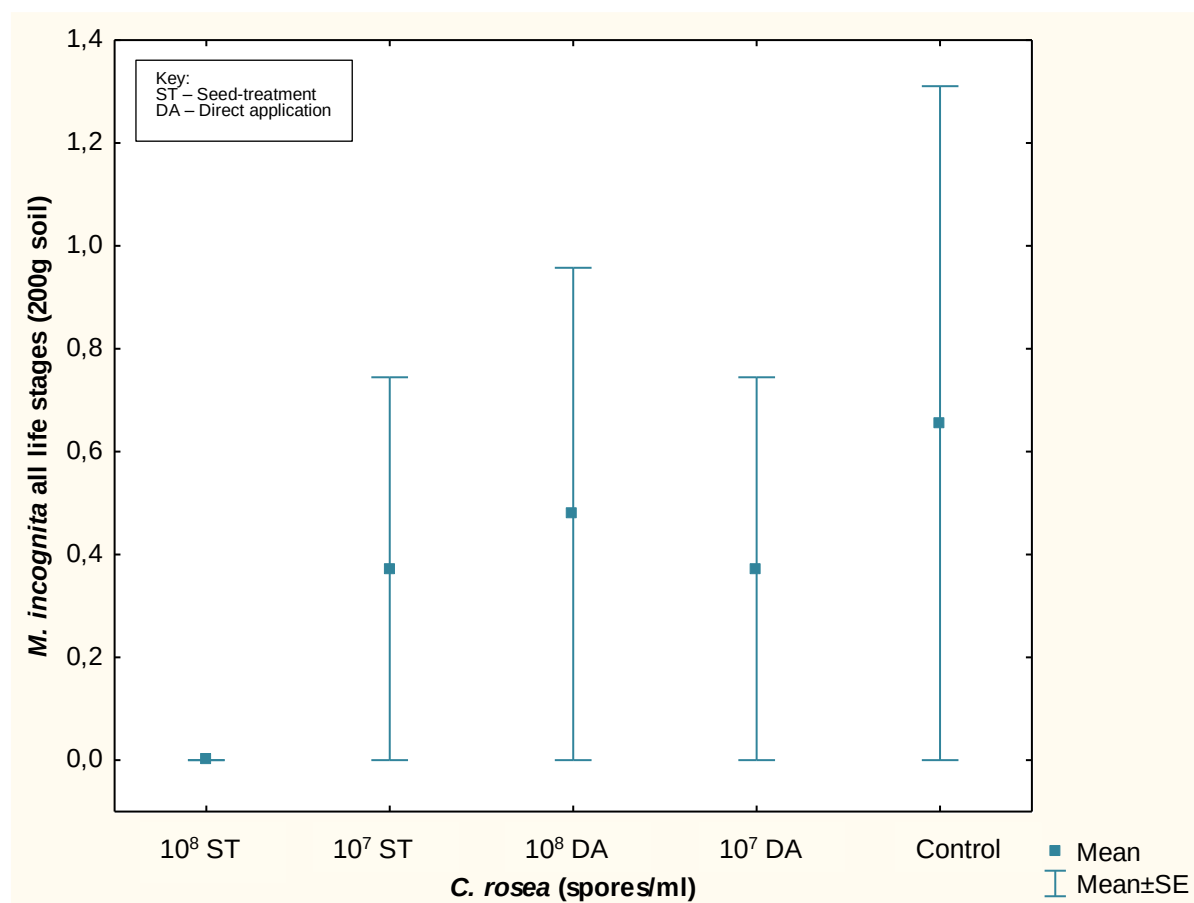


Figure 6: The effect of different concentrations (spores per ml) of *Clonostachys rosea* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 200 g soil) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.2.2. *Trichoderma asperellum*

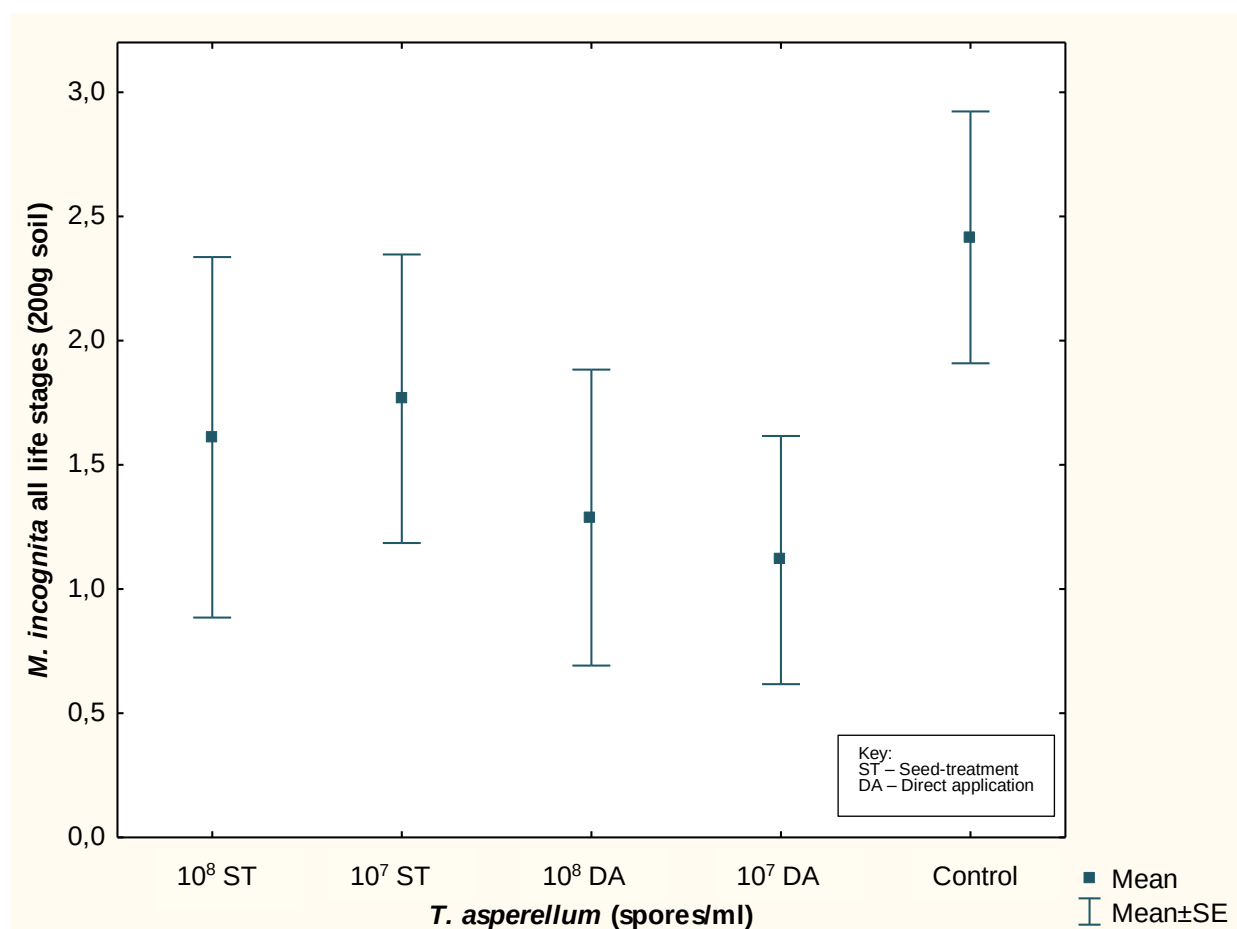


Figure 7: The effect of different concentrations (spores per ml) of *T. asperellum* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 200 g soil) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.2.3. *Trichoderma asperellum* kd

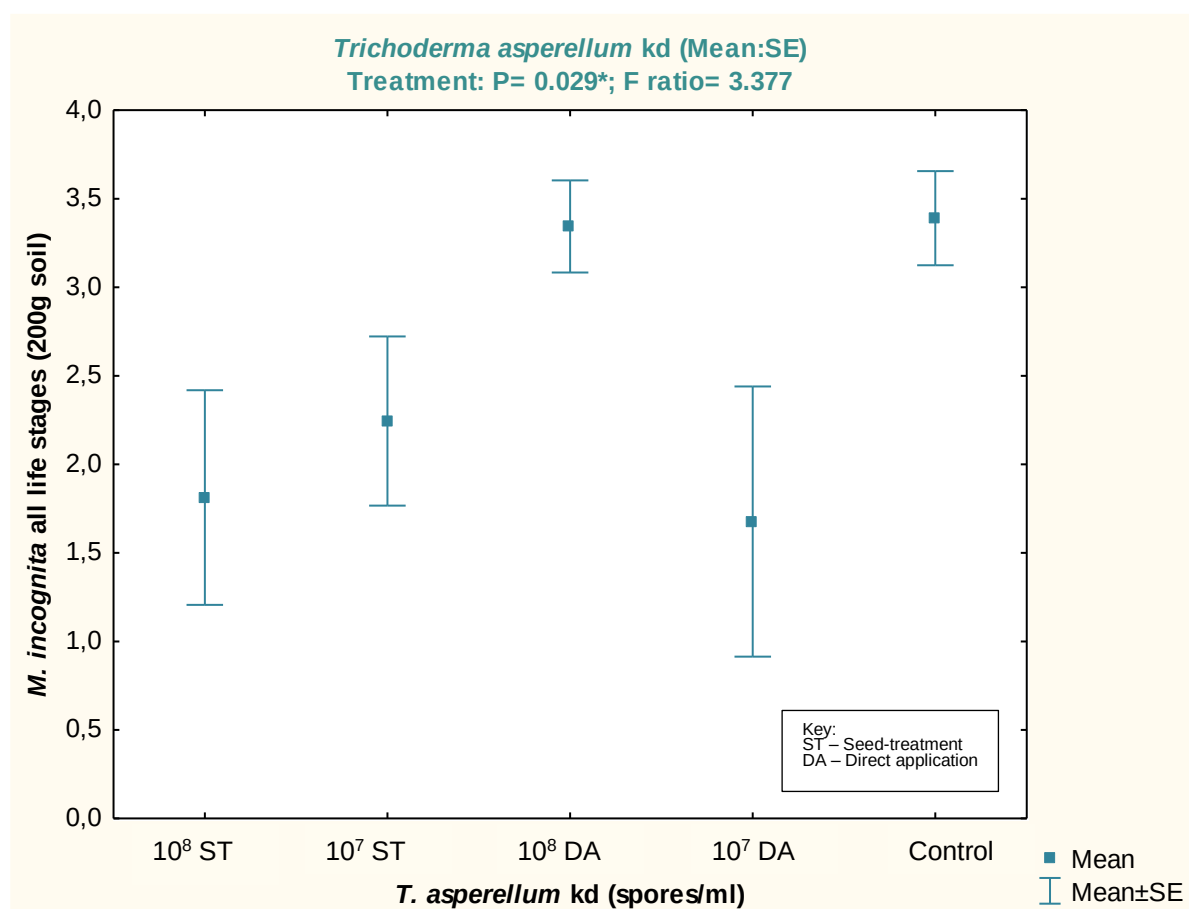


Figure 8: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* kd on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 200 g soil) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.2.4. *Trichoderma virens*

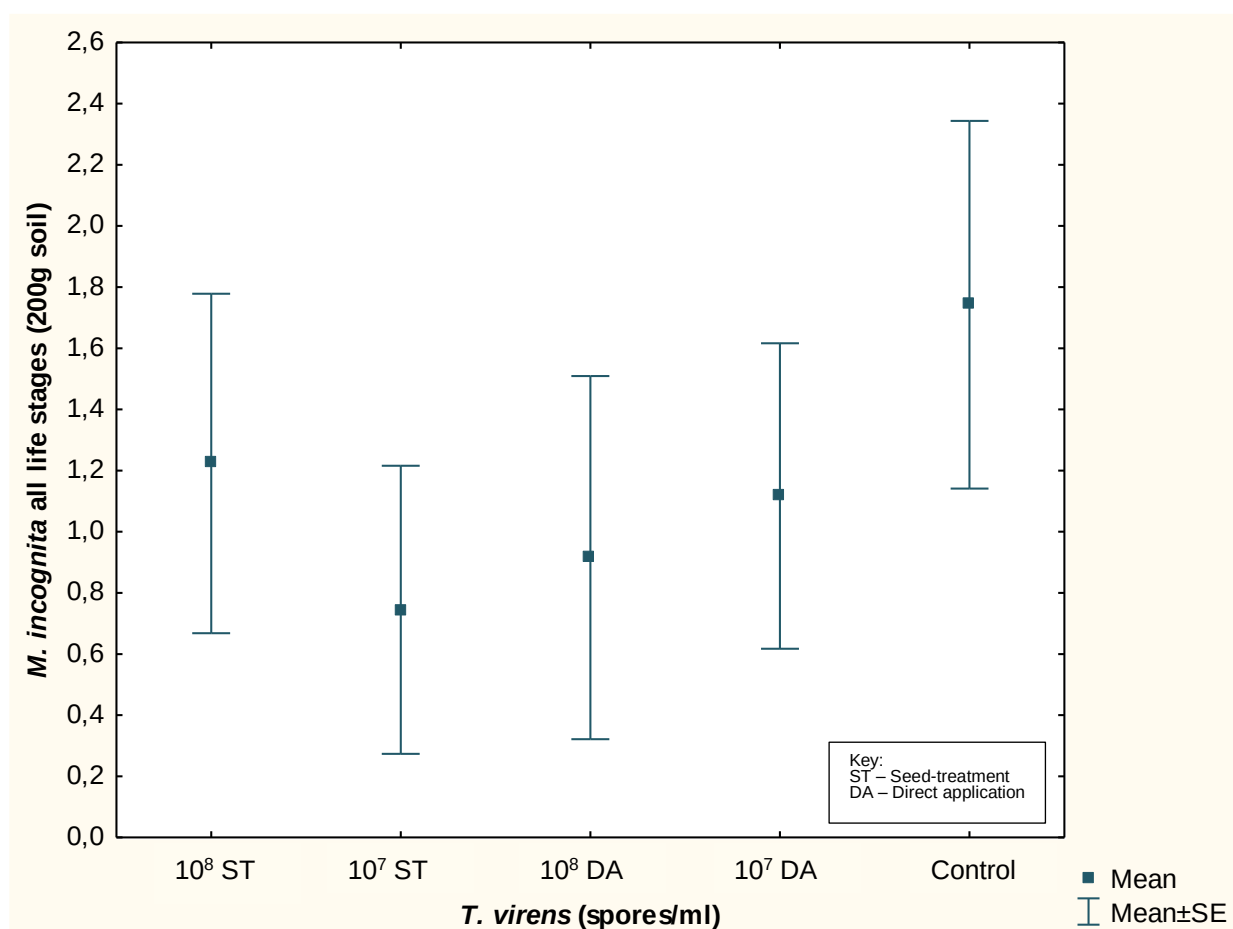


Figure 9: The effect of different concentrations (spores per ml) of *Trichoderma virens* on the reproduction of *Meloidogyne incognita* (referring to number of life stages per 200 g soil) under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.3. Plant parameter data: root mass 48 DAP

1.3.1. *Clonostachys rosea*

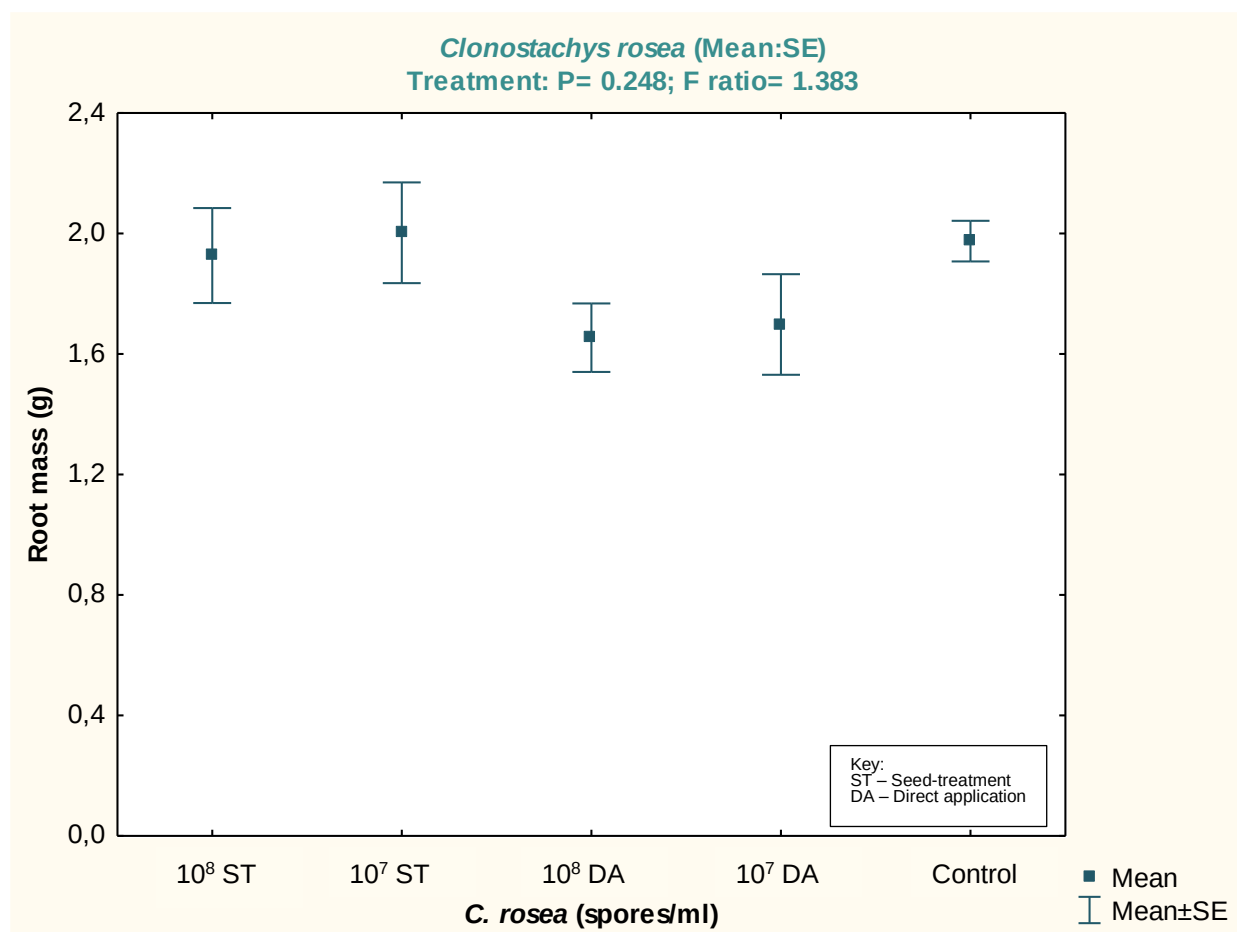


Figure 10: The effect of different concentrations (spores per ml) of *Clonostachys rosea* on the root mass under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.3.2. *Trichoderma asperellum* kd

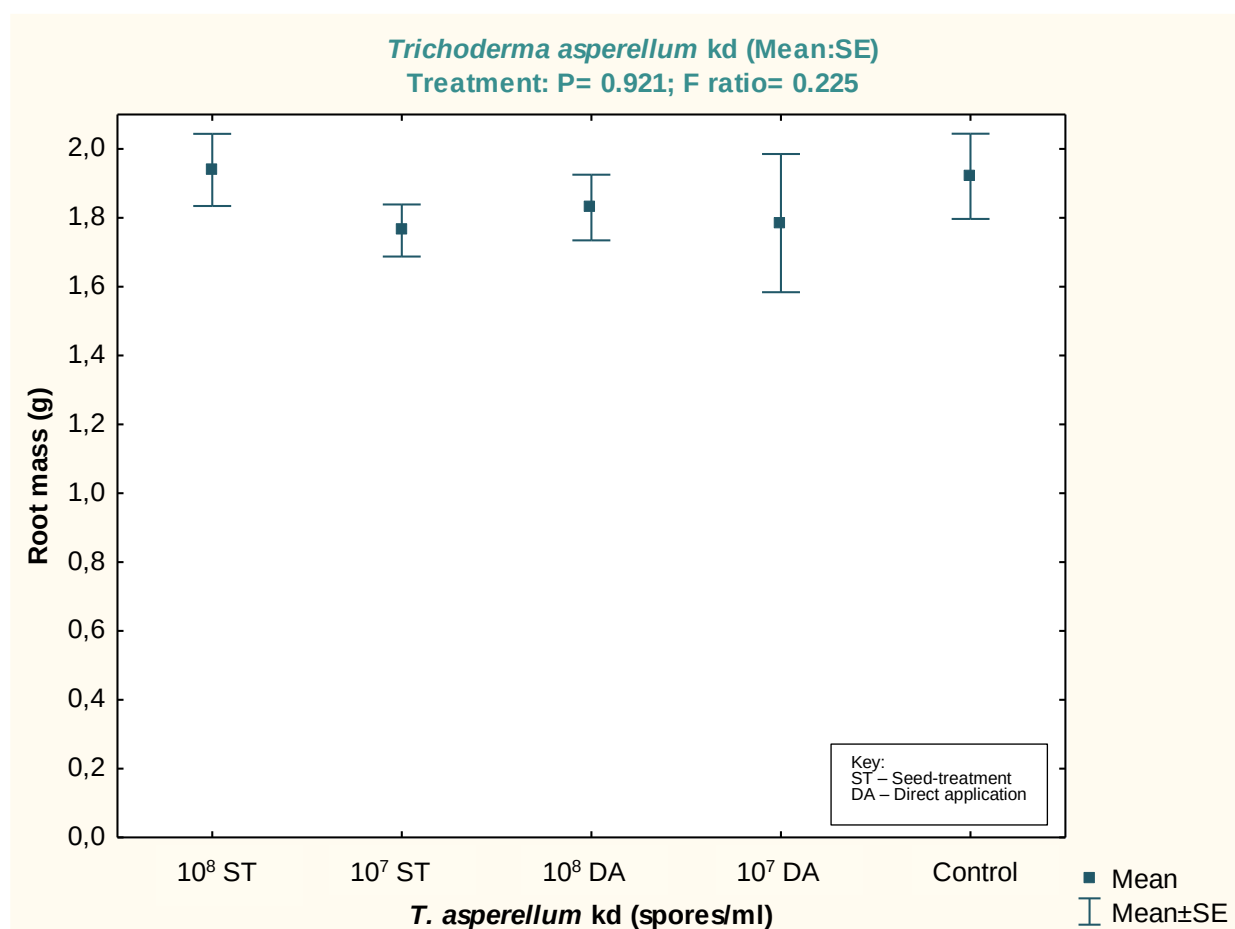


Figure 11: The effect of different concentrations (spores per ml) of *Trichoderma asperellum* kd on the root mass under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.3.3. *Trichoderma harzianum*

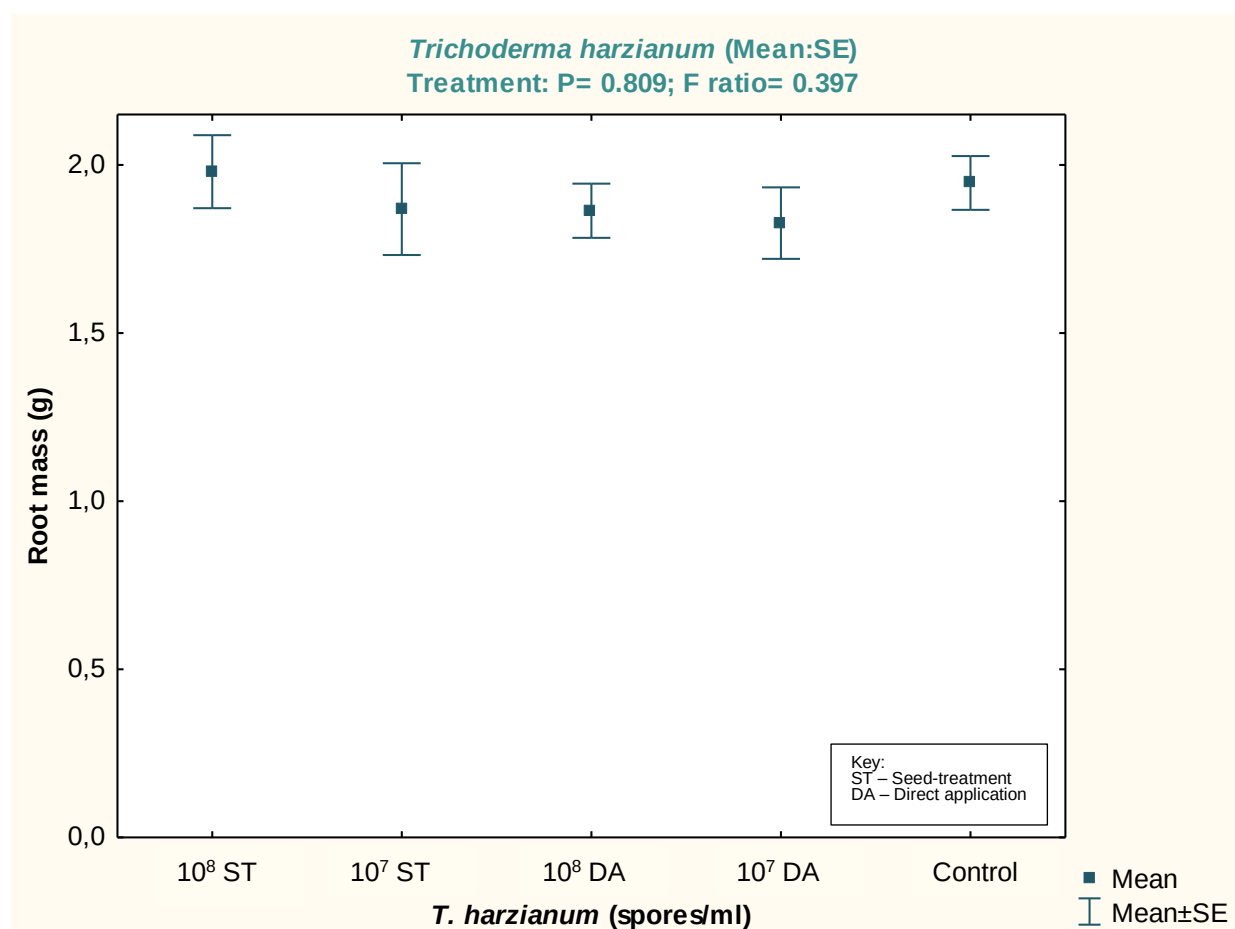


Figure 12: The effect of different concentrations (spores per ml) of *Trichoderma harzianum* on the root mass under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.3.4. *Trichoderma virens*

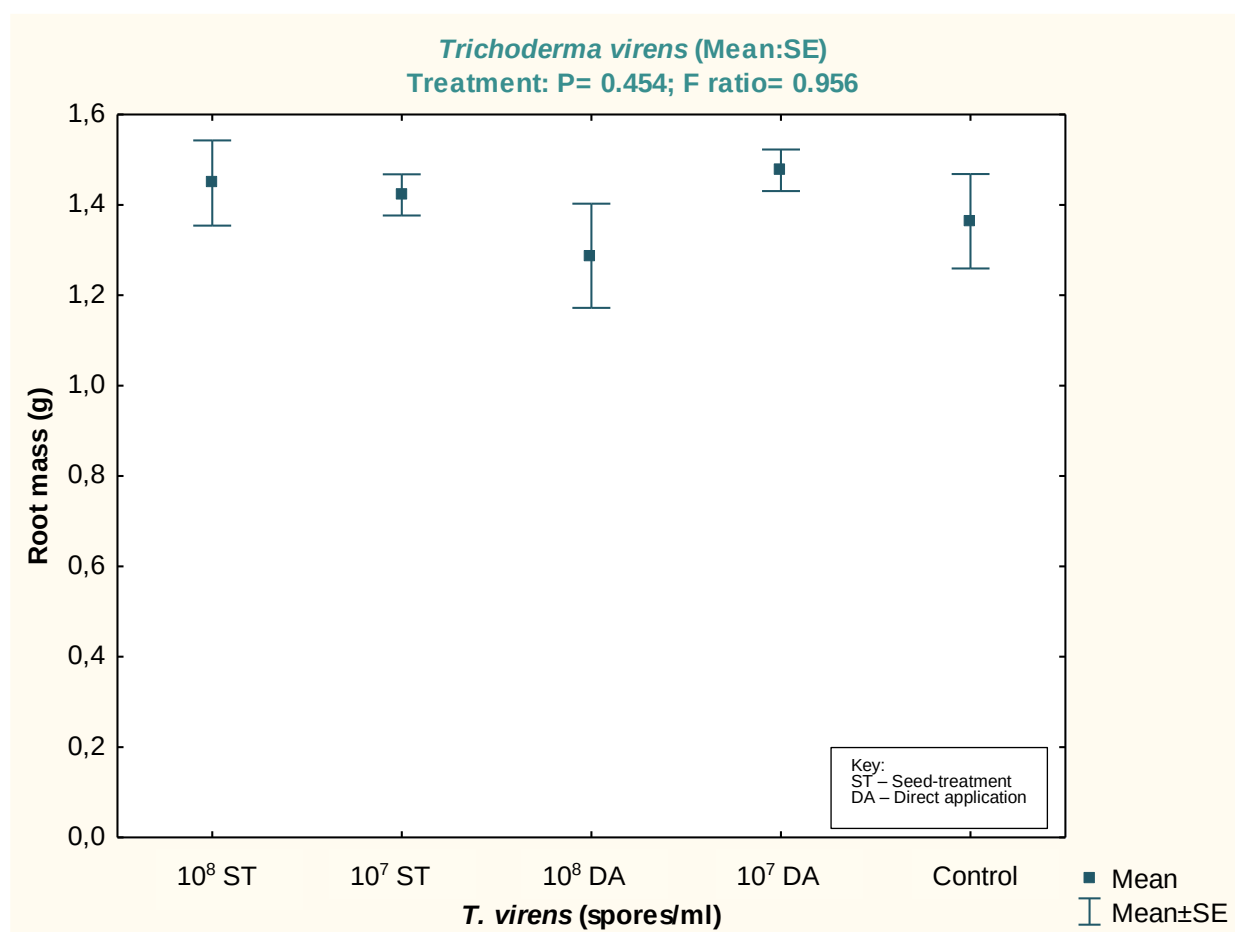


Figure 13: The effect of different concentrations (spores per ml) of *Trichoderma virens* on the root mass under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation using both a seed treatment (ST) and direct soil application (DA). An untreated control containing sterilized water was included.

1.4. Results: In vivo screening utilizing formulated fungal-based products: Products CR (a.i. *C. rosea* strain PHP 1701) + Eco-T® (a.i. *T. asperellum* strain kd)

1.4.1. Nematode soil data: 48 DAP

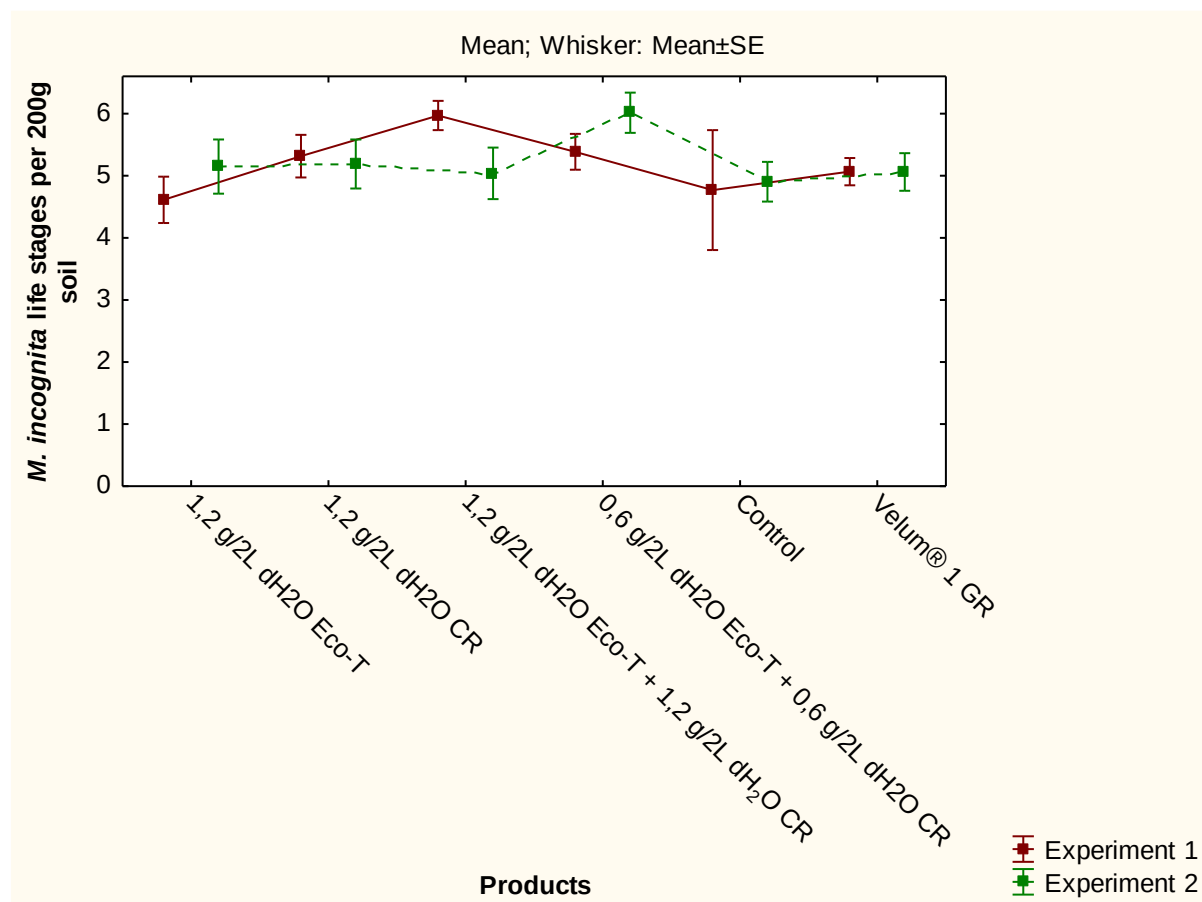


Figure 14: The effect of different concentrations of products CR and Eco-T® on the reproduction of *Meloidogyne incognita*, referring to number of life stages per 200 g soil, under *in vivo*, glasshouse conditions 48 days after planting, fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.

1.4.2. Plant parameter data: Root mass 48 DAP

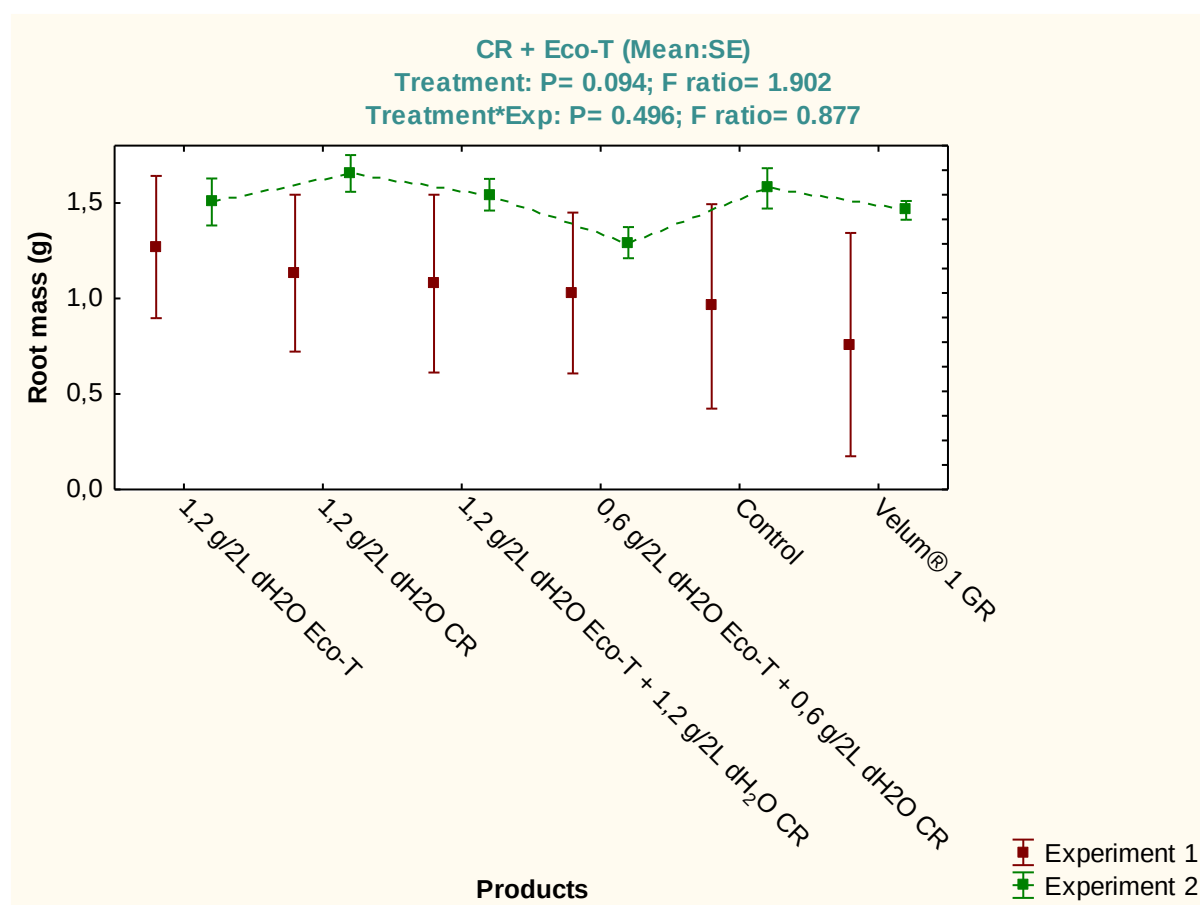


Figure 15: Root mass data (g) indicating the effect of different concentrations of products CR and Eco-T® under *in vivo*, glasshouse conditions 48 days after planting and inoculation with fungal and nematode inoculation. An untreated control containing sterilized water as well as Velum® 1GR as an upper control was included.