USE OF *TRICHODERMA* AND *BACILLUS* ISOLATES AS SEED TREATMENTS AGAINST THE ROOTKNOT NEMATODE, *MELOIDOGYNE JAVANICA* (CHITWOOD)

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

In the

Discipline of Plant Pathology

School of Agriculture, Earth and Environmental Science (SAEES)

College of Agriculture, Engineering and Science (CAES)

University of KwaZulu-Natal

Pietermaritzburg, South Africa

December 2015

UNIVERSITY OF
KWAZULU-NATAL ™

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THESIS SUMMARY

Biological control of plant parasitic nematodes, especially rootknot nematodes, has been a subject of intense research since the start of the 1900's. Bionematicides, however, have not received much attention because of the dominance of of agrochemical nematicides. Widespread de-registration and a complete phase out, in some cases, of major nematicides in the last decade has renewed the search for effective alternatives. Natural antagonists of plant parasitic nematodes such as bacteria and fungi, especially those resident in the plant root rhizosphere, have become candidates for the management of these pests. Though many bacterial and fungal strains have been evaluated as possible bionematicides, the development of consistent and cost effective formulations has been a challenge in the tropics. Products thus far commercialised for management of rootknot nematodes have targeted the egg and adult stage, and hence they have not prevented infection of plants by the mobile juvenile stages. This, coupled with the large quantities of these products required to successfully reduce plant damage and nematode reproduction, is a major limitation, especially for low value crops and for small scale farmers.

This study investigated the use of *Bacillus* spp. and *Trichoderma* spp. as seed treatments for the management of the rootknot nematode, *Meloidogyne javanica* Chitwood. The main goals were: to review the literature existing on management of rootknot nematodes; to screen and identify native *Bacillus* and *Trichoderma* strains for their *in vitro* efficacy against *M. javanica*; to determine the ability of these strains to reduce rootknot damage when applied as seed-treatments on tomato and soybean crops in the glasshouse; to investigate the ability of seed treatments to reduce rootknot damage and to improve yield oftomato and soybean in field trials inoculated with rootknot nematodes; and to determine the ability of selected biocontrol seed treatments to reduce the damage caused by the *M. javanica/Rhizoctoniasolani* disease complex on soybean.

A total of 111 *Trichoderma* and 70 *Bacillus* strains were isolated from the root zone of field crops and from animal pastures. They were screened against *M. javanica* second juvenile stages (J2s) *in vitro*. Out of these, the best five *Trichoderma* isolates and the best three*Bacillus* isolates were selected for further evaluation in the glasshouse. Eleven *Trichoderma* strains caused J2 mortality greater than50% and five *Bacillus* strains caused J2 mortality greater than 80% after

24 hours at 1×10^8 c.f.u. ml^{-1}. In dual culture assays with *Rhizoctonia solani*, a pathogen often associated with rootknot nematodes, four *Trichoderma* strains inhibited the mycelial growth of the pathogen.

Four *Trichoderma* strains (named C29, C59, C63 and C97), three *Bacillus* strains (named BC27, BC29 and BC31) and a commercial biocontrol agent, Eco-T®, were further evaluated against *M. javanica* in the glasshouse as seed treatments on soybean. All five *Trichoderma* strains, Eco-T®, and two *Bacillus* strains (BC27 and BC29) reduced root galling on soybeans and increased plant growth parameters ($P \leq 0.0001$). In field trials all the *Trichoderma* strains and a*Bacillus* strain (BC27) caused a significant increase in tomato yield and also caused a reduction in gall severity and nematode counts ($P \leq 0.0001$). All the test isolates also caused a reduction in gall severity on soybean in the field, although none of them caused a significant increase in either shoot and seed weight. In further glasshouse studies, four *Trichoderma* strains, (C29, C59, C63 and Eco-T®), reduced disease severity on soybean plants inoculated with both *M. javanica* and *Rhizoctonia solani* ($P \leq 0.0001$).

The use of bionematicides, especially as seed treatments, is a cost effective, safe and easy way to manage plant parasitic nematodes. The fungal and bacterial isolates evaluated in this study were able to reduce rootknot nematode damage while increasing yield in crops such as soybeans where no nematicides are currently registered and no rootknot resistant cultivars are currently available in South Africa. When combined with other integrated management tools, bionematicides could be a key component of crop production.

Keywords: Biological control, rootknot, *Trichoderma*, *Bacillus*, seed treatment

DECLARATION

I, Cleopas Chenai Chinheya, declare that the research reported in this thesis, except where otherwise indicated, and is my original work. This thesis has not been submitted for any degree or examination at any other university. This thesis does not contain any other persons' data, pictures, graphs or other.

This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted then their words have been re-written but the general information attributed to them has been referenced.

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ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to my wife, Ireen,

our children, Rufaro and Chenai,

my parents,

and my brothers and sisters.

To God, be the glory

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THESIS INTRODUCTION

Global food production must increase by 50% to meet the projected demand for food globally by 2050 (Chakraborty and Newton, 2011). An estimated 16% of all crops is lost globally each year due to plant diseases (Oerke, 2006). In developing countries, the problem of plant disease is worsened by the lack of resources dedicated to their study due in part to the difficulty of quantifying plant diseases and the resultant losses (Strange and Scott, 2005). Among the plant pests and diseases, nematodes are estimated to cause about 10% loss of global crop production, which is one third of the losses attributed to pests and diseases generally (Whitehead, 1998). The lack of nematology expertise in most countries, especially in developing countries (Luc et al, 1990; De Waele and Elsen, 2007, Jones et al, 2013), and the little attention paid to plant parasitic nematodes due to their insidious nature of attack, may imply even higher global yield loss estimates(Hassan et al, 2013). Most of the economic damage due to plant parasitic nematodes is attributed to two groups, cyst nematodes and rootknot nematodes (Abd-Elgawad and Askary, 2015). The latter group attack almost every species of higher plants and are responsible for the high yield losses in crops such as potato, soybeans, tomato and tobacco, among others (Sasser, 1980).

Fumigant and non-fumigant nematicides are still the most preferred method of controlling plant parasitic nematodes, in particular rootknot nematodes (Sikora and Fernández, 2005). However, most nematicides are gradually being withdrawn across the world due to the toxicity and their negative effects on the environment (Zasada et al, 2010; Onkendi et al, 2014). Host plant resistance has also been utilised for management of nematodes with varying success in different crops. However, the ability of some rootknot species to break resistance coupled with limited germplasm for some crops has restricted the use of this option (Cook and Starr, 2006; Karssen and Moens, 2006; Fourie et al, 2015). In recent years, significant attention has been focused on the use of microbial agents as possible alternatives to nematicides (Kerry, 1997; Hallman et al, 2009). In particular, isolates belonging to *Trichoderma* spp. and *Bacillus* spp. have been found to offer protection to rootknot nematode attack in many crops (Li et al, 2005; Singh and Siddiqui, 2010; Radwan et al, 2012, Zhang et al, 2014). The use of bionematicides especially if combined with other management options may offer better rootknot management options especially in resource poor countries.

The objectives of this study were to isolate *Trichoderma* and *Bacillus* strains from the root zone of plants and to evaluate them for their ability to control the rootknot nematode *Meloidogyne javanica* (Chitwood). The specific objectives were:

- To review the literature on the use of biological control agents against rootknot nematodes;
- To isolate and screen *Bacillus* isolates from the root zone of crops and animal pasture*in vitro*;
- To isolate and screen *Trichoderma* isolates from the root zone of crops and animalpasture*in vitro*;
- To select the best *Bacillus* and *Trichoderma* strains for use as bionematicides;
- To evaluate the efficacy of *Bacillus* isolates as seed treatments against *M. javanica* on soybean;
- To evaluate the efficacy of *Trichoderma* isolates as seed treatments against M. *javanica* on soybean;
- To evaluate the efficacy of selected *Bacillus* and *Trichoderma* isolates on tomato yield and *M. javanica* damage;
- To evaluate the efficacy of selected *Bacillus* and *Trichoderma* isolates on soybean yield and *M. javanica* damage;
- To investigate the effect of *Trichoderma* seed treatments on the *M. javanica-R. solani* disease complex, on soybean.

This dissertation is comprised of six discreet chapters: one literature review, followed by five research chapters. This is the dominant thesis format adopted by the University of KwaZulu-Natal because it facilitates the publication of research output more readily than the older monograph form of thesis. There is, therefore, some unavoidable repetition of references and introductory information between chapters.

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CHAPTER ONE

LITERATURE REVIEW

1.0   Introduction

Annually, plant parasitic nematodes are estimated to cause global crop losses of up to 14.6% of total crop production (Whitehead, 1998; Nicol et al, 2011) These crop losses, estimated at 157 billion USD (Hassan et al, 2013), are further compounded by an estimated 500 million USD spent on nematode control annually (Keren-Zur et al, 2000). Global crop losses to plant parasitic nematodes may even be higher because data is lacking from most countries which lack nematology expertise (Luc et al, 1990; De Waele and Elsen, 2007, Jones et al, 2013). Crop production losses in tropical and subtropical countries are estimated at 14,6% as compared to 8.8% in developed countries (Nicol et al, 2011), which is almost a third of the losses attributed to pests and diseases (Whitehead, 1998). Attention is seldom paid to nematodes because they are insidious pests with symptoms on attacked plants often resembling other abiotic stress factors (Hassan et al, 2013). Currently, about 4100 plant parasitic nematodes have been described (Decraemer and Hunt, 2006) and of these, 126 species from 33 genera are currently listed as regulated pests in one or more countries worldwide (Singh et al, 2013). Some of these plant parasitic nematodes live outside plant roots from where they cause damage and also transmit viruses. However, most of the economic damage in crops is attributed to sedentary endoparasites in the family Heteroderidae which comprises of two groups, the cyst nematodes and the rootknot nematodes (Williamson and Hussey, 1996; Abd-Elgawad and Askary, 2015). Sedentary nematodes have been managed using nematicides since the 1950s' (Moens et al, 2009). However pressure to reduce the use of nematicides because of health and environmental concerns has led to the development of antagonistic micro-organisms as biological control agents to these pests (Viaene et al, 2006).

Rootknot nematodes, *Meloidogyne* spp., which are widely distributed in tropics and subtropics, attack almost every species of higher plants and are the most damaging nematode genus globally (Sasser, 1980; Onkendi et al, 2014). In South Africa rootknot nematodes are the most common and most destructive nematode species and *Meloidogyne javanica* Chitwood is the most economically important species (Fourie et al, 2001a). Rootknot nematodes have been

recorded to attack and cause significant damage to most crops in South Africa, resulting in significant yield losses and total crop failure in some incidences (Fourie et al, 2001b; Onkendi and Moleleki, 2013). The success of rootknot nematodes is attributed to their reproductive capacity, endoparasitic nature as well as several physiological and biochemical adaptations during their life cycle (Moens et al, 2009).

1.1   Rootknot life cycle and damage

Rootknot nematodes are obligate sedentary endo-parasites, spending most of their active life stages feeding inside plant roots (Karssen and Moens, 2006). The life cycle of rootknot nematodes is illustrated in Fig 1. Depending on species and environmental conditions the life cycle generally lasts 3 – 6 weeks and comprises of four juvenile stages and the adult stage (Castagnone-Sereno et al., 2013). The adult female nematode lays eggs in a gelatinous matrix, composed of glycoproteins, which protects the eggs from extreme environmental conditions (Moens et al, 2009). This egg batch is normally laid on the surface of galls although it may be laid inside them. The life stages of the rootknot nematode are separated by moults and the first moult occurs inside the egg, giving rise to a second stage juvenile (J2), which hatches out of the egg. The second juvenile stage, which is the motile and infective stage, penetrates the roots above the growing tip and migrates to the meristematic tissues near the vascular tissues (Bird et al, 2009). Here the juvenile initiates a permanent feeding site by inducing adjacent cells to differentiate into specialised nurse cells known as giant cells (Moens et al, 2009). The J2 undergoes three successive moults into an obese adult female. Rootknot nematodes growth and reproduction is dependent on the giant cells for nutrition and hence they do not necessarily kill the host plant (Castagnone-Sereno et al., 2013).
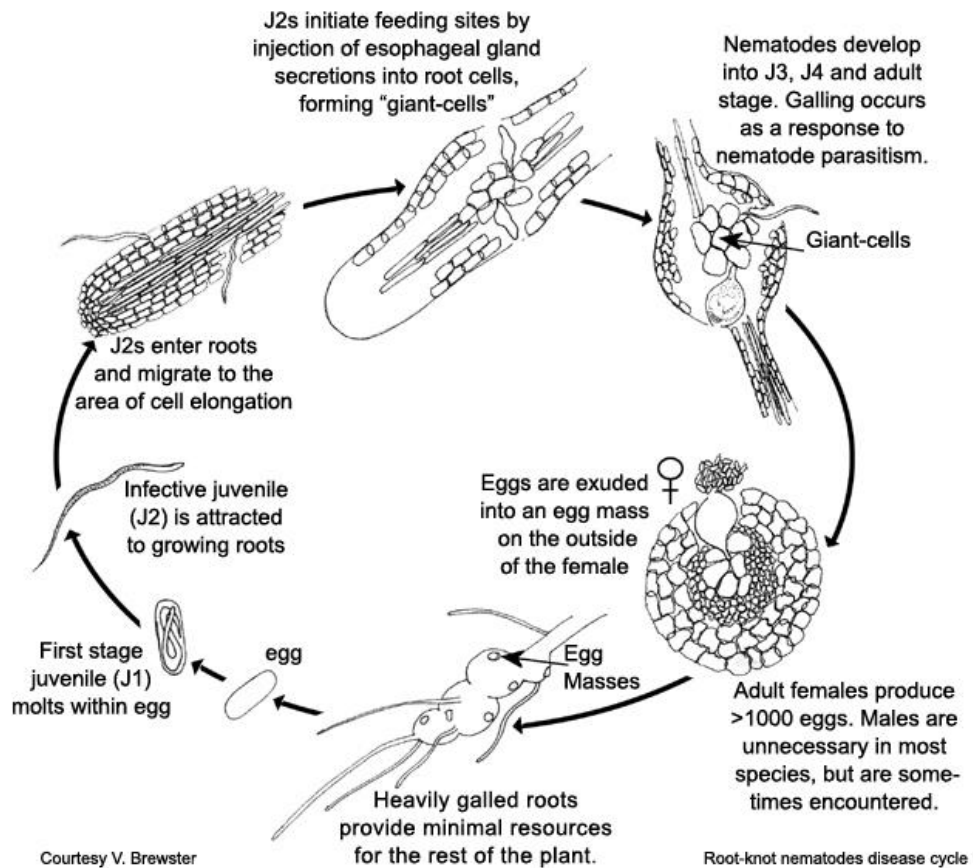
Fig 1 Life cycle of rootknot nematodes

(www.apsnet.org/edcenter/intropp/lessons/Nematodes/Pages/RootknotNematodes)

Susceptible host plants respond to nematode feeding by undergoing marked physiological and morphological changes, usually forming giant cells at the feeding sites (Moens et al, 2009). These giant cells, together with expansion and proliferation of nearby pericycleand cortical cells, results in the characteristic root-knot galls (Bird et al, 2009). Nutrient and water uptake is impaired due to these physiological changes that damage plant roots, ultimately resulting in reduced plant productivity (Castagnone-Sereno et al., 2013). Above ground symptoms of infected plants typically resemble those of plants with weakened root systems. These include reduced shoot biomass; paleness and yellowing of leaves and foliage; stunting and wilting; and reduction in yield (Moens et al, 2009). In some cases, however, infected plants may not exhibit symptoms above ground although yield reduction and distortions may occur, e.g., with root and tuber crops such as potatoes and carrots.

Management of rootknot nematodes is one of the important costs of production in Africa (Onkendi et al, 2014) and in other parts of the world (Wesemael et al, 2011) and hence several ways of managing nematodes have been investigated.

1.2 Rootknot Taxonomy

Rootknot nematodes were originally referred to as *Anguillula marioni* Cornu, 1879, then *Heterodera radicicola* (Greeff, 1872), Müller, 1884 and after 1932 *Heterodera marioni* (Cornu, 1879) Goodey, 1932 until Chitwood (1949) reinstated *Meloidogyne* (Kleynhans, 1991). The genus contains more than 80 nominal species (Subbotin and Moens, 2006).

Taxonomic Tree

Domain: Eukaryota

    Kingdom: Metazoa

        Phylum: Nematoda

           Family: Meloidogynidae

              Genus: *Meloidogyne*

Source: cabi.org/isc/datasheet/33246

1.3 Management of rootknot nematodes

The main aim of managing rootknot nematodes, as with all pests, is to reduce plant infection, thereby optimising yield with reduced production costs (Karssen and Moens, 2006). The use of chemicals, physical control, plant resistance and biological control have been used singly or integrated to manage rootknot nematodes with varying success (Whitehead, 1998).

Though the methods for the management of rootknot nematodes outlined above have been used with varying degrees of success, they also have their shortfalls. A critical evaluation of individual tools and the determination of cost-benefit ratios is important in the development of nematode integrated programmes for them to be adopted by farmers (Sikora et al, 2005). Rootknot nematodes are usually controlled effectively using a combination of methods rather than one measure (Whitehead, 1998). The main aim of IPM against nematodes is usually to

reduce the density of nematodes to levels below the economic injury level rather than eradication.

1.3.1 Physical control

This involves manipulating the habitat of the organism thereby making it unsuitable for survival. Rootknot nematodes are fragile organisms negatively affected by rapid changes in abiotic factors such as heat, moisture, oxygen availability and availability of host.

1.3.1.1 Steaming

Most soil plant pathogens, including nematodes and weeds are effectively controlled by steam sterilising at $80 - 100°C$ (Messenger and Braun, 2000, Samtani et al, 2012). Various steaming methods which include aerated steaming (van Loenen, 2003), negative pressure steaming and the Fink and Hood steaming technique (Messenger and Braun, 2000) have been successfully used to sterilise seedbed and glasshouse soil in horticulture. Steaming is mainly restricted to glasshouse and small fields because it is very expensive and it requires energy sources and specialised machinery (Lamberti, 1997). In addition, steaming leaves a biological "vacuum", thereby rendering the soil more prone to infestation by pathogens (Messenger and Braun, 2000). For rootknot nematodes steaming often fails to effectively supress the population because they can be found in deeper soil layers (Karssen and Moens, 2006).

1.3.1.2 Soil solarisation

Temperatures lethal to plant parasitic nematodes may also be attained by soil solarisation with a layer of plastic mulch, especially in areas with prolonged solar radiation (Hildalgo-Diaz and Kerry, 2007). Significant reduction in nematode infestation has been attained using solarisation alone (Nico et al, 2003; Oka et al, 2007) or in combination with organic amendments (Oka et al, 2007, Colombo et al, 2012, Marahatta et al, 2012), bio-nematicides (Giannakou et al, 2007) and nematicides (Chellemi et al, 1997). However, the cost of plastic mulch, the time required for effective solarisation, dependence on weather, soil texture and depth, and disposal methods for plastic mulch are significant drawbacks for solarisation methods (Messenger and Braun, 2000).

1.3.1.3 Biofumigation

Biofumigation is defined as a process that occurs when volatilecompounds with pesticidal properties are released during decomposition ofplant materials or animal products (Youssef, 2015). The main active compounds involved in biofumigation are glucosolinates (GSLs) and isothiocynates (ITCs), which are mostly produced when tissues of crucifers are hydrolysed

(Kruger et al, 2013). Some of these hydrolysis products, particularly the ITCs, are known to have broad biocidal activity including insecticidal, nematicidal, fungicidal, antibiotic and phytotoxic effects (Salem, 2014). Biofumigation is as an effective method for control of rootknot nematodes in various agrosystems (Anita, 2012; Youssef and Lashein, 2013; Edwards and Ploeg, 2014; Ismail, 2015). The large amount of organic matter to be transported to the field or the cost of cover crops to be incorporated into the soil, however, is the main limitation on the practical approach of this method (Hildalgo-Diaz and Kerry, 2007).

1.3.1.4 Crop rotation

Cultural control practices for rootknot nematodes emphasize the accurate identification, preferred hosts and environmental preferences (Viaene et al, 2006). This information is usually lacking in subsistence farming systems, especially in the subtropics where technical expertise is often lacking. Global population increases have resulted in less land being devoted to agriculture, consequently limiting the availability of land for effective rotations. Economic justification of rotations is often difficult due to the lack of cost benefit data.

Crop rotation, which involves alternating crops with non-hosts or poor hosts to the nematode species of interest, is one of the most extensively used cultural means of reducing rootknot nematode populations (Hildalgo-Diaz and Kerry, 2007). Soil infestation by *Meloidogyne* spp. may be significantly reduced by alternating crops with grasses, cereals, *Compositae* and other non-host plants (Whitehead, 1998).Because populations of the rootknot nematodes composed of multiple species are increasingly widespread, effects of crop rotation must be completely characterised (Hildalgo-Diaz and Kerry, 2007). Stirling (2013), found the integration of crop rotation, organic amendments, mulching and solarisation to be beneficial for vegetable production. Alternating resistant and susceptible rootknot cultivars together with sunnhemp also significantly reduced galling in tobacco (*Nicotiana tabacum* LINN.) (Mazarura et al, 2012). The alternative crop, however has to give substantial income and increase yield in the subsequent crop for this method to be successful (Starr et al, 2007). Also, different populations of the same *Meloidogyne* species may react differently to the same host plant, hence caution needs to be taken when extrapolating results from one location to another (Karssen and Moens, 2006).

1.3.2   Host plant resistance

Development of nematode resistant crops is ideally the most cost effective and environmentally effective method of managing crop losses (Dowler and van Gundy, 1984; Zasada et al, 2010). The availability of and access to plant germplasm collections containing genes for resistanceand rapid advances in plant science technologies have made host plant resistance (HPR) an important tool in management of nematodes (Roberts, 1992). There has been extensive research in the development of resistance in different crops targeting different pests in the nematode community (Wesemael et al, 2011). To this end, a number of individual plant resistance (R) genes have already been evaluated and are being efficiently used in crop improvement research programs (Gurunani et al, 2012). In wheat, soybean and potato crops HPR has been successfully used to manage various cyst nematode species (Dowler and van Gundy, 1984; Cook and Starr, 2006; Hildalgo-Diaz and Kerry, 2007). The *Mi* gene in tomato confers resistance to *Meloidogyne javanica*, *Meloidogyne incognita* and *Meloidogyne arenaria* and is extensively used in managing these species (Williamson and Hussey, 1996; Wesemael et al, 2011). However, *Meloidogyne* isolates capable of breaking this resistance have been reported from various parts of the world (Karssen and Moens, 2006).

The use of resistant cultivars has been relied upon in most crops for management of various *Meloidogyne* species. However, it is difficult to solely rely upon host resistance because rootknot nematodes have many species, races, biotypes and populations. Though polygenic genes have been utilised in some crops, emerging 'resistance breaking' rootknot strains often complicate the management of monogenic resistance in crops. Also available resistance is often linked to undesirable characteristics that make growing of resistant cultivars unattractive, and often the costs of developing resistant cultivars may not be justified by the scale of problem (Cook and Starr, 2006).

1.3.3   Chemical control

Nematicides have been relied upon for the control of rootknot nematodes in most cropping systems over the last five decades. Nematicides are mainly used to control rootknot nematodes in high value crops, and it is usually uneconomic to apply them on low value crops (Karssen and Moens, 2006). Cost, environmental concerns and toxicity to users have generally limited the use of nematicides in agriculture (Haydock et al, 2006). Frequent use of nematicides often leads to reduced efficacy because of accelerated microbial degradation (Whitehead, 1998).

Nematicides are chemical compounds that directly kill nematodes and they are applied mainly to limit plant damage (Haydock et al, 2006). Nematicide use in crop production can be traced back to the work with carbon disulphideby Kuhn in 1871 in efforts to control the sugarbeet nematode, *Heterodera schachtii* (Schmidt 1871)(Taylor, 2003). Since then nematicides have been the primary method of control for nematodes in various crops (Hildalgo-Diaz and Kerry, 2007). The nematode life cycle, which is largely restricted to the soil and plant roots, the relative impermeability of the nematode body surface and the limited access to the nematode oral route makes it difficult to effectively deliver chemical toxicants. In that respect most nematicides are broad spectrum and volatile compounds able to permeate through the soil (Chitwood, 2003). Nematicides fall into two main categories, fumigants and non-fumigants and are further classified according to their mode of action (Hildalgo-Diaz and Kerry, 2007).

Fumigants, which are compounds based on halogenated hydrocarbons and those that release methyl isothiocyanate, are mainly used as pre-plant treatments (Hildalgo-Diaz and Kerry, 2007). These compounds are usually applied as liquid formulations and turn to the gaseous phase in the soil. They are effective upon contact with adults, juveniles and eggs, together with other pests and weeds (Sikora et al, 2005). The fumigants include chloropicrin, 1,3-dichloropropene (1,3-D), methyl bromide, 1,2-dichloropropane (1,2-DBCP), 1,3-dichloropropene and 1,2-dichloropropane mixtures (DD), formaldehyde, Ethylene di bromide (EDB), metam sodium and dazomet. These products were developed in the first half of the 20th century (Haydock et al, 2006).

Methyl bromide, because of its broad spectrum of activity,was the dominant soil fumigant for managing plant-parasitic nematodes and other soil borne pests (Zasada et al, 2010). Methyl bromide was highly effective against rootknot nematodes as a pre-plant treatment in seedbeds and in the field. However,it is extremely damaging to the atmospheric ozone layer and, according to the 1997 Montreal protocol, its use was scheduled to be stopped in all countries by the year 2015 (Haydock et al, 2006; Zasada et al, 2010). Another frequently used fumigant, ethylene dibromide, has also been deregistered in the US and in most countries in the world (Chitwood, 2003).

Since the phase-out of methyl bromide the use of other fumigants have been explored while some have been reregistered for use in some countries, especially for high value crops

(Chitwood, 2003, Zasada, 2010). The fumigants, including methyl iodide, 1,3-D, metam sodium and dazomet, have been registered as pre-plant treatments for the control of rootknot nematodes on tobacco seedbeds and fields. However, possible carcinogenic properties in methyl iodide (Bolt and Gansewendt, 1993), 1,3-D (Klaunig et al, 2014) and metam sodium (Sugeng et al, 2013), as well as being highly toxic to users and non-target soil organisms, are issues of concern among users and consumers. Fumigants are also relatively ineffective in soils with a clay content above 15-20%, and with a high organic content (Noling, 2003).

The dominant non-fumigant nematicides are organophosphates and carbamates, which are applied to the soil at planting as granular or liquid formulations (Hildalgo-Diaz and Kerry, 2007). These have either contact or nemastatic properties against nematodes and usually immobilize nematodes when applied at higher rates (Sikora et al, 2005; Hildalgo-Diaz and Kerry, 2007). Non-fumigant nematicides, however, do not kill eggs at current recommended dosages, do not have broad spectrum activity and only provide protection to crops in the early phase of growth (Sikora et al, 2005).

Nematode management programs are still reliant on nematicides, whether used singly or in integration with other methods (Haydock et al, 2006). The global market of nematicides is about 250 000 tonnes of active ingredients annually (Haydock et al, 2006; Hildalgo-Diaz and Kerry, 2007) and is estimated to be worth US $300 million in the USA (Haydock et al, 2006).

Health and environmental risks associated with nematicides have led to significant efforts to find alternative nematode management options (Stirling and Stanton, 1997). Application of nematicides to the same soil continually also may lead to reduced persistence, a process known as accelerated microbial degradation (Haydock et al, 2006). In recent years some nematicides have been deregistered and restricted due to issues associated with underground water contamination, effects on non-target organisms, human safety and residues in food stuffs (Haydock et al, 2006). These developments have invigorated the research for alternative nematode management techniques that do not depend on chemical nematicides (Rodriguez-Kabana et al, 1987).

There has been a marked shift in research on management from nematicides to biological control (Sikora et al, 2005). Various studies have shown varying levels of success with the integration of biocontrol with crop rotation, soil disinfestation, soil amendments and green

manures and nematicides (Al-Rehiayani et al, 1999; Chen et al, 2000; Hildalgo-Diaz and Kerry, 2008). Biological control may not totally replace nematicides in the future. However, they remain an integral part of IPM strategies in developing agriculture (Kerry, 1997)

1.3.4   Biological control

In the context of managing plant parasitic nematodes biological control is defined as "the management of plant diseases and pests with the aid of living organisms" (Viaene et al, 2006). Microbial pathogens, endophytes and antagonists play a crucial role in the regulation of plant parasitic nematodes in various agroecosystems (Hallmann et al, 2009). The use of microbial agents for the control of nematodes has been the subject of intense research in recent years as a possible alternative to nematicides (Mankau, 1981, Kerry, 1997). Fungi and bacteria are an integral part of the soil community and both have shown potential as nematode antagonists (Akhtar and Malik, 2000).

1.3.4.1 Bacteria

Bacteria used in biocontrol of plant parasitic nematodes can be categorised into two groups, parasitic bacteria and non-parasitic rhizobacteria (Siddiqui and Mahmood, 1999). These two groups differ in their mode of action.

1.3.4.1.1 Parasitic bacteria

The genus *Pasteuria* contains the only obligate, endospore forming bacterial parasites of major importance to nematode control (Sikora, 1992).*Pasteuria* are obligate hyperparasites of plant-parasitic nematodes and have generated intense interest as a promising biocontrol agent (Cho et al, 2000; Preston et al, 2003).

Parasites of the genus *Pasteuria*have a similar life cycle in different hosts, which begins with bacterial spores attaching to nematode juveniles as they move in the soil (Viaene et al, 2006). These spores later germinate, form germ tubes that penetrate the developing juvenile and the germ tubes form primary colonies in the pseudocoelom (Chen and Dickson, 1998). Many daughter colonies that are formed from vegetative microcolonies form sporangia from which endospores are latter formed. The parasitised nematode survives but its fecundity will be greatly reduced with female adults containing as much as two million spores that are released into the

soil (Viaene et al, 2006; Tian et al, 2007). A single spore binding to the body wall of a (J2) may be enough to cause infection and propagation of the parasite (Preston et al, 2003).

Pasteuria spp. are host specific and are known to successfully parasitise rootknot species among others. The most common and widespread species, *Pasteuria penetrans* (Thorne 1940), is mainly parasitic on *Meloidogyne* spp., whilst *Pasteuria thornei*(Sayre and Starr, 1988); and *Pasteuria nishizawae* parasitise lesion and cyst nematodes, respectively (Viaene et al, 2006). *Pasteuria penetrans*significantly reduced galling caused by *Meloidogyne arenaria* (Neal) Chitwood in tomato *(Solanum lycopersicum* L.) (Cho et al, 2000). Similar studies on *M. arenaria* Race 1 also resulted in reduction and root galling and overwintering juvenile populations over two subsequent seasons (Chen et al, 1996). *Pasteuria penetrans*also reduced galling and egg mass counts in eggplant (*Solanum aethiopicum* L.) (Ahmad et al, 2007) and in soybean (*Glycine max* (L.) Merr.) (Sharma and Vivaldi, 1999).

Some of the key characteristics that make *P. penetrans*a successful biocontrol candidate are its ability to limit nematode reproduction, reduce infectivity of spore-bearing juveniles, persist in soil for long periods and its resistance to desiccation and extreme temperatures (Siddiqui and Mahmood, 1999). In addition, *P. penetrans* can be successfully integrated with some non-fumigant nematicides and other cultural control methods (Sikora et al, 1992; Siddiqui and Mahmood, 1999). Following the successful development of mass propagation methods of some *Pasteuria* isolates, a biocontrol product has since been commercialised for the control of soybean cyst nematodes (Wilson and Jackson, 2013). However, challenges still remain on the management of the broader community of plant parasitic nematodes because *Pasteuria* spp. have narrow host ranges. In addition, the development of low cost, mass production techniques remain a challenge, especially in developing countries (Gowen et al, 2008).

1.3.4.1.2 Rhizobacteria

Bacteria that are rhizosphere competent or that colonise the rhizosphere are commonly referred to as rhizobacteria. Kloepper and Schroth (1978) coined the term Plant Growth Promoting Rhizobacteria (PGPR) for rhizobacteria capable of enhancing plant growth. Globally, research into the use of PGPRs has increased greatly since then as they have gained importance in agriculture (Figueiredo et al, 2011). Many of these PGPRs may also supress plant disease (Siddiqui et al, 2007).

The early phase of root penetration by nematodes is important because it often has an impact on the final degree of plant damage (Sikora et al, 2008). Early protection of the rhizosphere of plants with rhizobacteria is therefore important because it targets the vulnerable juvenile stage of nematodes (Kerry, 2001). Metabolites produced by some bacteria, especially *Burkholderia* spp., *Pseudomonas* spp. and *Bacillus* spp. interfere with nematode behaviour, feeding and reproduction, thereby reducing penetration and damage in plants (Viaene et al, 2006).

A number of *Pseudomonas* spp. have potential as biocontrol agents for the management of rootknot nematodes. Timper et al (2009) used seed treatments of various crops to supress *M. incognita* populations. In peas (*Pisum sativum*L.), apart from reducing *Meloidogyne incognita* Chitwood galling and reproduction, *Pseudomonas* isolates also increased plant growth (Siddiqui et al, 2009). Rhizosphere competent *Pseudomonas* isolates, isolated from suppressive soils, also increased tomato plant growth while reducing *M. incognita* damage and reproduction (Singh and Siddiqui, 2010). *In vitro* studies on the efficacy of *Pseudomonas fluorescens* UTPF5 killed *M. javanica* second stage juvenile after 24 hours (Bagheri et al, 2014). Several investigations have also been carried out on the potential of *Bacillus* spp. as rhizobacterial antagonists of rootknot nematodes (Table 1).

Various mechanisms employed by rhizobacteria to reduce nematode damage and reproduction in plants have been suggested and include: (1) regulating nematode behaviour; (2) interfering with nematode-host recognition; (3) competition for nutrients; (4) plant growth promotion; (5) induced systemic resistance (Ongena and Jacques, 2008; Siahpoush et al, 2011; Adam et al, 2014) and (6) production of by-products that inhibit egg hatching, reduce juvenile survival and/or kill nematodes directly (Lian et al, 2007; Peng et al, 2011; Zhang et al, 2012; Oliveira et al, 2014).

1.3.4.1.3 Bacteria (Inundative Applicationsversus Seed Treatments)
The majority of the research on the use of rhizobacteria as biocontrol agents of rootknot nematodes have been focused on inundative approaches where bacterial cell suspensions or culture filtrates are applied to the root zone *in-situ* (Sikora, 1992). However, this approach has performed poorly in field trials because of the inconsistencies in product distribution as well as the costs involved in treating 2500 tons of bulk soil at 25cm deep per hectare (Sikora et al,

2008). In addition, this method of application increases production costs, reduces profit margins and is uneconomical, especially for low value crops (Sikora and Pocasangre, 2006). Economically viable product application methods such as root dips and seed treatments have since been found to be more promising, because they reduce the quantity of biocontrol agent that needs to be applied (Mahdy, 2002).

1.3.4.2 Nematophagous fungi

Nematophagous fungi vary in their characteristics which include: obligate parasites which survive on nematodes; facultative parasites which sometimes feed on nematodes; and other fungithatare intermediate between the two (Viaene et al, 2006). These fungi vary in the nematode-life stage they attack and also in mechanisms involved.

Table 1 Studies on biocontrol of rootknot nematodes by *Bacillus* spp.

Bacillus spp.	*Meloidogyne* spp	Effect of bacteria on nematode	Reference
B. thuringiensis	*M. hapla*	Reduced galling on lettuce	Chen et al, 2000
B. cereus	*M. javanica*	Reduced penetration and development on tomato	Oka et al, 1993
B. megaterium	*M. graminicola*	Reduced penetration and development on rice	Padgham and Sikora, 2007
B. firmus	*M. incognita*	Larval mortality and reduced egg hatching *in vitro*	Mendoza et al, 2009
B. firmus	*M. incognita*	Larval mortality and reduced egg hatching *in vitro*. Reduced damage *in vivo*	Terefe et al, 2009
B. pumilus	*M. javanica*	Larval mortality and reduced egg hatching *in vitro*. Reduced damage *in vivo*	Siddiqui et al, 2007
B. subtilis	*M. javanica*	Larval mortality and reduced egg hatching *in vitro*. Reduced damage on cowpea and mash bean	Dawar et al, 2008
B. subtilis	*M. javanica*	Seed treatment or drench reduced damage and larval population in rhizosphere.	Li et al, 2005
B. subtilis	*M. incognita*	Increased shoot weight of tomatoes	Siddiqui and Akhtar, 2009
Bacillus isolates	*M. incognita*	Increased shoot weight and reduce tomato galling	Singh and Siddiqui, 2010
B. subtilis *B. thuringiensis*	*M. incognita*	Formulation increased yield and reduced tomato damage in the field	Khan and Tarannum, 1999
B. thuringiensis	*M. incognita*	Reducing galling in soybean when concomitantly applied with reduced oxamyl rates	El-Sherif and Isamil, 2009
B. subtilis	*Mixed species*	Increased shoot weight and reduced tomato galling	Prakob et al, 2009
B. thuringiensis	*M. javanica*	Increased shoot weight and reduced soybean galling	Mahdy et al, 2006
B. pumilus	*M. incognita*	Increased shoot weight and reduced soybean galling	Akhtar and Siddiqui, 2008
B. firmus	*M. incognita*	Promoted plant growth while reducing damage and reproduction	Xiong et al, 2015

1.3.4.2.1 Nematode trapping fungi

Among the many organisms studied for biological control of nematodes, nematode trapping fungi are the most widely studied (Stirling et al, 1998).

Nematode-trapping fungi have been described and classified mostly in *Arthrobotrys* Corda, *Dactylaria* Sacc, and *Dactylella* Grove since 1930 (Swe et al, 2011). Nematode trapping fungi immobilise nematodes using non-adhesive traps or sticky structures, usually produced on mycelia before they infect their host (Fig 2) (Viaene et al, 2006).

Various studies have been carried out to evaluate the suitability of various nematode trapping fungi as biocontrol option for rootknot nematodes. Jaffee and Muldoon (1995) found the nematode trapping fungi, *Monocrosporum ellipsosporum* (Preuss) R.C. Cooke and C.H. Dickinson to supress *M. javanica* penetration of roots in field soils. In a study to determine the influence of soil management on nematode trapping fungi, *Athrobotrys dactyloides* Drechsler and *Nematoctonus leiosporus*Drechsler were more frequent in organic than convention soils. However, suppression of *M. javanica* was not influenced by these fungi (Jaffee et al, 1998). Similarly, *A. dactyloides* and *N. leiosporus* did not reduce the level of damage to tomato roots, although they successfully colonised the rhizosphere of the plants (Persson and Jansson, 1999). However, formulations of *A. dactyloides* produced on solid phase media significantly reduced galling on tomatoes in glasshouse experiments using field soil, by as much as 57 - 96% (Stirling et al, 1998). Bordallo et al (2002) found *A. dactyloides*to aggressively colonise barley roots without hindering root growth.

Despite many years of relatively intensive research on nematode trapping fungi, progress on their commercialisation has been limited mainly because of inconsistent field performances, which has been the result of the loss of virulence and insufficient quality control in pre-application steps (Viaene et al, 2006; Swe et al, 2011). This in part is because little is known about their growth and development in the soil, as well as the factors that determine when they switch between the saprophytic and the parasitic phase (Viaene et al, 2006). A further problem is that they tend to produce relatively few, large conidia, which makes their commercialization difficult.
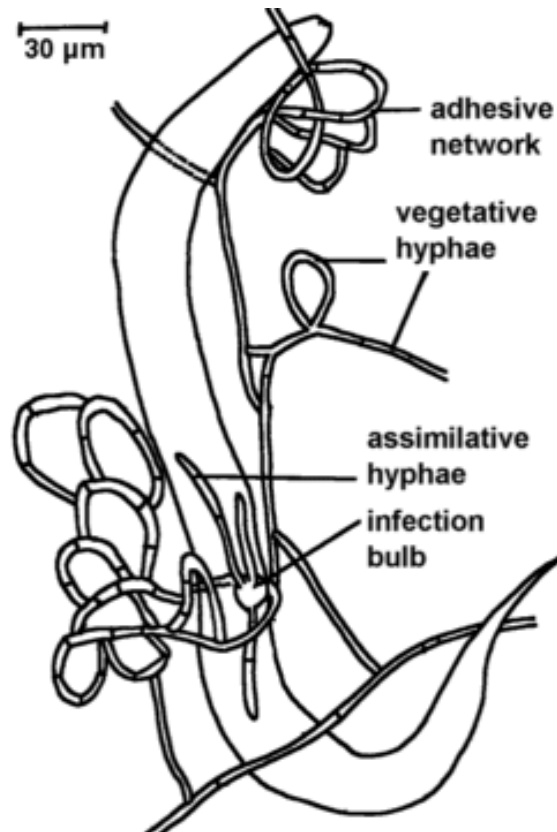
Fig 2 Nematode trapping hyphae trapping nematodes

(Source: http://www.biological-research.com/philip-jacobs%20BRIC/ar-olig.htm)

1.3.4.2.2 Arbuscular Mycorrhizae

Mycorrhizal fungi form a symbioticassociation with plant roots where the fungus acquires carbohydrates while in turn supplying the plant with beneficial products such as minerals and hormones (Siddiqui and Mahmood, 1995). Both arbuscular mycorrhizal fungi and plant parasitic nematodes share plant roots for resources, hence several studies have been undertaken to evaluate the possibility of the former mitigating the negative effects of the latter (Hol and Cook, 2005).

Arbuscular mycorrhizal fungi and root nodule bacteria have been found to reduce the severity of nematode damage in various legume crops (Siddiqui and Mahmood, 1995). Inoculating soybeans with chlamydospores of *Glomus macrocarpus* Tul. and Tul. significantly increased growth parameters while reducing galling by *M. incognita* (Kellam and Schenk, 1980). Planting cereal crops after legumes can increase the levels of arbuscular mycorrhizal fungi and reduce nematode densities (Bagayoko et al, 2000). Various *Glomus* spp. suppress plant parasitic nematode densities while increasing growth in peach (*Prunus persica* L.) rootstocks (Calvet et

al, 2001), tomato (Talavera et al, 2001), banana (*Musa acuminata* L.) (Elsen et al, 2003) and cucumber (*Cucumis sativus* L.) (Zhang et al, 2008). Both native strains and pre-inoculation with mycorrhizae also reduced colonisation and reproduction of *P. penetrans* in pioneer dune grass (De La Peña et al, 2006). Hol and Cook (2005) found that plants colonised by arbuscular mycorrhizal fungi were damaged more by ectoparasitic nematodes than by endoparasites, and of the endoparasites, rootknot nematodes were supressed more than cyst nematodes.

Investigations into the possible mechanism behind plant parasitic nematode suppression in pioneer dune grass concluded that a systemic plant response was not involved (De La Pena et al, 2006). However, Elsen et al (2008) found that induced systemic resistance was involved in reducing infection in banana due to *Radopholus similis* (Cobb) Thorne and *Pratylenchus coffeae* Zimmermann. Mycorrhizal root exudates in tomato were also implicated as the mechanism behind the reduction in penetration by *M. incognita* (Vos et al, 2012).

1.3.4.2.3 Pochonia chlamydosporia (synonym Verticillium chlamydosporium) (Goddard) Zare and Gams

Pochonia chlamydosporia is a facultative parasite of nematode eggs that colonises eggs using appressoria developed from undifferentiated hyphae on the eggshell (Kerry, 2001). The pathogenicity of *P. chlamydosporia*to nematodes is mediated by the production of proteinases (Segers et al, 1996), chitinases, esterases, lipases and serine proteinase VCP1 (Esteves et al, 2009) and a nematoxin, phomalactone (Viaene et al, 2006). Rootknot juveniles (J2) emerging from eggs of *M. incognita* and *M. javanica* have also been observed to be parasitised by *Verticillium leptobactrum* Gams (Regaieg et al, 2011). Full strength culture filtrates of *Verticillium chlamydosporium* were found to cause up to 65.5% mortality on *M. javanica*in *vitro* (Mukhtar and Pervaz, 2003). When applied as soil treatments*V. chlamydosporium* also significantly reduced penetration, galling and egg masses of *M. hapla* in lettuce (Viaene and Abawi, 2000), okra (Chaya and Rao, 2013), *Phaseolus vulgaris* L. (Sharf et al, 2014), cucumber (Viggiano et al, 2014) and carrots (Bontempo et al, 2014). In simulated field conditions *V. chlamydosporium* applied as soil treatments alone or in combination with the symbiotic bacterium *Photorhabdus luminescens*(Poinar and Thomas 1979) Boemare et al. (1993)significantly reduced gall formation and egg masses on cucumber (Zakaria et al, 2013). Bourne and Kerry (1998) found that *V. chlamydosporium*effectively colonised the plant rhizosphere thereby parasitizing 90% of exposed *M. incognita* and *M. javanica* eggs. However, they found that in large galls formed on tomatoes there was a reduction in egg infection because the fungus could not access eggs laid deep inside plant tissues. The efficacy of *V.*

chlamydosporium was therefore found to be influenced by host plant species (Bourne et al, 1996; Bourne and Kerry, 1998; Kerry, 2001). Another challenge with *V. chlamydosporium* is that the method of application using broadcast applications, as used in most studies, is not economical in large scale situations (Kerry, 2001). Some of the aspects that have made this egg-parasite one of the most studied include: its cosmopolitan distribution; its robust rhizosphere competency; the accessibility of strains from herbarium collections and that it is easily cultured in the laboratory (Manzanilla-Lopez et al, 2013).

1.3.4.2.4 Purpureocillium lilacinus (formerly Paecilomyces lilacinus) (Thom) Samson

Purpureocillium lilacinus (PL) is perhaps one of the most studied nematode biocontrol agents with studies having been done on *Meloidogyne* spp, *Heterodera avenae* (Schmidt) Franklin, *R. similis* and *Tylenchulus semipenetrans* Cobb and *Rotylenchulus reniformis* Linford and Oliveira (Viaene et al, 2006). Though early studies on PL efficacy indicated no nematicidal properties (Hewlett et al, 1988), latter strains yielded more positive results (Cabanillas et al, 1989). Studies with Strain PL251 yielded significant nematode suppression leading to the development and commercialisation of the product, especially in subtropical and tropical regions (Viaene et al, 2006). Khan et al (2006) found PL 251 to reduce both damage and densities of *M. javanica* on tomato, *H. avenae* on barley and *R. similis* on banana. Sufficient reduction of *M. incognita* galling on tomato was found to be established with a minimum dose of 1×10^6 c.f.u.g^{-1} of soil (Kiewnick et al, 2006). On cotton PL 251 was found to be comparable to aldicarb for the control of *R. reniformis* (Castillo et al, 2013). Mukhtar et al (2012) also established significant suppression of rootknot galling on okra using a different strain of *P. lilacinus*.

1.3.4.2.5 Fusarium spp.

Species of the genus *Fusarium* are cosmopolitan, being found in most soils and plant roots (Sikora et al, 2008). Studies on banana found non-pathogenic isolates of endophytic *Fusarium* spp. isolated from the roots to be antagonistic to *R. similis* (Niere et al, 1999). Dababat and Sikora (2007b) found *Fusarium oxysporum* Strain 162 (Fo 162) to significantly reduce *M. incognita* damage on tomato and attributed this to induced systemic resistance. In another study, Paparu et al (2009) combined the application of two non-pathogenic *Fusarium* strains to banana, and this resulted in a reduction in damage by burrowing nematodes and banana weevils. Mendoza and Sikora (2009) further demonstrated that the combination of Fo 162, with either the egg parasite *P. lilacinus* or the antagonist *Bacillus*, significantly reduced *R. similis* damage on banana, and more than when the former was applied alone. Similarly, Fo 162 has also been found to reduce damage in cucurbits (Menjivar et al, 2011) and tomato (Martinuz et al, 2013).

In field studies, Waweru et al (2014) found single applications of three different *F. oxysporum* isolates to significantly reduced damage on banana caused by *Pratylenchus goodeyi* Sher and Allen and *Helicotylenchus multicinctus* (Cobb) Golden. In addition to induced systemic resistance being the mechanism behind the reduced nematode damage and densities (Dababat and Sikora, 2007a; Martinuz et al, 2012), volatile substances produced by *Fusarium* spp. have also been found to be involved (Freire et al, 2012).

1.3.4.2.6 Trichoderma spp.

Species of the filamentous ascomycete genus *Trichoderma* are frequently isolated from diverse habitats (Vinale et al, 2008; Druzhinina et al, 2011). Many *Trichoderma* isolates act as biological control agents (Benítez et al, 2004). The ability of *Trichoderma* isolates to rapidly colonise the rhizosphere, produce numerous spores, and release a wide range of enzymes has made them prime candidates in the search for nematode antagonists because the bulk of plant parasitic nematodes are found in this zone (Harman et al, 2004; Verma et al, 2007; Vinale et al, 2008).

Trichoderma isolates have been found to inhibit eggs from hatching and to immobilise second stage juveniles in a number of *in vitro* studies (Sharon et al, 2001; Bokhari, 2009; Sharon et al, 2009; Szabóet al, 2012; Zhang et al, 2015). Daragó et al (2013) also found various *Trichoderma* spp. to reduce populations of *Xiphinema index*Thorne and Allen *in vitro*. Multiple modes of action have been cited in many studies as being behind the nematicidal abilities of *Trichoderma* isolates (Verma et al, 2007). Zhang et al (2015) reported that *Trichoderma longibrachiatum* Rifai conidia adhered to and parasitised the surface of juveniles, thereby deforming or completely dissolving them. They attributed this to proteases. Proteinase-transformed *Trichoderma* isolates displayed enhanced efficacy against both eggs and juveniles when compared to wild type strains (Sharon et al, 2001). Sharon et al (2009) also found that carbohydrate residues such as fucose, on the surface of the nematode and fungal conidia, are involved in antibody and lectin-mediated improved parasitism.

Apart from reducing nematode damage severity, *Trichoderma* isolates have been reported to enhance growth parameters on various crops. On tomato, different isolates have been found to increase shoot weight while reducing rootknot galling and reproduction (Sahebani and Hadavi, 2008; Affokpon et al, 2011; Radwan et al, 2012; Jamshidnejad et al, 2013; Elgorban et al, 2014). *Trichoderma* isolates were also reported to reduce *M. graminicola* damage on rice (Le et al,

2009) and *Heterodera avenae* infection on wheat (Zhang et al, 2014). Shennawy et al (2012) found the combination of *T. koningii* and *Bacillus megaterium* to be more effective in reducing rootknot-*Fusarium* disease complex in potato than individual biocontrol agents.

1.3.4.2.7 Other fungal biocontrol agents

Some of the emerging fungal biocontrol agents that have also received significant consideration include the yeasts (*Saccharomyces* spp.), *Muscodor albus*Woropong, Strobell and Heiss, *Lecanicillium* spp, the entomopathogens *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium* spp., and *Penicillium* spp. Different yeast isolates, especially *Saccharomyces cerevisiae*, have been found to have nematicidal properties often comparable to conventional nematicides in field trials (Ismail et al, 2005; Hashem et al, 2008; Karajeh, 2013). Volatiles produced by *M. albus*, a plant endophyte with antibacterial and antifungal properties, have shown efficacy on different plant parasitic nematodes (Grimme et al, 2007; Riga et al, 2008; Strobel, 2011). The entomopathogenic fungi *B. bassiana* and *Metarhizium* spp have also been found to have nematicidal effects in several studies (Sun et al, 2006; Liu et al, 2008; Zhao et al, 2013)

1.3.5 Inundative vs Endophytes

The majority of studies on the efficacy of various bionematicides have been based on inundative applications in both controlled environments and in the field (Oyekanmi et al, 2007; Goswami et al, 2008; Abd-El-Khair and El-Nagdi, 2014). Relatively few isolates have, however, been tested successfully in the field as seed dressing, with the majority of those tested being rhizobacteria (Oostendorp and Sikora, 1989; Wahla et al, 2012; Munif et al, 2013), some fungi (Khan et al, 2005; Athman, 2006), and some combinations of bacteria and fungi (Haseeb and Khumar, 2012). Although inundative approaches, especially drenches, have been successful in greenhouse and field trials, the amount of biocontrol product required to treat large commercial fields, and the costs and the technology involved are their major limiting factor (Athman et al, 2006; Viaene et al, 2006; Sikora et al, 2008). This is especially true for ovicidal products because the contact time between the antagonist and nematode pest needs to be prolonged, thus they fail to reduce damage. In cases where isolates are larvicidal and rhizosphere competent, penetration of infective juveniles is significantly reduced because the rhizosphere becomes the first line of defence and parasitism continues inside the root. The success of commercial bionematicides has largely depended on effective formulations capable of persisting in the rhizosphere to protect plants in the early growth phases.

Inundative approaches where cell suspensions or culture filtrates are applied to the root zone have largely been used to manage rootknot nematodes. However, this approach has performed poorly in field trials because of the inconsistencies in product distribution, as well as the costs involved in treating approximately 2500 tons of bulk soil (25-cm deep x 100m x 100m per hectare) (Sikora et al, 2008). In addition, this method of application increases production costs, reduces profit margins and is uneconomical, especially for low value field crops (Sikora and Pocasangre, 2006). Economically viable product application methods such as root dips and seed treatments have since been found to be more viable because they reduce the quantity of biocontrol agent that needs to be applied without reducing the level of control provided (Mahdy, 2002).

1.3.6 *Global bionematicide developments*

In the last decade the major global pesticide companies have shown significant interest in bionematicides and a number of them have since acquired companies producing and marketing these products. In 2009 the global pesticide market was estimated at USD 43 billion with estimates of 5 – 8% growth p.a. In this market, USD 1.6 billion is estimated to be spent on bionematicides (Trainer et al, 2014). Though the major proportion of this amount belongs to the major pesticide companies, smaller industries in developing countries have started to develop bionematicides.

1.3.6.1 Bionematicides registered for *Meloidogyne* spp.

1.3.6.1.1 Bacillus firmus (Wilson and Jackson, 2013)

This bionematicide was initially developed and marketed by AgroGreen® before it was acquired by Bayer CropScience in 1997. Currently, two *Bacillus firmus* products are being marketed as bionematicides by Bayer CropScience as Nortica® and VOTiVO™, with the former being applied as a drench and the latter as a seed treatment. VOTiVO is registered for applications in combination with PONCHO® (an insecticide), for the control of *Meloidogyne* spp. on soybean.

1.3.6.1.2 Pasteuria penetrans (Wilson and Jackson, 2013)

After initial complications in development of mass production technologies, a patent was filed in 2004 by a US based company, Pasteuria Biosciences, for the *in vitro* production of *Pasteuria* spp. Pasteuria Bioscience went ahead to produce and market a product Econema® for the control of *Belonolaimus* spp. on turf. In 2012 Syngenta acquired Pasteuria Bioscience and went

on to launch another seed treatment nematicide, CLARIVA™ in 2014, aimed at controlling soybean cyst nematodes.

1.3.6.1.3 *Purpureocillium lilacinus (Tranier et al, 2014)*

Originally developed from Isolate PL251 found in the Philippines, BIO-ACT® was marketed by Prophyta (Pty) Ltd until the company was acquired by Bayer CropSciences in 2013. It is an ovicidal product used for the control of various endoparasitic nematodes: *Meloidogyne* spp., *Globodera* spp., *Heterodera* spp., *Pratylenchus* spp. and *Radopholus similis*. BIO-ACT is formulated as a water dispersible granule and hence can be applied via various irrigation systems. In South Africa PL Gold® was produced and sold as a licensed version of PL251 by BCP (Pty) Ltd. BCP (Pty) Ltdwas acquired by Becker-Underwood, who were subsequently bought by BASF, who subsequently stopped producing this product.

1.3.6.1.4 *Trichoderma spp. (Woo et al, 2014)*

Sales of *Trichoderma* containing products have been growing exponentially in the last 5 years, with more than 250 products available across the world. Of these, nine products are found in five African countries, registered as fungicides and growth stimulants. Though a notable few *Trichoderma* products have been registered on other continents for control of nematodes, there is currently no nematicidal product of *Trichoderma* registered in Africa. However, research efforts are underway to evaluate various *Trichoderma* strains as bionematicides, especially as seed treatments, due their versatility, rhizosphere competency, ease of production and use for other beneficial attributes to plants.

1.3.6.1.5 *Entomopathogens Beauveria bassiana and Metarhizium spp. (Tranier et al, 2014)*

A number of registered entomopathogenic fungal isolates and some isolates under evaluation have been found to have nematicidal properties. *Beauveria bassiana* products BOTANICARD® and MYCOTROL®, and *Metarhizium anisopliae*Strain F52, all registered as bioinsecticides, are also being evaluated for their nematicidal properties. Some of these entomopathogens are root endophytes, which makes them ideal candidates for multiple pest control.

1.3.7   Limitations of biological control

Like all microorganisms, biological control agents are affected by environment and edaphic factors. Some of these that have been shown to influence the survival, reproduction and activity of biocontrol agents include pH, temperature and other antagonistic organisms residing in the

rhizosphere (Sayre and Walter, 1991; Barbercheck, 1992; Luambano et al, 2015). Uncertainty about the unpredictable effects on non-target organisms is also an issue limiting the release of biological control agents (Hoeschle-Zeleden et al, 2013). Mass production faculties and formulation technologies for most BCA's are not readily available, especially in developing countries. Adequate and coherent legislature for the production and use of BCA's is still a challenge among African countriesespecially in regards to registration barriers and quality control issues because they are poorly understood by both policy makers and regulatory agencies (Hoeschle-Zeleden et al, 2013).

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CHAPTER TWO

BIOLOGICAL CONTROL OF THE ROOTKNOT NEMATODE, *MELOIDOGYNE JAVANICA* (CHITWOOD) USING *BACILLUS* ISOLATES, ON SOYBEAN

Abstract

In this study, the biocontrol potential of *Bacillus* isolates was investigated in laboratory and greenhouse experiments. Five out of 70 bacterial isolates from the root-zone of crops and a goat pasture caused second stage juvenile (J2) mortality greater than 50% *in vitro* after 24 hours.Three of the five selected isolates (BC27, BC29 and BC31) which were isolated from the root-zone of a goat pasture caused J2 mortality greater than 80% at 10^8spores ml^{-1}*in vitro* after 24 hours, with BC27 causing100% J2 mortality after 3 hours. Seed treatment of soybean with Isolates BC27 and BC29 caused a reduction in rootknot galling and egg mass counts (P≤0.0001) and also caused a significant increase in shoot weight (P≤0.0001), when compared to the Control.Blast analysis revealed that the two select isolates, BC27 and BC29,hadsimilar sequences to *Bacillus* spp. T2 and *Bacillus* spp. KT18, as listed on the Gen-Bank, respectively.

2.1 Introduction

Soybean (*Glycine max* (L.) Merr.) has a high protein and oil content. It also has a high nitrogen fixing potential and hence it may play an important role in rotation systems, especially in Africa (Sinclair et al, 2014). Soybean production is affected by plant parasitic nematodes, resulting in significant yield losses (Oyekanmi and Fawole, 2010). The sedentary nematodes, *Meloidogyne* spp. (rootknot nematodes) and *Heterodera glycines* (Ichinohe) (soybean cyst nematode), are some of the most important nematodes affecting soybean production (Sikora et al, 2005; Oyekanmi et al, 2007; Doucet et al, 2007).

Sustainable production of soybean in South Africa is threatened by a build-up of various rootknot nematode species in all growing regions (Fourie et al, 2015). There are currently no registered nematicides on soybeans in South Africa (Croplife, 2015). Development of resistant cultivars is currently being pursued but none are currently available (Fourie et al, 2013). Furthermore, successful management of rootknot nematodes in soybean cropping systems

cannot be done using host plant resistance alone (Fourie et al, 2015). Biological control using *Bacillus* isolates has been successfully used to manage rootknot nematodes in other crops (Li et al, 2005; Singh and Siddiqui, 2010; Flor-Peregrin et al, 2014; Ramezani Moghaddam et al, 2014) and one of these isolates, *Bacillus firmus* Bredemann and Weder Isolate N1, has already been commercialised for use on field-grown vegetable crops (Copping, 2009).

The early phase of root penetration by nematodes is important because it often has an impact on the final degree of plant damage (Sikora et al, 2008). Early protection of the rhizosphere of plants with rhizobacteria is therefore important because the bacteria target the vulnerable juvenile stage of nematodes (Kerry, 2001). Metabolites produced by some bacteria, especially *Burkholderia* spp., *Pseudomonas* spp. and *Bacillus* spp., interfere with nematode behaviour, feeding and reproduction, thereby reducing penetration and damage in plants (Viaene et al, 2006).

Bacillus spp. are endospore forming, hence they are able to survive for prolonged periods under unfavourable conditions (Singh and Siddiqui, 2010). Various mechanisms employed by rhizobacteria to reduce nematode damage and reproduction in plants have been suggested and include regulating nematode behaviour, interfering with nematode-host recognition, competition for nutrients, plant growth promotion, induced systemic resistance (Ongena and Jacques, 2008; Siahpoush et al, 2011; Adam et al, 2014), and production of exudates that inhibit egg hatching, reduce juvenile survival and/or kill nematodes directly (Lian et al, 2007; Peng et al, 2011; Zhang et al, 2012; Oliveira et al, 2014).

Inundative approaches where bacterial cell suspensions or culture filtrates are applied to the root zone have largely been used to manage rootknot nematodes. However, this approach has performed poorly in field trials because of the inconsistencies in product distribution, as well as the costs involved in treating approximately 2500 tons of bulk soil (25-cm deep x 100m x 100m per hectare) (Sikora et al, 2008). In addition, this method of application increases production costs, reduces profit margins and is uneconomical, especially for low value field crops (Sikora and Pocasangre, 2006). Economically viable product application methods such as root dips and seed treatments have since been found to be more viablebecause they reduce the quantity of biocontrol agent that needs to be applied without reducing thelevel of control provided (Mahdy, 2002).

The aim of this study was to isolate, screen and identify nematicidal *Bacillus* isolates that worked as biocontrol agents for *M. javanica*, using *in vitro* selection and *in vivo*field testing of their efficacy when applied as seed treatmentson soybean.

2.2 Materials and methods

2.2.1 Preparation of nematode inoculum

Meloidogyne javanica inoculum was initially obtained from the Unit for Environmental Sciences and Management, North West University, Potchefstroom, South Africa, and maintained on tomato (*Solanum lycopersicum* L.) cv. Moneymaker. Three week old seedlings grown on composted bark were inoculated with second stage juveniles (J2) and maintained in the glasshouse at $25 \pm 2°C$. At least four weeks after inoculation, or when inoculum was required, tomato roots were uprooted, washed with tap water to remove attached soil and chopped into 2-5 cm pieces. Eggs were extracted by shaking the roots in 1% sodium hypochlorite in a glass jar for 3 minutes. The resulting suspension was then washed through a series of sieves (1000 μm, 330 μm, 190 μm, 100 μm and 38 μm) with eggs being collected from the bottom sieve (38 μm). Eggs were thoroughly washed to remove residual sodium hypochlorite and then hatched in Baermann trays at $25 \pm 2°C$ in the incubator. Second stage juveniles were collected from the trays after 3 – 5 days and used for both *in vitro* and *in vivo* trials.

2.2.2 In vitro screening of bacterial isolates for nematicidal activity against M. javanica juveniles.

Soil samples were collected from the root zone of grass plants in sheep and goat pastures (which carry a high load of gastrointestinal nematodes), and various field crops at the University of KwaZulu-Natal research farm. A 1 g subsample was suspended in sterile distilled water and heated at 80°C in a water bath for 15 minutes. Upon cooling, serial dilutions were made up to 10^3 before one ml aliquots were plated in triplicate onto Nutrient Agar plates using the spread plate technique. Plates were incubated at 28°C for 72 hours before sub-culturing. Distinct colonies were streaked onto tryptone soy agar (TSA) (Merck®) and incubated at 28°C for 3 days. Subsequent bacterial isolates were then stored in 30% glycerol at -80°C. In total 70 bacterial isolates were subjected to *in vitro* assays with *M. javanica* juveniles.

For the first batch of *in vitro* screening, isolates were cultured on TSA before transferring single colonies into flasks with 20 ml of sterile tryptone soy broth (TSB) (Merck). Flasks were

incubated at 28°C for 24 hours with agitation in a shaking incubator. The isolates were then centrifuged at 10 000 rpm at 4°C, the supernatant discarded and the bacterial spores re-suspended in sterile ¼ strength Ringer's solution. Serial dilutions were made up to 10^3 before 1 ml of the bacterial isolates and 1 ml of nematode suspension containing 30 – 35 juveniles were transferred into 5 cm^3 polystyrene wells and incubated at 25°C in triplicate. After 24 hours the number of dead juveniles were counted using a stereomicroscope (Olympus BX41). Nematodes were considered dead if they did not move on probing with a fine needle.

For the second batch of *in vitro* screening, isolates were cultured on TSA before transferring single colonies into flasks with 20 ml of sterile Tryptone Soy Broth (TSB) (Merck). Flasks were incubated at 28°C for 24 hours with agitation in a shaking incubator. The isolates were then centrifuged at 10 000 rpm at 4°C, the supernatant discarded and the bacterial spores re-suspended in sterile ¼ strength Ringer's solution. The spore suspension was counted using a haemocytometer and the concentration was adjusted to 10^8 spores ml^{-1}. One ml of the bacterial isolates and one ml of nematode suspension containing 30 – 35 juveniles were transferred into 5 cm^3 polystyrene wells and incubated at 25°C in triplicate. After 24 hours the number of dead juveniles were counted.

2.2.3 Effects of bacterial isolates on the control of M. javanica in glasshouse experiments
Soybean seeds were surface sterilised by rinsing them in 3% sodium hypochlorite for 5 mins, then rinsing them in sterile distilled water five times. A bacterial spore-sticker suspension was made up by adding 1.0g of carboxymethyl cellulose (CMC) to 50 ml of spore suspension (10^8 ml^{-1}) in a 200ml flask. The suspension was shaken (150 rpm) in a rotary shaker (Model GFL 3005, Labortechnik, Germany). A batch of 120 seeds were added to each flask and allowed to soak in the spore-sticker suspension for about an hour with constant swirling. The treated soybean seeds were then air-dried in petri dishes under a laminar flow overnight (16 – 18hrs) (Yobo et al, 2010).

Soybean seeds were planted in the middle of 12 cm diameter pots filled with composted pine bark (Potting Mix, National Plant Foods (Pty) Ltd, Camperdown, South Africa) and maintained in the glasshouse with uniform irrigation and fertigation. At first true leaf stage plants were inoculated with 1 000 J2 nematodes in three shallow pencil holes around the plant. Each

treatment was replicated five times and the trial was repeated twice. The trial was terminated at 42 days after nematode inoculation. The plants were washed free of adhering soil, fresh root and shoot weights as well as galls were assessed. The egg masses were then stained with erioglaucine before counting.

2.2.4 *Identification of the best bacterial isolates*
Genomic identification of the best bacterial isolates was done using 16S rRNA sequence analysis. Characterisation was done at Inqaba Biotechnical Industries (www.inqaba.co.za).

2.2.5 *Statistical analysis*
Data was subjected to analysis of variance (ANOVA) using SAS 9.3 and means were separated using Duncan's multiple range test.

2.3 Results

2.3.1 *In vitro screening of bacterial isolates against M. javanica juveniles*
A total of 70 bacterial isolates were screened against *M. javanica* J2s under *in vitro* conditions after the initial spore suspension was diluted to 10^3 using serial dilutions. Of these, only five isolates caused mortality greater than 50%, constituting 7.1% of all isolates (Table 2.1). Three isolates (BC27, BC29 and BC31) from the rhizosphere of grass in goat pastures caused larval mortality greater than 80% after 24 hours and were selected for the second screening phase.

In the second *in vitro* screening the bacterial spore suspension was adjusted to 10^8 spores ml^{-1} using a haemocytometer and tests were conducted in wells. At 3 hours after commencement of the experiment Isolate BC27 had caused juvenile mortality of 100% and was significantly better than all other treatments (P < 0.001). Larvae did not recover upon either being probed or with further dilution of the bacterial suspension.

Table 2.1 Percent mortality of *M. javanica* J2s in a bacterial spore suspension after 24 hours

Isolate	Source	Mortality[a]	Isolate	Source	Mortality
BC1	Tomato	38	BC36	Goat pasture	31
BC2	Tomato	25	BC37	Goat pasture	0
BC3	Tomato	3	BC38	Goat pasture	10
BC4	Tomato	26	BC39	Goat pasture	26
BC5	Tomato	0	BC40	Goat pasture	43
BC6	Tomato	28	BC41	Goat pasture	5
BC7	Tomato	2	BC42	Goat pasture	17
BC8	Tomato	24	BC43	Goat pasture	30
BC9	Tomato	30	BC44	Goat pasture	1
BC10	Tomato	12	BC45	Goat pasture	29
BC11	Tomato	42	BC46	Goat pasture	38
BC12	Squash	0	BC47	Goat pasture	2
BC13	Pepper	2	BC48	Sheep pasture	42
BC14	Pepper	33	BC49	Sheep pasture	1
BC15	Pepper	26	BC50	Sheep pasture	25
BC16	Pepper	1	BC51	Sheep pasture	0
BC17	Pepper	7	BC52	Sheep pasture	39
BC18	Pepper	15	BC53	Sheep pasture	17
BC19	Pepper	18	BC54	Cattle pasture	24
BC20	Pepper	25	BC55	Cattle pasture	2
BC21	Tobacco	0	BC56	Cattle pasture	62
BC22	Tobacco	30	BC57	Cattle pasture	10
BC23	Soybean	4	BC58	Cattle pasture	0
BC24	Soybean	26	BC59	Cattle pasture	32
BC25	Goat pasture	0	BC60	Cattle pasture	1
BC26	Goat pasture	35	BC61	Cattle pasture	5
BC27	Goat pasture	100	BC62	Cattle pasture	26
BC28	Goat pasture	4	BC63	Cattle pasture	0
BC29	Goat pasture	100	BC64	Cattle pasture	55
BC30	Goat pasture	0	BC65	Cattle pasture	5
BC31	Goat pasture	87	BC66	Cattle pasture	0
BC32	Goat pasture	32	BC67	Cattle pasture	37
BC33	Goat pasture	0	BC68	Cattle pasture	28
BC34	Goat pasture	3	BC69	Cattle pasture	0
BC35	Goat pasture	39	BC70	Cattle pasture	34
			Control	Water	2

[a]Mean % mortality of three replications.

Isolate BC29 caused significantly higher mortality than Isolate BC31, although both caused mortality greater than 50% after 3 hours (P < 0.001). At 24 hours both BC27 and BC29 caused 100% mortality and were significantly more effective than Isolate BC31, although the latter caused significantly higher mortality than the Control (Table 2.2). All three isolates were consequently selected for further screening in the glasshouse on soybean.

Table 2.2   Second *in vitro* screening of selected bacterial isolates on *M. javanica* J2s

	% Mortality	
Isolate	3hrs	24hrs
BC27	100.00a[f]	100.00**a**
BC29	87.56**b**	100.00**a**
BC31	64.06**c**	84.18**b**
Control	0.50**d**	1.09**c**

[f]Mean of 10 replications followed by the same letters in the same column are not significantly different at P < 0.0001 based on Duncan's multiple range test.

2.3.2   *Efficacy of three bacterial isolates against M. javanica in glasshouse experiments*

Two bacterial isolates (BC27 and BC29) significantly reduced the number of galls formed and the number of egg masses compared to the Untreated Control (Table 2.3). Treatment of soybean seeds with the bacterial isolate BC29 resulted in the highest reduction in galling (83.71%) and egg mass formation (86.48%). Isolates BC27 and BC29 had a considerable effect on infection and subsequently reproduction of *M. javanica* on soybeans. Both isolates also significantly increased soybean root and shoot weight and both parameters were significantly higher than for the Control (Table 2.3). Isolate BC31 was comparable to the Control treatments for both nematode inoculated and non-inoculated plants. There was also a significant difference for both shoot and root weight between nematode-inoculated and non-inoculated plants for isolates BC27 and BC29. However, Isolates BC27 and BC29 had the highest increments for root and shoot weight when nematode inoculated or un-inoculated plants were compared alone. Root and shoot weight in the Control treatments and in treatments BC31 did not significantly differ with nematode inoculation (P=0.001).

2.3.3 Blast and identification of selected bacterial isolates

The analysis of nucleotide sequences of the 16S rRNA gene sequences using BLAST analysis revealed that Isolates BC27 and BC29 exhibited sequences similar to *Bacillus* spp. T2 (Accession HQ418499.1, E-value 2E51) and *Bacillus* spp. KT18 (Accession KJ734022.1, E-value 0E00) listed in the Gen-Bank, respectively.

Table 2.3   Effect of selected bacterial isolates on the biocontrol of *M. javanica* on soybeans *in vivo*

Treatments	Root galling	Egg masses	Root weight	Shoot weight
BC27	-	-	16.89**a**	23.58**a**
BC29	-	-	17.59**b**	27.69**b**
BC31	-	-	8.93**c**	17.71**c**
Control	-	-	7.62**c**	16.23**c**
M. javanica + BC27	55.60**b**[g]	42.9**b**	13.36**b**	20.48**b**
M. javanica + BC29	23.0**a**	21.1**a**	16.16**a**	25.71**a**
M. javanica + BC31	119.40**c**	127.3**c**	6.91**c**	15.11**c**
M. javanica	141.20**d**	156.1**d**	6.59**c**	14.59**c**

[g]Mean of 10 replications followed by the same letters in the same column are not significantly different at $P < 0.0001$ based on Duncan's multiple range test.

2.4   Discussion

Rootknot nematodes *M. incognita* (Kofoid and White) Chitwood, *M. javanica* and *M. arenaria*(Chitwood) are important pests limiting soybean production (Sikora et al, 2005; Oyekanmi and Fawole, 2010). The use of nematicides to manage rootknot nematodes in soybean cultivation is not economically sustainable (Sikora et al, 2005). Numerous bacterial isolates, among them *Pseudomonas* spp. and *Pasteuria* spp., have been found to have nematicidal properties against rootknot nematodes (Cho et al, 2000; Siddiqui et al, 2009; Timper et al, 2009; Singh and Siddiqui, 2010; Bagheri et al, 2014). A number of *Bacillus* isolates have also been screened and found to have nematicidal properties against *M. javanica in vitro* (Ashoub and Amara, 2010; Park et al, 2014) and *in vivo* (Oliveira et al, 2007; Wei et al, 2014; Xiong et al, 2015).

In this studybacterial isolates recovered from the rhizosphere of crops did not exhibit desirable pathogenicity*in vitro* and hence they were not selected for further evaluation. Three isolates, two of which were identified as *Bacillus* spp., were found to be the most effective isolates against *M. javanica* juveniles *in vitro*. These isolates were isolated from the rhizosphere of grass of goat pastures.These pastures may therefore have potential to harbour competent biocontrol agents against nematodes. The goats on these pastures are frequently infested with gastrointestinal nematodes.

Total J2 mortality was observed as a result of the bacterial spore suspensions of one isolate, BC27, as early as 3 hours after inoculation, although both BC27 and BC29 caused 100% mortality after 24 hours. Isolate BC31 caused significantly lower J2 mortality than the other two isolates, although it was significantly higher than the Control. This makes the isolates ideal biocontrol agents because they may exhibit nematicidal activity upon application in the rhizosphere, thereby protecting host plants in their early growth stages. *Bacillus* isolates have been found to produce proteolytic enzymes, which are responsible for nematode mortality (Chantawannakul et al, 2002; Tian et al, 2007; Mohammed et al, 2008).

The biocontrol efficacy of both *Bacillus* isolates, BC27 and BC29 in glasshouse experiments was significantly higher than in the Control plots. Although Isolate BC29 caused significantly lower J2 mortality than Isolate BC27 *in vitro* at 3 hours, *in vivo*, Isolate BC29 caused a greater reduction in galls and egg masses than Isolate BC27. This may be due, in part, to the ability of these isolates to colonise the rhizosphere of soybean plants. Similar trends were observed *in vitro* and *in vivo* for Isolate BC31, with the isolate causing a significant reduction in galls and egg masses but less than Isolates BC27 and BC29. The size of galls observed for plants treated with Isolate BC31 were also larger and had coalesced, while for the plants treated with Isolates BC27 and BC29, the galls were small and discrete.

Isolates BC27 and BC29 significantly increased soybean root and shoot weight for both rootknot inoculated and non-inoculated plants when compared to the Control. Similar results have been observed with other *Bacillus* isolates on different crops (Oliveira et al, 2007, Xiong et al, 2015). These attributes would enhance the value of these isolates, providing nematode

control and enhanced growth. Fourie et al (2013) noted that both nematode suppression and yield results need to be considered when making a decision on rootknot management options. Based on this notion, Isolate BC31 would therefore not be an ideal biocontrol candidate because it resulted in biomass similar to the Controls.

Although the mechanisms involved in reducing gall formation and egg masses were not conducted in this study, Adam et al (2014) found that *Bacillus subtilis* isolates repelled rootknot juveniles and also induced systemic resistance in tomato plants, thereby reducing nematode reproduction. Padgham and Sikora (2007) also found an isolate of *Bacillus megaterium* to reduce root penetration and migration of *M. graminicola* to the root zone of rice plants.

Some *Bacillus* isolates suppress plant diseases in addition to rootknot nematodes and hence provide new possibilities for plant disease management (Adam et al, 2014). This, compounded with the ability of dry spores to be applied directly to seed as a commercial seed coating, and their prolonged shelf-life, makes *Bacillus* isolates ideal biocontrol agents for rootknot nematode management. This is especially so in crops such as soybeans where nematode management options are limited by costs. However, the ability of the *Bacillus* isolates in this study to persist in the rhizosphere, their interaction with other soil microbes, their field efficacy and the consistency of results in field trials all need to be studied further.

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CHAPTER THREE

BIOLOGICAL CONTROL OF THE ROOTKNOT NEMATODE, *MELOIDOGYNE JAVANICA* (CHITWOOD), ON SOYBEAN, USING *TRICHODERMA* ISOLATES

Abstract

In this study, the biocontrol potential of the filamentous fungus, *Trichoderma*, was investigated in laboratory and greenhouse experiments. *In vitro* studies of 111 *Trichoderma* strains showed that conidial suspensions of 11 strains isolated from the root zone of field crops and from animal pastures caused *M. javanica* second stage (J2) mortality greater than 50% after 24 hours. Under greenhouse conditions seed treatments of soybean with 10^8 conidia ml^{-1} significantly increased plant biomass and reduced the number of galls and egg masses. The analysis of nucleotide sequences of the ITS1 region using BLAST analysis revealed that the effective *Trichoderma* isolates exhibited sequences similar to *T. spirale*, *T. harzianum* and *T. virens*.

3.1 Introduction

Rootknot nematodes, *Meloidogyne* spp., are widely distributed in the tropics and subtropics. They are the most damaging nematode genus globally and cause crop losses of up to 157 billion USD annually (Sasser, 1980; Nicol et al, 2011; Onkendi et al, 2014). Rootknot nematodes have been recorded to attack and cause significant damage to most crops in South Africa, resulting in significant yield losses and total crop failure in some incidences (Fourie et al, 2001b; Onkendi and Moleleki, 2013). *Meloidogyne javanica* Chitwood is one of the most common and economically important nematode species in South Africa (Fourie et al, 2001a; Fourie et al, 2015).

Management of rootknot nematodes is one of the important costs of production in Africa (Onkendi et al, 2014) and in other parts of the world (Wesemael et al, 2011) and hence many ways of managing nematodes have been investigated. Sedentary nematodes have been managed using nematicides since the 1950s' (Moens et al, 2009). However pressure to reduce the use of nematicides because of health and environmental concerns has led to the development of antagonistic micro-organisms as biological control agents to these pests (Viaene et al, 2006).

Research efforts have pursued the development of resistance in different crops to plant parasitic nematodes (Wesemael et al, 2011).). In wheat, soybean and potato crops, host plant resistance has been used to manage various cyst nematode species (Dowler and van Gundy, 1984; Cook and Starr, 2006; Hildalgo-Diaz and Kerry, 2007). However, there are currently no registered resistant soybean cultivars in South Africa (Fourie et al, 2015). Microbial pathogens, endophytes and antagonists play a crucial role in the regulation of the populations of plant parasitic nematodes in various agroecosystems (Hallmann et al, 2009). The use of microbial agents for the control of nematodes has been the subject of intense research in recent years as a possible alternative to nematicides (Mankau, 1981; Kerry, 1997). Fungi and bacteria are an integral part of the soil community and both have shown potential as nematode antagonists (Akhtar and Malik, 2000).

Species of the filamentous ascomycete genus *Trichoderma* are readily isolated from various ecological habitats (Vinale et al, 2008; Druzhinina et al, 2011) and many of these isolates have been found to be good biological control agents (Benítez et al, 2004). *Trichoderma* isolates have been found to be among the best biocontrol agents for controlling nematodes because of their ability to rapidly colonise the rhizosphere, produce numerous spores, and release a wide range of enzymes, since the bulk of plant parasitic nematodes are found in the rhizosphere (Harman et al, 2004; Verma et al, 2007; Vinale et al, 2008).

In a number of *in vitro* studies, *Trichoderma* isolates have been found to prevent nematode eggs from hatching, and to immobilise second stage juveniles (J2s) (Sharon et al, 2001; Bokhari et al, 2009; Sharon et al, 2009; Szabó et al, 2012; Zhang et al, 2015). Apart from reducing nematode damage severity, *Trichoderma* isolates have been reported to enhance growth parameters on various crops. On tomato (*Solanum lycopersicum* L.), different isolates have been found to increase shoot weight while reducing rootknot galling and reproduction (Sahebani and Hadavi, 2008; Affokpon et al, 2011; Radwan et al, 2012; Jamshidnejad et al, 2013; Elgorban et al, 2014). *Trichoderma* isolates were also reported to reduce *Meloidogyne graminicola* (Golden and Birchfield) damage on rice (*Oryza sativa* L.) (Le et al, 2009) and wheat (*Triticum aestivum* L.) (Zhang et al, 2014). Shennawy et al (2012) found the combination of *Trichoderma koningii* Oudem and *Bacillus megaterium* (de Barry) to be more effective in

reducing rootknot-*Fusarium* disease complex in potato (*Solanum tuberosum* L.) than the individual biocontrol agents.

This study was conducted to identify nematicidal *Trichoderma* isolates with the potential to be biocontrol agents against *M. javanica* through *in vitro* selection and to determine their *in vivo* efficacy on soybean when applied as seed treatments.

3.2   Materials and methods

3.2.1   Preparation of nematode inoculum

Meloidogyne javanica inoculum was initially obtained from the Unit for Environmental Sciences and Management, North West University, Potchefstroom, South Africa, and maintained on tomato cv. Moneymaker. Three week old seedlings grown on composted bark were inoculated with second stage juveniles (J2) and maintained in the glasshouse at $25 \pm 2°C$. At least four weeks after inoculation, or when inoculum was required, tomato roots were uprooted, washed with tap water to remove attached soil and chopped into 2-5 cm pieces. Eggs were extracted by shaking the roots in 1% sodium hypochlorite in a glass jar for 3 minutes. The resulting suspension was then washed through a series of sieves (1000 μm, 330 μm, 190 μm, 100 μm and 38 μm), with eggs being collected from the bottom sieve (38 μm). Eggs were thoroughly washed to remove residual sodium hypochlorite and then hatched in Baermann trays at $25 \pm 2°C$ in the incubator. Second stage juveniles were collected from the trays after 3–5 days and used for both *in vitro* and *in vivo* trials.

3.2.2   In vitro screening of Trichoderma isolates for nematicidal activity against M. javanica juveniles.

Soil samples were collected from the root zone of sheep and goat pastures, and various field crops at the University of KwaZulu-Natal research farm. A 1 g sub-sample was suspended 100 ml of sterile distilled water and serial dilutions were made (10^3) (Yobo et al, 2010). One ml (1ml) aliquots of each dilution were plated in triplicate onto a *Trichoderma* selective media (TSM) (Askew and Laing, 1993) using the spread plate technique. The plates were then incubated at $28\pm1°C$ for 7 days in the dark before colonies resembling *Trichoderma* were subcultured onto Potato Dextrose Agar (PDA) (Merck®). These subcultures were also incubated at $28\pm1°C$ for 7 days in the dark, each isolate coded, and then stored in 30% glycerol

at -80°C. In total 111 *Trichoderma* isolates were subjected to *in vitro* assays with *M. javanica* juveniles.

For the first round of *in vitro* screening, isolates were cultured on PDA at 28±1°C for 1-2 weeks, depending on sporulation. The plates were then flooded with 10 ml of sterile ¼ strength Ringer's solution and the mycelia scraped off the medium. The resultant suspension was filtered through cheese cloth into McCartney bottles. Serial dilutions of the conidial suspension were made up to 10^1 before 1 ml of the *Trichoderma*isolates and 1 ml of nematode suspension, containing approximately 40 juveniles, were transferred into 5 cm^3 polystyrene wells and incubated at 25°C in triplicate. After 24 hours the number of dead J2s were counted using a stereomicroscope (Olympus BX41). Nematodes were considered dead if they did not move on probing with a fine needle.

For the second round of *in vitro* screening, isolates were cultured on PDA at 28±1°C for 1-2 weeks, depending on sporulation. The plates were then flooded with 10 ml of sterile ¼ strength Ringer's solution and the mycelia scraped off the medium. The resultant suspension was filtered through cheese cloth into McCartney bottles. The conidia suspension was counted using a haemocytometer and the concentration adjusted to 10^8 conidia ml^{-1}. One ml of the *Trichoderma* isolates and 1 ml of nematode suspension, containing approximately 100 juveniles, were transferred into 5 cm^3 polystyrene wells and incubated at 25°C in triplicate. After 24 hours the number of dead J2s were counted.

3.2.3 *Effects of 11 Trichoderma isolates on M. javanica in glasshouse experiments*

The Round-up Ready soybean cultivar, DM5953RSF, used in this study was obtained from Pannar Seeds (Pty) Ltd, Greytown. Soybean seeds were surface sterilised by rinsing in 3% sodium hypochlorite for 5 mins, then rinsing in sterile distilled water five times. A fungal spore-sticker suspension was made up by adding 1.0g of carboxymethyl cellulose (CMC) to 50ml of conidial suspension (10^8 ml^{-1}) in a 200ml flask. The suspension was shaken (150 rpm) in a rotary shaker (Model GFL 3005, Labortechnik, Germany) for one hour. A batch of 120 seeds were added to each flask and allowed to soak in the spore-sticker suspension for about an hour with constant swirling. The treated soybean seeds were then air-dried in petri dishes under laminar flow overnight (16 – 18 hrs) (Yobo et al, 2010).

Soybean seeds were planted in the middle of 12 cm diameter pots filled with composted pine bark (Potting Mix, National Plant Foods, Camperdown, South Africa) and maintained in the glasshouse with uniform irrigation and fertigation. At the first true leaf stage plants were inoculated with 1 000 J2s in three shallow pencil holes around the plant. Each treatment was replicated five times and the trial was conducted twice. The trial was terminated at 42 days after nematode inoculation. The plants were washed free of adhering soil before assessments were made of the fresh root and shoot weights and nematode gall numbers. The egg masses were stained with erioglaucine before counting.

3.2.4   Identification of selected *Trichoderma* isolates
Identification of the best *Trichoderma* isolates was done using 16S rRNA sequence analysis. Characterisation was done at Inqaba Biotechnical Industries (www.inqaba.co.za).

3.2.5   Statistical analysis
Data was subjected to analysis of variance (ANOVA) using SAS 9.3 and means were separated using Duncan's multiple range test.

3.3   Results

3.3.1   In vitro screening of Trichoderma isolates against M. javanica juveniles
In the first screening phase a total of 111 *Trichoderma* isolates and one commercial strain, EcoT® were screened against *M. javanica* J2s under *in vitro* conditions after the conidia suspension was diluted to 10^1 using serial dilutions. Of these, 11 isolates and EcoT® caused J2 mortality greater than 50% after 24 hours, which amounted to 9.9% of the isolates screened (Table 3.1).

In the second *in vitro* screening the concentration of each of the selected isolates was adjusted to 10^8 conidia ml^{-1} before evaluation. All *Trichoderma* isolates evaluated caused mortality greater than 50% and were all significantly better than the Control (water) (P < 0001). The following isolates caused mortality greater than 70%: C5, C16, C29, C36, C59, C63 and C97 (Table 3.2). However, only Isolates C29, C59, C63 and C97 were selected for further evaluation in the glasshouse because they sporulated better than the other four isolates (C5, C11, C16 and

C36). The commercial biocontrol agent EcoT® was included in further studies because it is already registered for control of other root pathogens, and was known to have some nematicidal properties.

3.3.2 *Efficacy of Trichoderma isolates against M. javanica in glasshouse experiments*

The five *Trichoderma* isolates under evaluation, including the commercial biocontrol agent EcoT®, significantly reduced the number of galls and egg masses compared to the Control ($P \leq 0.0001$) (Table 3.3). Amongst the *Trichoderma* isolates, Isolate C29 caused the greatest reduction in nematode damage while Isolate C97 caused the least reduction. However, although all isolates caused a significant increase in root and shoot weight compared to the Control, Isolates C29 and C97 caused the greatest shoot weight ($P \leq 0.0001$) (Table 3.3).

Table 3.1 Percent mortality of *M. javanica* J2s resulting from *in vitro* treatments with fungal conidial suspensions.

Isolate	Source	Mortality[a]%	Isolate	Source	Mortality[a]%	Isolate	Source	Mortality[a]%
C1	F millet[b]	35	C40	Soybean	3	C79	Goat past	47
C2	F millet	13	C41	Soybean	3	C80	Goat past	1
C3	Peas	7	C42	Tobacco	1	C81	Goat past	3
C4	Peas	33	C43	Peas	7	C82	Goat past	7
C5	Peas	64	C44	F millet	2	C83	Goat past	5
C6	Peas	1	C45	F millet	2	C84	Goat past	0
C7	Peas	1	C46	F millet	5	C85	Goat past	2
C8	Peas	8	C47	Tomato	3	C86	Goat past	57
C9	Peas	12	C48	Pepper	5	C87	Goat past	0
C10	Peas	27	C49	Pepper	1	C88	Goat past	7
C11	Peas	70	C50	Pepper	5	C89	Goat past	11
C12	Pepper	5	C51	Pepper	4	C90	Goat past	9
C13	Pepper	61	C52	Tomato	2	C91	Goat past	6
C14	Pepper	4	C53	Tomato	2	C92	Goat past	1
C15	Tomato	3	C54	F millet	9	C93	Goat past	0
C16	Tomato	77	C55	F millet	3	C94	Goat past	2
C17	Tomato	6	C56	Tomato	58	C95	Goat past	3
C18	Tomato	5	C57	Tomato	3	C96	Goat past	6
C19	Tomato	3	C58	Tomato	3	C97	Goat past	81
C20	Squash	24	C59	Pepper	86	C98	Goat past	2
C21	Squash	3	C60	Pepper	5	C99	Goat past	1
C22	Squash	8	C61	Pepper	5	C100	Goat past	6
C23	Soybean	8	C62	Sheep past.	3	C101	Cattle past.	3
C24	Tobacco	27	C63	Sheep past.	83	C102	Cattle past	3
C25	Tobacco	9	C64	Sheep past.	2	C103	Cattle past.	1
C26	Tobacco	35	C65	Sheep pas[t].	4	C104	Cattle past	2
C27	Bean	13	C66	Sheep past.	3	C105	Cattle past.	35
C28	Bean	53	C67	Sheep past.	3	C106	Cattle past	1
C29	Tobacco	95	C68	Sheep past.	4	C107	Cattle past.	3
C30	Tobacco	1	C69	Sheep past.	5	C108	Cattle past	4
C31	Peas	2	C70	Sheep past.	4	C109	Cattle past.	6
C32	Peas	4	C71	Sheep past.	6	C110	Cattle past	3
C33	Peas	1	C72	Sheep past.	2	C111	Cattle past	1
C34	Peas	4	C73	Sheep past.	9	EcoT®		64
C35	Soybean	6	C74	Goat past.	6	Control	Water	2
C36	Soybean	74	C75	Goat past.	6			
C37	Soybean	1	C77	Goat past.	5			
C38	Soybean	2	C78	Goat past.	3			
C39	Soybean	3	C76	Goat past	3			

[a]Mean % mortality of three replications.   [f]Finger millet   [t] Pasture

Table 3.2   Second *in vitro* screening of selected *Trichoderma* isolates against *M. javanica* J2s

Isolate	% Mortality
C29	94.7**a**
C59	84.7**b**
C5	82.7**b**
C97	82.2**b**
C16	74.3**c**
C36	72.0**cd**
C63	71.5**de**
C11	69.2**e**
C13	60.8**f**
C28	55.2**g**
C56	51.5**h**
EcoT®	50.9**h**
Control	1.5**i**

[f]Means of 10 replications followed by the same letters in the same column are not significantly different at $P < 0.0001$ based on Duncan's multiple range test.

Table 3.3 Efficacy of selected *Trichoderma* isolates for the biocontrol of *M. javanica* on soybean plants *in vivo*

Treatments	Galls counts	Egg mass counts	Root weight (g)	Shoot weight (g)
C29	-	-	16.46**b**	28.35**a**
C59	-	-	14.79**cd**	25.07**c**
C63	-	-	14.50**d**	23.67**d**
C97	-	-	17.75**a**	28.65**a**
EcoT®			16.16**b**	27.19**b**
Control			8.43**g**	22.59**e**
M. javanica + C29	23.80**e**	42.10**e**	15.96**b**	26.78**b**
M. javanica + C59	43.20**d**	63.0**d**	13.61**e**	22.48**e**
M. javanica + C63	68.70**c**	74.8**c**	12.17**f**	21.73**e**
M. javanica +C97	79.8**b**	90.30**b**	18.25**a**	27.30**b**
M. javanica+ EcoT®	70.10**c**	63.20**d**	15.25**c**	24.72**c**
M. javanica	140.60**a**	164.60**a**	6.07**h**	17.67**f**

[g]Means of 10 replications followed by the same letters in the same column are not significantly different at P < 0.0001 based on Duncan's multiple range test.

3.3.3 *Genomic identification of selected fungal isolates*

An analysis of nucleotide sequences of the ITS1 region using BLAST analysis revealed that Isolates C59 and C63 exhibited sequences similar to *T. spirale* Isolate ATT15 (Accession HQ607861.1, E-value 0E00); Isolate C29 resembled *T. harzianum* Isolate A1S (Accession KJ767087.1, E-value 0E00); and Isolate C97 resembled *T. virens* Isolate FT-333 (Accession KJ739790.1, E-value 0E00), listed in Gen-Bank™.

3.4   Discussion

Several fungal biocontrol agents antagonistic to plant parasitic nematodes have been successfully registered and commercialised across the world in the last decade. Isolates of *Purpureocillium lilacinus* (Thom) Samson and the entomopathogens *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium* spp. have been registered for the control of rootknot nematodes in some countries (Tranier et al, 2014; Croplife 2015). Although the production and sale of biocontrol products containing*Trichoderma* have also grown exponentially, only one

isolate is currently registered for the control of nematodes in Africa (Woo et al, 2014; Croplife, 2015).

In this study native *Trichoderma* strains were isolated and evaluated for their nematicidal activity against *M. javanica*. Twelve isolates, including a commercial isolate, were found to cause significant mortality to *M. javanica* J2s *in vitro*. Of these, four isolates were chosen because of their efficacy and sporulation (C29, C59, C63 and C97). The strains were isolated from the root zone of tobacco and pepper and from the root zone of kikuyu grass of sheep and goat pastures, respectively (Table 3.1). Other strains pathogenic to nematodes were previously isolated from the same habitats (Pambuka 2014, unpublished), which indicates that they are suitable habits for bionematicidal agents. The commercial Isolate EcoT®, although it caused significantly less mortality than the other four isolates (C29, C59, C63 and C97), was also tested on the merits of its control of other phytopathogens, which would be an added advantage because rootknot nematodes are frequently found in association with other plant pathogens. Similar evaluations *in vitro* have found many *Trichoderma* isolates to cause mortality of rootknot nematodeJ2s (Sharon et al, 2009; Bhokari, 2009; Elgorban et al, 2014).

All the five *Trichoderma* isolates selected for glasshouse studies as seed treatments significantly reduced the galling and egg masses on soybean. Isolate C29 caused a greater reduction in galling and egg mass production than the other isolates, while EcoT® caused the least reduction. Furthermore, galls observed in plants treated with Isolate C29 were much smaller and were discrete, while for all the other treatments, most of the galls were coalesced. Several other *Trichoderma* isolates have been found to cause reductions in galling and egg mass production when applied as drenches (Sahebani and Hadavi, 2008; Shennawy et al, 2012; Szabó et al, 2012; Jamshidnejad et al, 2013). However, it needs to be noted that inoculating plants with nematodes at first true leaf stage does not fully represent field conditions because nematodes will already be present in the soil at seeding.

In addition to reducing the number of galls and egg masses, all the test isolates significantly increased both root and shoot weight of the soybean plants. Although Isolate C97 caused a smaller reduction in gall and egg mass counts, it stimulated plant growth, resulting in the

greatest root and shoot weights, resulting in weights similar to those resulting from treatment with Isolate C29, the most nematicidal of the isolates. These observations are similar to finding in other studies where *Trichoderma* isolates also enhanced growth parameters (Le et al, 2009; Affokpon et al, 2011; Zhang et al, 2014).

Several modes of action have been cited as the basis forthe nematicidal abilities of *Trichoderma* isolates (Verma et al, 2007). Zhang et al (2015) reported that the conidia of an isolate of *Trichoderma longibrachiatum* Rifai adhered to and parasitised the surface of juvenile nematodes, thereby deforming or completely dissolving them, and attributed this to proteases. Protease-transformed *Trichoderma* isolates displayed enhanced efficacy against both nematode eggs and juveniles when compared to wild type strains (Sharon et al, 2001).

The ability of *Trichoderma* isolates to rapidly colonise the rhizosphere, produce numerous spores, and release a wide range of enzymes has made them prime candidates in the search for nematode antagonists because the bulk of plant parasitic nematodes are found in this zone (Harman et al, 2004; Verma et al, 2007; Vinale et al, 2008).Prospects for *Trichoderma* strains as bionematicides, especially as seed treatments, are good due their versatility, rhizosphere competency, ease of production and their use for other beneficial effects on plants (Woo et al, 2014). However, reproducibility and consistency of results with *Trichoderma* isolates, especially in field trials, are important factors to be considered for them to be successful commercially.

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CHAPTER FOUR

USE OF *TRICHODERMA* AND *BACILLUS* ISOLATES TO PROTECT TOMATO PLANTS GROWN IN SOIL INFESTED WITH *MELOIDOGYNE JAVANICA* (CHITWOOD)

Abstract

Some*Trichoderma* and *Bacillus* isolates reduce the incidence and severity of rootknot galling resulting in improved yields from tomato plants. In this study, five *Trichoderma* isolates and one *Bacillus* isolate were evaluated for their efficacy against *M. javanica* in the field. All the isolates were applied as seed treatments at 10^8 spores ml^{-1} and seedlings were raised in speeding trays. The tomato seedlings were transplanted into soils pre-infested with *M. javanica*. The six isolates under evaluation all caused a reduction in galling severity and improved fruit yield when compared to the Control. Seed treatments with Isolates C29 and EcoT®+C97 resulted in the highest yield increases and caused the greatest reductions in nematode damage in nematode infested plots when compared to non-nematode infested plots. The results provide evidence for the effectiveness of biocontrol agents in providing protection against *M. javanica* on tomato.

4.1 Introduction

Vegetables are high value cash crops and are also a major component of human diets globally (Abbasi et al, 2002). Tomatoes are an important source of vitamins and contribute about 24% of vegetable production in South Africa (Grandillo and Chetelat, 2011). Plant parasitic nematodes, chief among them rootknot nematodes, are a major constraint in tomato production especially for field tomatoes where significant resources are needed for their management (Sikora and Fernández, 2005).

Fumigant and non-fumigant nematicides are still the most common method of nematode management for vegetables especially under intensive cultivation (Sikora and Fernández, 2005). However, most nematicides are being gradually withdrawn from the market across the world (Zasada et al, 2010; Onkendi et al, 2014). Host plant resistance, particularly through the use of the *Mi* gene, has been used globally for rootknot nematode control (Cook and Starr, 2006). The *Mi* gene in tomato confers resistance to *Meloidogyne javanica*Chitwood,

Meloidogyne incognita Chitwood and *Meloidogyne arenaria* Chitwood and is extensively used in managing these species (Williamson and Hussey, 1996; Wesemael et al, 2011). However, the *Mi* gene is not effective at temperatures above 28°C (Cook and Starr, 2006) and some *Meloidogyne* biotypes capable of breaking this resistance have been reported to be present various regions of the world (Karssen and Moens, 2006).

The use of microbial agents for the control of nematodes has been the subject of intense research in recent years as a possible alternative to nematicides (Mankau, 1981; Kerry, 1997). Fungi and bacteria are an integral part of the soil community and both have shown potential as nematode antagonists (Akhtar and Malik, 2000).*Trichoderma* and *Bacillus* isolates have been found to reduce rootknot nematode damage on tomato (Goswami et al, 2008; Nzanza et al, 2012; Elgorban et al, 2014; Ramezani Moghaddam et al, 2014) and other vegetables (Bhokari, 2009; Loganathan et al, 2010; Affokpon et al, 2011; Park et al, 2014). In most of these studies inundative applications of biocontrol agents have been used. This study sought to evaluate the efficacy of *Trichoderma* and *Bacillus* isolates, previously evaluated in the greenhouse, against *M. javanica* as seed treatments to tomato in field trials.

4.2   Materials and methods

A field experiment was conducted during the December/April 2013/2014 season at Ukulinga research farm, a facility of KwaZulu-Natal University, Pietermaritzburg, South Africa (29°24′E; 30°24′S). Soils at the site are classified as Westleigh form (Table 4.1).

Table 4.1   Soil physical and chemical properties for field trial.

pH	% Clay	Total cations cmol.L^{-1}	% Acid saturation	% Organic Carbon
4.44	37	16.84	1	2.9

4.2.1 Nematode inoculum, biocontrol agents and seed source

Meloidogyne javanica inoculum was initially obtained from the Unit for Environmental Sciences and Management, North West University, Potchefstroom, South Africa, and maintained on tomato (*Solanum lycopersicum* L.) cv. Moneymaker. Field infestation was done by inoculating the respective plots with freshly chopped, infested tomato roots. The infested roots were evenly spread in the planting furrows. The tomato cultivar Moneymaker® was used in this study, sourced from Starke Ayres Pty (Ltd), Pietermaritzburg.

Tomato seeds were surface sterilised by rinsing in 3% sodium hypochlorite for 5 mins, then rinsing in sterile distilled water five times. A spore-sticker suspension was made up by adding 1.0g of carboxymethyl cellulose (CMC) to 50ml of either bacterial or fungal spore suspension (10^8 ml^{-1}) in a 200ml flask. The suspension was shaken (150 rpm) in a rotary shaker (Model GFL 3005, Labortechnik, Germany) for one hour. A batch of 120 seeds were added to each flask and allowed to soak in the spore-sticker suspension for about an hour with constant swirling. The treated tomato seeds were then air-dried in petri dishes under laminar flow overnight (16 – 18 hrs). Seeds were then sown in 240 cell trays and transplanted into the field at four leaf stage. Two *Trichoderma* isolates Eco-T and C97 were also applied as a combination due to their ability to sporulate better than all other isolates in this study.

4.2.2 Nematode assessments

At 120 days after planting, the roots and rhizosphere of 5 plants per plot were sampled. Roots and soil from each treatment were thoroughly combined, a 200 cm^3 soil sub-sample and a 50 g sub-sample obtained, after which samples were stored at 4°C until nematode extraction. Nematodes were extracted from 200g soil samples following the decanting and sieving method (Hooper et al, 2005), followed by a modified Baermann tray method (Hooper et al, 2005). Nematodes (eggs and J2) were also extracted from 1g root samples following maceration of 1 cm root pieces in 0.5% NaOCl (Hooper et al, 2005). The resulting suspension was then washed through a series of sieves (1000 μm, 330 μm, 190 μm, 100 μm and 38 μm) with eggs and J2s being collected from the bottom sieve (38 μm). Galling severity was assessed on a scale of 0 – 8 (Daulton and Nusbaum, 1969) where: 0 = free of galls; 1 = trace, less than 5; 2 = 6-25 galls; 3 = 26-100 galls; 4 = moderate, gall numerous, mostly discrete; 5 = moderately heavy, numerous, many coalesced; 6 = heavy, galls very numerous, coalesced, root growth slightly

retarded; 7 = very heavy, mass invasion, slight root growth; 8 = extremely heavy, mass invasion, no root development.

4.2.3   Yield assessments

Yield assessments were done by picking and weighing of ripe fruit, with a final picking done at 120 days after planting.Fruit were picked from 20 plants in the mid two rows in each four row plot. Percent yield loss increase/decrease was calculated for each treatment using the equation: {(mean yield of treated plots/yield of Control) × 100} – 100.

4.2.4 Trial design, layout and data analysis

The experimental treatments were in a randomised complete blocks design laid out in a split plot arrangement. There were two factors in the main plot, rootknot nematodes and no rootknot nematodes. There were 9 subplot factors; Control (no sticker), Control (with sticker), EcoT®, Isolate C29, Isolate C59, Isolate C63, Isolate C97, Isolate C97+EcoT® and Isolate BC27. Inter row and intra row spacing was 80cm and 40 cm, respectively, and each plot consisted of four rows with 10 plants each. There were three replications for each treatment. Data was subjected to analysis of variance (ANOVA) using SAS 9.3 and means were separated using Duncan's multiple range test.

4.3   Results

4.3.1   Nematode assessments

Seed treatments with both *Trichoderma* and *Bacillus* isolates caused a significant decrease in both galling severity and nematode counts, when compared with the Control. Treatments with Isolates C29 and EcoT®+C97 caused reductions in gall severity of 45% and 40%, respectively, while Isolates C59, C63 and C97 caused reductions in gall severity by between 21 – 23% (Table 4.2). The biocontrol agent EcoT® on its own reduced gall severity by 34% while the *Bacillus* isolate BC27 reduced galling by 21%. However, only Isolates C29and EcoT®+C97 caused a significant reduction in nematode counts in the roots and in the soil. The *Bacillus* Isolate BC27 caused a significant reduction in J2 and eggs in the roots but not in the soil. There were insignificant rootknot nematode juvenile populations in the un-infested plots. Other plant parasitic nematode genera were encountered in the un-infested plots but the populations were also insignificant.

Table 4.2 Rootknot nematode data for biocontrol agents in both nematode infested and non-nematode infested plots

Treatments	Galling index	Eggs and J2 counts in roots (1g)	J2 counts in soil (200cm³)
Control	0.10g	5.0e	1.33d
Control (Sticker)	0.10g	4.0e	2.00d
EcoT®	0.00g	0.0e	0.00d
C29	0.00g	0.0e	0.00d
C59	0.23g	7.3e	0.00d
C63	0.03g	0.0e	0.00d
C97	0.00g	0.0e	0.00d
C97+EcoT®	0.00g	0.0e	0.00d
BC27	0.20g	0.0e	0.00d
Control + *M. javanica*	5.30a	1521.7ab	558.3a
Control (Sticker) + *M. javanica*	4.97b	1580.0a	580.0a
EcoT® + *M. javanica*	3.50d	1435.0ab	421.7b
C29 + *M. javanica*	2.90f	1105.3d	131.3c
C59 + *M. javanica*	4.10c	1431.7ab	461.0ab
C63 + *M. javanica*	4.20c	1356.7bc	460.0ab
C97 + *M. javanica*	4.13c	1458.3ab	541.0ab
C97+EcoT®+ *M. javanica*	3.20e	1146.7d	215.3c
BC27+ *M. javanica*	4.20c	1241.7cd	521.7ab
	F value = 471.6 $P \leq 0.001$ CV = 8.19	F value = 153.9 $P \leq 0.001$ CV = 14.52	F value = 42.44 $P \leq 0.001$ CV=30.35

Means of 3 replications followed by the same letters in the same column are not significantly different at $P < 0.0001$ based on Duncan's multiple range test.

4.3.2 Yield assessments

Seed treatment of tomato with *Trichoderma* and *Bacillus* isolates significantly increased yield irrespective of nematode infestation, when compared to the Control. The highest yield increase was observed in nematode infested plots planted to C29 treated seed with a yield increase of 66% while the highest yield reduction was observed in the nematode infested Control plots (Table 4.3).

Rootknot nematode infestation caused a reduction in yield in both the Control plants and the C59 treated plants. There was, however, no significant difference in yield between nematode infested and non-nematode infested plots for plants treated with Isolates C63, C97, BC27 and EcoT®. Seed treatments with Isolates C29 and EcoT®+C97 resulted in higher yield increases in nematode infested plots than the yields from non-infested plots.

Table 4.3 Yield data (kg) for biocontrol agents in both nematode infested and non-nematode infested plots

Treatments	Yield (kg)	Yield increase (+)/Yield decrease (-1)
Control	15.4ef	
Control (Sticker)	14.6f	-5
EcoT®	16.7def	+8
C29	18.6cde	+21
C59	22.1b	+44
C63	19.8bcd	+29
C97	19.7bcd	+28
C97+EcoT®	16.4def	+6
BC27	17.8def	+16
Control + *M. javanica*	9.4g	-39
Control (Sticker) + *M. javanica*	7.6g	-51
EcoT® + *M. javanica*	16.4def	+7
C29 + *M. javanica*	25.5a	+66
C59 + *M. javanica*	17.4def	+13
C63 + *M. javanica*	19.6bcd	+27
C97 + *M. javanica*	21.5bc	+40
C97+EcoT®+ *M. javanica*	22.5b	+46
BC27+ *M. javanica*	19.2bcd	+25
	F value = 18.55	
	P ≤ 0.001	
	CV=9.86	

[g]Means of 3 replications followed by the same letters in the same column are not significantly different at P < 0.0001 based on Duncan's multiple range test.

In C59 a yield decline was observed between inoculated and and no-inoculated plots which indicated that this strain was impacted negatively by *M. javanica* and although nematode numbers were lower than the Control this was not sufficient. EcoT® and C63 reduced the severity of damage although yield was similar. Isolates BC27 and C97 showed a yield increase between inoculated and non-inoculated plots although not significant while C97+EcoT® and C29 showed a significant yield increase.

4.4   Discussion

Seed treatment of tomato seed with *Trichoderma* and *Bacillus* isolates caused a reduction in gall severity, rootknot nematode density and an increase in yield in the field. *Trichoderma* Isolate C29 and a combination of Isolate C97 and EcoT® caused the greatest reduction in galling severity in this study. These results confirm previous results (Chapter 2 and 3) in *in vitro* and greenhouse studies where the *Bacillus* and *Trichoderma* isolates used in this study also showed nematicidal properties. In results from another study (Chapter 6), only *Trichoderma*

Isolate C97 was compatible with the biocontrol agent EcoT® and hence it was the only fungal combination evaluated in this study.

Similar results using *Trichoderma* and *Bacillus* isolates were also found in other studies. Dababat and Sikora (2007a) found that drenching of tomato seedlings with selected *Trichoderma* isolates caused reduction in the incidence and severity of the rootknot nematode *M. incognita*. Goswami et al (2008) also found that the application of a powder formulation of a *Trichoderma* isolate reduced *M. incognita* populations on tomato. Other *Trichoderma* and *Bacillus* isolates were also found to cause reductions in galling and egg mass production when applied as drenches to various crops (Sahebani and Hadavi, 2008; Shennawy et al, 2012; Szabó et al, 2012; Jamshidnejad et al, 2013; Abd-El-Khair and El-Nagdi, 2014). Xiong et al (2015) found drenching tomatoes with *Bacillus* strain YBf-10 reduced galling. Oliveira et al (2007) found metabolites of *Bacillus megaterium* Strain 54-06 reduced rootknot damage of coffee plants, while Wei et al (2014) also found drenching with another *Bacillus* Strain Jdm2 provided protection for up to 48% three months after planting tomato. Drenching with *Bacillus cereus* Isolate C1-7 was found to cause complete inhibition of root galling and egg mass development on carrot and tomato (Park et al, 2014). A commercial biocontrol agent BioNem® (*Bacillus* spp.) was also found to cause a reduction in rootknot galling on tomato by 75 – 84% and also to increase shoot weight by 20 – 24% (Terefe et al, 2009).

However, in this study it was also observed that seed treatments with Isolates C29 and EcoT®+C97 resulted in yield increase in nematode infested plots when compared to non-infested plots. It could be possible that the biocontrol agents were parasitic on the nematodes thereby deriving nutrition and this could have led to increased multiplication of the biocontrol agents. This, in-turn, would result in more benefits to the treated plants with nematodes than the un-infested plots.

Several modes of action have been cited as the basis for the nematicidal abilities of *Trichoderma* isolates (Verma et al, 2007). Zhang et al (2015) reported that the conidia of an isolate of *Trichoderma longibrachiatum* Rifai adhered to and parasitised the surface of juvenile nematodes, thereby deforming or completely dissolving them, and attributed this to proteases. Protease-transformed *Trichoderma* isolates displayed enhanced efficacy against both nematode eggs and juveniles when compared to wild type strains (Sharon et al, 2001).

The majority of studies on the efficacy of various bionematicides have been based on an inundative application in both controlled environments and in the field (Oyekanmi et al, 2007; Goswami et al, 2008; Abd-El-Khair and El-Nagdi, 2014). There are limited studies on seed treatment of tomato seeds with bionematicidal biocontrol agents in field trials and therefore comparison of results obtained in this study with similar work was a challenge. Although inundative approaches, especially drenches, have been successful in greenhouse and field trials, the amount of biocontrol inoculum required to treat large commercial fields, the costs and the technology involved are their major limiting factor (Athman et al, 2006; Viaene et al, 2006; Sikora et al, 2008). This is especially so for ovicidal products because the contact time between the antagonist and nematode pest needs to be prolonged or they will not reduce damage. In cases where the biocontrol agents are larvicidal and rhizosphere competent, penetration of infective juveniles is significantly reduced because the rhizosphere becomes the first line of defence and parasitism continues inside the root.

The success of commercial bionematicides has largely depended on effective formulations capable of persisting in the rhizosphere that operate by protecting plants in the early growth phases. Like all microorganisms biological control agents are affected by environmental factors and these influence their efficacy, resulting in inconsistent results. Edaphic factors that have been found to influence the survival, reproduction and activity of biocontrol agents include pH, temperature and other antagonistic organisms residing in the rhizosphere (Sayre and Walter, 1991; Barbercheck, 1992; Luambano et al, 2015). This study, therefore needs to be repeated both spatially and temporally, to gather further data on the efficacy of the isolates being tested.

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CHAPTER FIVE

USE OF *TRICHODERMA* AND *BACILLUS* ISOLATES AS SEED TREATMENTS TO PROTECT SOYBEAN PLANTS GROWN IN SOIL INFESTED WITH *MELOIDOGYNE JAVANICA* (CHITWOOD)

Abstract

Plant parasitic nematodes cause significant yield losses to soybean and options for their management are currently limited. Some nematicidal biocontrol agents, which include isolates of *Trichoderma* and *Bacillus,* reduce the severity and damagecaused by nematodes to crop plants, thereby improving yield. In this study, the biological control potential of five *Trichoderma* isolates and one *Bacillus* isolate was evaluated as seed treatments against *M. javanica* in the field. All of the six isolates under evaluation caused a reduction in galling severity and nematode rootknot nematode counts when compared with the Control. Seed treatment with EcoT®+C97 caused the highest reduction in galling severity. The test isolates, however, did not cause any improvement on soybean shoot biomass and seed weight. The results of this study indicate the potential of using biocontrol agents for effective management of rootknot nematodes in soybean using seed treatment.

5.1   Introduction

Plant parasitic nematodes are important plant pests causing significant yield losses globally (Whitehead, 1998; Nicol et al, 2011). These losses to plant parasitic nematodes may be higher than current estimates because data is lacking from many countries that lack nematology expertise (Luc et al, 1990; De Waele and Elsen, 2007; Jones et al, 2013). Most of the economic damage in crops is attributed to sedentary endoparasites in the family Heteroderidae, which comprises of two groups, the cyst nematodes and the rootknot nematodes (Williamson and Hussey, 1996; Abd-Elgawad and Askary, 2015).

The Javanese rootknot nematode (*Meloidogyne javanica* Chitwood) is the most economically important plant parasitic nematode of soybean in South Africa and occurs in most soybean (*Glycine max* (L.) Merr.) producing provinces of South Africa where it poses a threat to the

sustainable production of the crop (Fourie et al, 2001; Fourie et al, 2015). Currently where are no registered chemical nematicides in South Africa (Croplife, 2015; Fourie et al, 2015). This is compounded by the limited availability of host plant resistance among local soybean cultivars (Fourie et al, 2013; Fourie et al, 2015). Integrated nematode management remains one of the most important tools for rootknot nematode management (Haydock et al, 2006). Biocontrol agents are an economically feasible and safe alternative for the management of rootknot nematodes. *Trichoderma* spp. and *Bacillus* spp., among other biocontrol agents, have been found to be effective against plant parasitic nematodes (Haydock et al, 2006; Shennawy et al, 2012).

Trichoderma spp. have been found to have nematicidal properties as well as the ability to antagonise phytopathogenic fungi (Yobo et al, 2010; Affokpon et al, 2011; Elgorban et al, 2014). *Trichoderma* isolates are readily isolated from the soil and roots of plants in diverse habitats (Druzhinina et al, 2011), produce numerous conidia and readily colonise the root zone of most plants (Harman et al, 2004; Verma et al, 2007; Vinale et al, 2008). Several *Trichoderma* isolates are registered as bionematicides across the globe (Woo et al, 2014). However, only one isolate is currently registered in South Africa as a bionematicide, on carrot and citrus (Croplife, 2015). Most of these *Trichoderma*-based bionematicides are restricted to inundative applicationsthat are costly because large quantities of product have to be applied in order to treat the entire volume of soil in which crop roots will be found, and nematodes could be present. Development of seed treatments of bionematicides is currently being pursued by the global agrochemical industry as a more cost effective, targeted approach.

Bacteria that are rhizosphere competent or can colonise the rhizosphere are commonly referred to as rhizobacteria (Sikora et al, 1992). Globally, research into the use of plant growth promoting rhizobacteria has increased greatly and they have gained importance in agriculture (Figueiredo et al, 2011). Many of these rhizobacteria also supress plant diseases (Siddiqui et al, 2009). Metabolites produced by some *Bacillus* isolates have been found to be nematicidal and to reduce nematode damage to plants. *Bacillus* isolates survive extended periods of extreme environmental conditions by producing endospores. This attribute gives them extended shelf-livesand makes them ideal for seed treatments. This study sought to evaluate the efficacy of

Trichoderma and *Bacillus* isolates, previously evaluated in the greenhouse, against *M. javanica*when applied as seed treatments to soybean in field trials.

5.2   Materials and methods

A field experiment was conducted during the December/April 2013/2014 season at Ukulinga research farm, a facility of KwaZulu-Natal University, Pietermaritzburg, South Africa (29°24´E; 30°24´S). Soils at the site are classified as Westleigh form (Table 5.1).

Table 5.1   Soil physical and chemical properties for field trial.

pH	% Clay	Total cations cmol.L^{-1}	% Acid saturation	%Organic Carbon
4.44	37	16.84	1	2.9

5.2.1   Nematode inoculum, biocontrol agents and seed source

Inoculum of *Meloidogyne javanica* was initially obtained from the Unit for Environmental Sciences and Management, North West University, Potchefstroom, South Africa, and maintained on tomato (*Solanum lycopersicum* L.) cv. Moneymaker. Afield infestation was established by inoculating the respective plots with freshly chopped, infested tomato roots. The infested roots were evenly spread in the planting furrows. Soybean seeds were treated with biocontrol agents as described in Chapter 3. The Round-up Ready soybean cultivar, DM5953RSF, used in this study was obtained from Pannar Seeds (Pty) Ltd, Greytown.

5.2.2   Nematode assessments

At 110 days after planting the roots and rhizosphere of 5 plants per plot per treatment were sampled. Roots and soil from each treatment were thoroughly combined, a 200 cm^3 soil sub-sample and a 50 g plant root sub-sample was obtained, after which the samples were stored at 4°C until nematode extraction. Nematodes were extracted from the soil following the decanting and sieving method (Hooper et al, 2005), followed by a modified Baermann tray method (Hooper et al, 2005). Nematodes (eggs and J2) were also extracted from 1g root samples following maceration of 1 cm root pieces in 0.5% NaOCl (Hooper et al, 2005). The resulting suspension was then washed through a series of sieves (1000 μm, 330 μm, 190 μm, 100 μm

and 38 μm) with eggs and J2s being collected from the bottom sieve (38 μm). Galling severity was assessed on a scale of 0 – 8 (Daulton and Nusbaum, 1969) where: 0 = free of galls; 1 = trace, less than 5; 2 = 6-25 galls; 3 = 26-100 galls; 4 = moderate, gall numerous, mostly discrete; 5 = moderately heavy, numerous, many coalesced; 6 = heavy, galls very numerous, coalesced, root growth slightly retarded; 7 = very heavy, mass invasion, slight root growth; 8 = extremely heavy, mass invasion, no root development.

5.2.3 Yield assessments

Yield assessments were done by weighing of plant shoots at 110 days after planting and seed weight at harvest. The shoots from 5 plants per plot per treatment and seed from 10 plants per plot per treatment were oven dried at 70°C for 3 days before weighing. Percent yield loss increase/decrease was calculated for each treatment using the equation: {(mean yield of treated plots/yield of Control) × 100} – 100.

5.2.4 Trial design, layout and data analysis

The experimental design was a randomised complete blocks design laid out in a split plot arrangement. There were two factors in the main plot, rootknot nematode inoculation and no rootknot nematode inoculation. There were 9 subplot factors: Control (no sticker), Control (with carboxymethyl cellulose CMC sticker), EcoT®, Isolate C29, Isolate C59, Isolate C63, Isolate C97, Isolate C97+EcoT® and Isolate BC27. Inter-row and intra-row spacing were 10cm and 45 cm, respectively, and each plot consisted of four rows with 10 plants each. There were three replications for each treatment. Data was subjected to analysis of variance (ANOVA) using SAS 9.3 and means were separated using Duncan's multiple range test.

5.3   Results

5.3.1 Nematode assessments

The overall mean score for the galling in soybean plants was less than 2; on the scale of 0 – 8 which translates to between 5 and 25 galls. Seed treatments with both *Trichoderma* and *Bacillus* isolates caused a significant decrease in both galling severity and nematode counts, when compared with the Control (Table 5.2). Treatment with a combination of Isolates EcoT®+C97 caused the greatest reduction in gall severity (56%). The biocontrol agent EcoT® on its own reduced gall severity by 44% while the *Bacillus* isolate BC27 reduced galling by 38%.

Table 5.2 Rootknot nematode data for soybean plants treated with biocontrol agents in both nematode infested and non-nematode infested plots

Treatments	Galling index	Eggs and J2 counts in roots	J2 counts in soil
Control	0.1f	15g	10e
Control (Sticker)	0.0f	10g	5e
EcoT®	0.0f	0g	0e
C29	0.0f	5g	4e
C59	0.0f	12g	10e
C63	0.0f	4g	0e
C97	0.0f	0g	0e
C97+EcoT®	0.0f	0g	0e
BC27	0.0f	0g	0e
Control + *M. javanica*	1.6ab	740a	221b
Control (Sticker) + *M. javanica*	1.8a	646b	269a
EcoT® + *M. javanica*	0.9cde	405d	136c
C29 + *M. javanica*	0.8de	183f	119c
C59 + *M. javanica*	1.0cd	492c	252ab
C63 + *M. javanica*	1.1c	430cd	287a
C97 + *M. javanica*	1.4b	318e	152c
C97+EcoT® + *M. javanica*	0.7e	281e	85d
BC27 + *M. javanica*	1.0cd	157f	149c
	F=48.99	F=109.54	F=82.50
	P≤0.0001	P≤0.0001	P≤0.0001
	CV=27.09	CV=19.87	CV=21.49

[g]Means of 3 replications followed by the same letters in the same column are not significantly different at P < 0.0001 based on Duncan's multiple range test.

All treatments caused a significant reduction in the J2 and egg counts in soybean roots when compared to the nematode inoculated Control. All other isolates, except Isolates C59 and C63, caused significant reductions in the nematode counts in soil. There were insignificant rootknot nematode juvenile populations in the un-infested plots. Other plant parasitic nematode genera were encountered in the un-infested plots but the populations were also insignificant.

5.3.2   Yield assessments

Seed treatment of soybean with *Trichoderma* and *Bacillus* isolates did not have a significant effect on either dry shoot weight or seed weight (Table 5.3). However, seed treatment with the *Bacillus* Isolate BC27caused the greatest increase in shoot weight in plots not inoculated with nematodes. On the other hand, the greatest reduction in shoot weight was observed in Control plots where the seed was only treated with a carboxymethyl cellulose sticker.

Table 5.3 Soybean shoot weight and grain yield for soybean treated biocontrol agents, and planted in both nematode infested and non-nematode infested plots

Treatments	Shoot weight (g)	Yield increase (+)/ Yield decrease(-1)	Seed weight (g)	Yield increase (+)/ Yield decrease (-1)
Control	113.3	-	653.9	-
Control (Sticker)	93.7	-17.3	651.0	-0.4
EcoT®	124.3	+9.7	780.4	+19.4
C29	117.8	+4.0	699.1	+6.9
C59	105.7	-6.7	566.8	-13.3
C63	83.0	-26.7	587.8	-10.1
C97	105.6	-6.7	568.3	-13.1
C97+EcoT®	110.4	-2.6	597.5	-8.6
BC27	127.2	+12.3	666.9	+2.0
Control + *M. jav*	118.7	+4.8	529.3	-19.1
Control (Sticker) + *M. jav*	96.9	-14.5	550.2	-15.9
EcoT® + *M. jav*	122.0	+7.7	766.8	+17.3
C29 + *M. jav*	109.5	-3.4	640.8	-2.0
C59 + *M. jav*	106.5	-6.0	700.9	+7.2
C63 + *M. jav*	116.5	+2.8	726.7	+11.1
C97 + *M. jav*	107.1	-5.5	671.4	+2.7
C97+EcoT® + *M. jav*	114.2	+0.8	571.9	-12.5
BC27 + *M. jav*	113.3	0	628.0	-4.0
	P = 0.524		P = 0.84	
	CV=34.49		CV=21.80	
	F = 29.54		F = 0.64	

[g]Means of 3 replications followed by the same letters in the same column are not significantly different at P < 0.0001 based on Duncan's multiple range test.

Seed treatments with EcoT® caused an increase in seed weight of 17.3% in nematode infested plots and 19.3% in un-infested plots. Overall, small increases in yield parameters were observed in this study, even under nematode infestation.

5.4 Discussion

In this study one *Bacillus* isolate (BC27), four *Trichoderma* isolates (C29, C59, C63 and C97), a registered *Trichoderma*-based biocontrol agent (EcoT®) and a combination of EcoT® and C97 all caused reductions in galling severity when compared with the Control. Seed treatment with EcoT®+C97 caused the greatest reduction in galling severity, followed by Isolate C29. All the test isolates caused a reduction in egg and J2 counts in the roots, and J2's in the soil. However, none of the seed treatments with biocontrol agents caused a significant yield increase.

The results of the nematode assessments in this study confirmed the results obtained in two glasshouse studies using the same isolates (Chapter 2). Oyekanmi et al (2007) found an isolate of *Trichoderma pseudokoningii* Rifai to cause a reduction in galling severity when the isolate was applied to soybean seedlings prior to planting in the field. Similarly, Olabiyi et al (2013) found field applications of *Trichoderma* Isolate T22 to reduce rootknot nematode population when applied to soybean plots. Seed treatment using *Trichoderma* isolates has been found to cause a significant reduction in root galling severity on chickpea (*Cicer arietinum* L.) (Haseeb and Kumar, 2012), cowpea (*Vigna unguiculata* [L.] Walp)(Nama et al, 2015), cucumber (*Cucumis sativus* L.) (Yan et al, 2011) and okra (*Abelmoschus esculentus* (L.) Moench) (Lal and Rana, 2013). Multiple modes of action have been cited in various studies as being the basis for the nematicidal abilities of *Trichoderma* isolates (Verma et al, 2007). Zhang et al (2015) reported that the conidia of *Trichodermalongibrachiatum*Rifai adhered to and parasitised the surface of juveniles, thereby deforming or completely dissolving them, and attributed this to excreted proteases. Although there are few studies on seed treatment of soybean with bacterial isoaltes other studies have also found other *Bacillus* isolates to control rootknot nematodes in field trial with other crops (Chen et al, 2000; Abd-El-Khair and El-Nagdi, 2014; Flor-Peregrínet et al, 2014).

However, seed treatments did not cause any significant yield differences in this study, contrary to results obtained in prior glasshouse trials (Chapter 2). Although seed treatments with isolates such as EcoT® caused a yield increase of 17.3% in nematode infested plots, the yield increase was not significant. Other studies, however, have found the use of *Trichoderma* and *Bacillus* isolates to cause an increase in yield parameters under nematode infestation (Le et al, 2009; Radwan et al, 2012; Shennawy et al, 2012; Jamshidnejad et al, 2013; Elgorban et al, 2014; Zhang et al, 2014). The low nematode counts and galling severity observed here may suggest that the cultivar used in this study is not highly susceptible to *M. javanica*, or the environmental conditions were not conducive for nematode infection. In addition, there was a large variation in yield parameters as evidenced by the low R-square values ($R^2 = 0.31$ for shoot weight and $R^2 = 0.23$ for seed weight). Spatial and temporal replication of the trial, especially in sandy soils and with a higher initial nematode density, may be required to elucidate more measurable yield responses to the treatments. The high clay content in soils used in this experiment are not favourable for nematode multiplication and hence could have contributed to the low level of damage observed. The sticker used in this study (CMC) resulted in lower yields. This also, may

have hindered the efficacy of the bionematicides used in this study. The inoculum level and inoculation method in this study may also have contributed to lower damage. This becomes apparent when the results obtained in this study are compared to the glasshouse experiments.

Further work on these and other bionematicides, especially as seed treatments, is important as evidenced by the interest shown by major pesticide companies, which have been acquiring companies marketing these products in the last ten years. In 2009 the global pesticide market was estimated at USD 43 billion, with estimates of 5 – 8% growth per annum.Of this market, USD 1.6 billion is estimated to be spent on bionematicides (Tranier et al, 2014). Though the major proportion of this amount is accounted for by major pesticide companies, smaller companies in developing countries have started to develop bionematicides. For crops such as soybeans for which the use of nematicides is uneconomical, the use of bionematicides as seed treatments, especially when combined with host plant resistance, may offer a cost effective and sustainable nematode management option.

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CHAPTER SIX

SCREENING OF *TRICHODERMA* ISOLATES AGAINST *RHIZOCTONIA SOLANI* (KÜHN)-*MELOIDOGYNE JAVANICA* (CHITWOOD) ROOT-ROT DISEASE COMPLEX OF SOYBEAN

Abstract

The biocontrol potential of five *Trichoderma* strains was evaluated in laboratory and greenhouse studies against *Rhizoctonia solani* and *Meloidogyne javanica*. Four biocontrol test isolates (EcoT®, C29, C59 and C63) were highly inhibitory to *R. solani* mycelial growth. Isolates C29 and C63 completely overgrew the *R. solani* on solid media. Seed treatment of soybean with the four *Trichoderma* strains (EcoT®, C29, C59 and C63)caused a significant increase in seedling survival and shoot mass (P≤0.0001), while reducing the number of galls in the glasshouse. Seed treatment with Isolate C97did not causea reduction in galling and seedling survival, and shoot mass was comparable with the inoculated Control.

6.1   Introduction

Soybean is a major crop in many countries (Wrather et al, 2001) and remains one of the most valued oilseed crops in the world (Singh and Shivakumar, 2010). Soybean production, however, is affected by soil borne pathogens, which include rootknot nematodes (Sikora et al, 2005; Oyekanmi et al, 2007; Oyekanmi and Fawole, 2010) and damping off diseases (Wrather et al, 2001; Singh and Shivakumar, 2010). Soybean infestation with rootknot nematodes may also predispose the plant to infection by other plant pathogens such as *Rhizoctonia solani* (Kühn) (Whitehead, 1998).

Management of plant disease complexes is usually difficult, and although chemical control options exist, reduction of one pathogen may not necessarily resolve the problem of the disease interaction (Back et al, 2002). Nematicide and fungicide use for the control of nematodes and soil borne pathogens, especially in soybean, is not ideal because of costs, environmental contamination and toxicity (Mahdy et al, 2006; Fourie et al, 2015). Microbial agents, endophytes and antagonists offer a more sustainable approach to the management of both plant

parasitic nematodes and soil borne pathogens in various crops (Kerry, 2001; Hallman et al, 2009). Among these, a number of bacterial isolates and fungal isolates have shown potential as nematode antagonists (Akhtar and Malik, 2000).

Isolates of species belonging to the filamentous ascomycete genus *Trichoderma* have been found to be effective biocontrol control agents (Benítez et al, 2004; Vinale et al, 2008; Druzhinina et al, 2011). Various *Trichoderma* isolates reduce rootknot nematode damage in many crop plants (Sahebani and Hadavi, 2008; Affokpon et al, 2011; Radwan et al, 2012;Yobo et al, 2013; Zhang et al, 2014) and some isolates also cause a reduction in *Rhizoctonia solani* incidence and damage to various plants (Montealegre et al, 2014; Singh et al, 2014). Some *Trichoderma* isolates have also been found to control the rootknot-root rot disease in soybeans (Mahdy et al, 2006). *Trichoderma* isolates are suitable as biocontrol agents because of their ability to rapidly colonise the rhizosphere, produce numerous spores, and to release wide range of enzymes, since the bulk of plant parasitic nematodes are found in this zone (Harman et al, 2004; Verma et al, 2007; Vinale et al, 2008; Zhang et al, 2014).

This study was aimed at evaluating the efficacy of *Trichoderma* isolates against the disease complex caused by rootknot nematodes, *Meloidogyne javanica* (Chitwood), and a root-rot fungus, *Rhizoctonia solani*Kühn, on soybean.

6.2 Materials and methods
6.2.1 Pathogens and inoculum preparation
Meloidogyne javanica inoculum was initially obtained from the Unit for Environmental Sciences and Management, North West University, Potchefstroom, South Africa, and maintained on tomato (*Solanum lycopersicum* L.) cv. Moneymaker. Three week old seedlings grown on composted bark were inoculated with second stage juveniles (J2) and maintained in the glasshouse at 25 ± 2°C. At least four weeks after inoculation, or when inoculum was required, tomato roots were uprooted, washed with tap water to remove attached soil and chopped into 2-5 cm pieces.

Rhizoctonia solani was obtained from theUKZN Plant Pathology Culture Collection Accession code: UKZNPPRS1and maintained on PDA. Fungal inoculum for glasshouse trials was obtained by culturing the isolate on barley seeds. Barley seeds (150g) were soaked overnight in water in a 1L Erlenmeyer flask before they were sterilised for two consecutive days for 15 minutes at 121°C. The seeds were then inoculated with *R. solani* mycelial plugs and incubated at 25-28°C for 7 days. The contents of the flasks were then air-dried and kept in sterile bags before inoculation.

6.2.3   Source of Trichoderma isolates
Five *Trichoderma* isolates, four of them isolated from the root-zone of field plants and animal pasture, and one registered for the control of phytopathogens (EcoT®) were used in this study. The isolates used in this study were previously identified as *T. spirale*(Isolates C59 and C63),*T. harzianum*(Eco-T® and Isolate C29) and *T. virens*(Isolate C97).

6.2.4   Dual culture assay
The antagonistic potential of the *Trichoderma* isolates was evaluated by dual culture assay on 90mm petri dishes with PDA (Yobo et al, 2005). Bioassays were performed by placing colonised 4mm agar blocks of a selected *Trichoderma* isolate and the pathogen on PDA. The plugs were placed 4cm apart on opposite sides of a 9cm Petri dish. Three replications were used and the bioassay was repeated three times and the plates were incubated in the dark at 28°C for 5 days. Controls for the *Trichoderma* isolates and the pathogen were set up on PDA.

After incubation for 5 and 7 days, respectively, the dual culture plates were assessed for antibiosis, antagonism and/or invasion potential. The degree of antagonism of each isolate towards *R. solani* was rated according to Bell et al. (1982) on a scale of 1–5;

1 = *Trichoderma* completely overgrew *R. solani* and covered the entire medium surface;

2 = *Trichoderma* overgrew at least two thirds of the medium surface;

3 = *Trichoderma* and *R. solani* each colonised at least approximately one-half of the medium surface and neither organism appear to dominate each other;

4 = *R. solani* colonised at least two-thirds of the medium surface and appear to withstand encroachment by *Trichoderma*;

5 = *R. solani* completely overgrew the *Trichoderma* and occupied the entire medium surface.

Trichoderma isolates with a mean score ≤ 2 were considered to be highly antagonistic, otherwise not highly antagonistic.

6.2.5   In vivo screening of Trichoderma isolates on R. solani-M. javanica root disease complex

Soybean seeds were surface sterilised by rinsing them in 3% sodium hypochlorite for 5 mins, then rinsing them in sterile distilled water five times. A fungal spore-sticker suspension was made up by adding 1.0 g of carboxymethyl cellulose (CMC) to 50 ml of a conidial suspension (10^8 ml^{-1}) in a 200 ml flask. The suspension was shaken (150 rpm) in a rotary shaker (Model GFL 3005, Labortechnik, Germany). A batch of 120 seeds were added to each flask and allowed to soak in the spore-sticker suspension for about an hour with constant swirling. The treated soybean seeds were then air-dried in petri dishes under laminar flow overnight (16 – 18 hrs) (Yobo et al, 2010).

Ten soybean seeds were planted in the middle of 12 cm diameter pots filled with composted pine bark (Potting Mix, National Plant Foods, Camperdown, South Africa) and maintained in the glasshouse with uniform irrigation and fertigation. The media in each pot was pre-inoculated and evenly mixed with 5 g of 1-2 cm root pieces infested with nematodes and 10 *R. solani* infested barley seeds. Each treatment was replicated three times. The trial was terminated at 4 weeks after nematode inoculation. The plants were washed free of adhering soil and shoot weights and number of galls assessed.

6.2.6   Statistical analysis

Data was subjected to analysis of variance (ANOVA) using SAS 9.3 and means were separated using Duncan's multiple range test.

6.3   Results

6.3.1   *In vitro assays*

Mycelial growth inhibition varied among the *Trichoderma* test isolates (Table 6.1). Isolates C29 and C63 caused maximum inhibition of *R. solani* mycelial growth (Fig 6.1). These isolates (C29 and C63) caused complete replacement of *R. solani* mycelia after an initial deadlock. EcoT® and Isolate C59 caused partial replacement of *R. solani* mycelia after an initial deadlock while Isolate C97 did not cause replacement after an initial deadlock.

Table 6.1   *In vitro* antagonism of *Trichoderma* isolates to *R. solani*

Isolate	Bell rating	Invasion ability	Antibiosis
EcoT®	3	2	+
C29	3	1	+
C59	3	2	+
C63	2	1	+
C97	3	3	_

Fig 6.1 Interactions between *Trichoderma* isolates and *Rhizoctonia solani* on PDA at 25°C
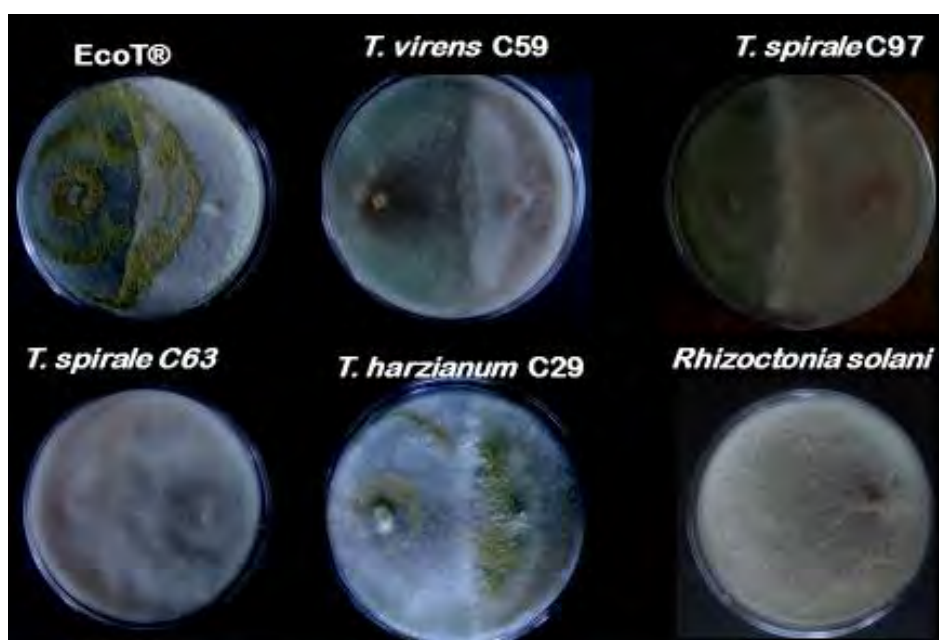
NB: All *Trichoderma* isolates growing from left to right and *R. solani* from opposite direction.

All the five *Trichoderma* isolates under evaluation, including the commercial biocontrol agent EcoT®, caused a significantreduction in the number of galls and caused an increase in seedling survival and shoot weight compared to the Control (P < 0.001) (Table 6.2). Furthermore, amongst the *Trichoderma* isolates, Isolate 63caused the greatest reduction in nematode damage while Isolate C97 caused the least reduction.

Table 6.2   Biological control of the rootknot nematode and root-rot disease complex on soybean

Isolate	% Survival	Fresh weight	Number of galls
Un-inoculated Control	90.0a	11.03a	0e
Inoculated Control (*M. jav + R. sol*)	43.3cd	4.76e	115.0a
EcoT®	56.7bc	6.19d	89.2b
C29	60.0bc	6.52cd	81.8b
C59	70.0ab	7.48c	63.3c
C63	70.0ab	9.61b	49.7d
C97	40.0d	4.80e	120.0a
	F=8.16 P≤0.0006, CV=18.75	F=8.16 P≤0.0001, CV=18.75	F=8.16 P≤0.0001, CV=18.75

6.4   Discussion

Plants are exposed to many pathogens, and their roots usually harbour many diverse microorganisms whose combined action causes significant damage (Karsen and Moens, 2007). Many nematode-fungus disease complexes involve rootknot nematodes, although other nematode species maybe involved (Back et al, 2002). Management of nematode-fungal disease complexes is complicated because management options are often effective on only one pathogen. The rootknot and root-rot disease complex caused by the rootknot nematode,*M. javanica,* and the root-rot fungus,*R. solani,*hasreceived significant attention in many crops including soybean (Agu 2002; Mahdy et al, 2006; Safiuddin and Shahab, 2012; Rizvi et al, 2015). Management of nematodes and nematode-fungi disease complexes on soybean using chemical control options is not feasible because of cost implications. The use of bionematicides

and biocontrol agents to manage phytopathogens offers a more cost effective, less toxic and more environmentally friendly option, especially for low value crops such as soybeans.

In this study, five *Trichoderma* strains were evaluated for their *in vitro* antagonism to *R. solani* as well as their effectiveness in controlling the rootknot and root-rot disease complex on soybean. Of these, four strains (C29, C59, C63 and EcoT®) were found to be highly antagonistic to *R. solaniin vitro*. Isolates C29 and C63 also caused maximum inhibition of *R. solani* mycelial growth with complete replacement of *R. solani* mycelia after an initial deadlock. Research elsewhere has also shown the ability of other*Trichoderma* strains to inhibit the growth of *R. solani* mycelia *in vitro* to varying degrees (Parizi et al, 2012; Olabiyi and Ruocco, 2013; Montealegre et al, 2014; Kotasthane et al, 2015). Unpublished results also found the four *Trichoderma* strains were also highly antagonistic *in vitro*to pathogenic strains of two other fungal pathogens, *Alternaria solani* (Ellis and G. Martin) L.R. Jones and Grout and *Fusarium solani* (von Martius)Saccardo.

In the glasshouse, sowing soybean seed into pine bark media pre-inoculated with *R. solani* and *M. javanica* resulted in poor germination, poor survival, reduced shoot weight and significantly higher galling in the inoculated Control. Seed treatment with all the *Trichoderma* isolates, except C97, improved soybean seedling survival, increased shoot weight and caused a reduction in galling when compared to the inoculated Control. Isolates C29 and C63 caused the greatest reduction in galling, and the greatest shoot weight and seedling survival. Biocontrol agent EcoT® combined with Isolate C59 also caused a significant increase in shoot weight and a reduction in galling. Mahdy et al (2006) also found seed treatment with a *Trichoderma* isolate caused an increase in seedling survival while reducing galling and disease severity in soybean. *Trichoderma* isolates have also been found to cause a reduction in rootknot and root-rot disease severity in other crops (Siddiqui et al, 1999; Bano et al, 2011). However, pine bark media favours the growth of saprophytic antagonists and hence may not represent ideal field conditions.

This study demonstrated the potential of four test *Trichoderma* strains to provide protection from two soybean pathogens, *R. solani* and *M. javanica*. Seed treatment with

*Trichoderma*strains may therefore offer a cost effective means of disease management in soybean, especially when used in combination with host plant resistance. The compatibility of these strains with commonly used agrochemicals such as fungicides to control soybean rust and their efficacy and reproducibility in field trials need to be evaluated further. Full registration processes would have to be followed before a nematicidal biocontrol product could be released to farmers.

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A Thesis Overview:

Major Findings and Their Implications

Introduction

Plant parasitic nematodes are a major constraint affecting crop production, especially in the tropics. Among the various groups of plant parasitic nematodes, rootknot nematodes (*Meloidogyne* spp.) are the most important nematode genera globally affecting most crop plants and resulting in significant yield losses. Rootknot nematodes, *Meloidogyne* spp., which are widely distributed in the tropics and subtropics, attack almost every species of higher plants and are the most damaging nematode genus globally (Sasser, 1980; Onkendi et al, 2014).Various approaches are being used for the management of plant parasitic nematodes,including chemical, physical, cultural methods and host plant resistance. However, pressure to reduce the use of chemical nematicides because of health and environmental concerns, and the shortage of nematode resistant germplasm has led to the development of antagonistic micro-organisms as biological control agents to these pests. Some of the most promising isolates that have been utilised and registered as biocontrol agents against rootknot nematodes belong to the genera *Trichoderma* and *Bacillus*.

The majority of the research and development of bionematicides havelargely been based on an inundative application in both controlled environments and in the field. However, relatively few isolates havebeen tested successfully in the field as seed dressing. Although inundative approaches, especially drenches, have been successful in greenhouse and field trials, the amount of biocontrol inoculum required to treat large commercial fields, the costs and the technology involved are their major limiting factor (Athman et al, 2006; Viaene et al, 2006; Sikora et al, 2007). The success of commercial bionematicides has largely depended on effective formulations capable of persisting in the rhizosphere to protect plants in the early growth phases.

The present research was therefore undertaken with the overall objective of developing seed treatments using*Trichoderma* and *Bacillus* based biocontrol agents for the management of *M. javanica*. Consequently, the research focused on: 1) Isolation, *in vitro* and *in vivo* screening of

Bacillus isolates against *M. javanica*; 2) Isolation, *in vitro* and *in vivo* screening of *Trichoderma*isolates against *M. javanica*; 3) The evaluation of the efficacy of soybean seed treatments with selected biocontrol agents on *M. javanica* damage and crop yield; 4) The evaluation of the efficacy of tomato seed treatments with selected biocontrol agents to reduce *M. javanica* damage and to increase crop yield; 5) To screen *Trichoderma* isolates against the *M. javanica* and *Rhizoctonia solani* disease complex of soybean. This overview presents a summary of the major findings and implications thereof.

Chapter 2:   Biological control of the rootknot nematode, *Meloidogyne javanica* (Chitwood) using *Bacillus* isolates, on soybean.

Major findings

- Five out of 70 bacterial isolates from the root-zone of crops and goat pasture grasses caused second stage juvenile (J2) mortality greater than 50% *in vitro* after 24 hours.

- Three of the five selected isolates (BC27, BC29 and BC31), which were isolated from the rootzone of grass in a goat pasture, caused J2 mortality greater than 80% at 10^8 spores ml^{-1}*in vitro* after 24 hours, with BC27 causing 100% J2 mortality after 3 hours.

- Seed treatment of soybean with Isolates BC27 and BC29 caused a reduction in rootknot galling and egg mass counts (P≤0.0001) and also caused a significant increase in shoot weight (P≤0.0001), when compared to an inoculated Control.

- Blast analysis revealed that the two selected isolates, BC27 and BC29, exhibited similar sequences to *Bacillus* spp. T2 and *Bacillus* spp. KT18, listed on the Gen-Bank, respectively.

Implications

- A small proportion of bacterial isolates associated with the root-zone of plants have potential bionematicidal properties.

- Seed treatment of soybean, as opposed to inundative application of biocontrol agents, may offer a more cost effective, farmer and environmental friendly method of application of biocontrol agents.

- Members of the genus *Bacillus* are potential bionematicides and in the spore form have long shelf lives.

Chapter 3: **Biological control of the rootknot nematode,** *Meloidogyne javanica* **(Chitwood) using** *Trichoderma* **isolates on soybean.**

Major findings

- 11 out of 111 *Trichoderma* strains isolated from the root zone of field crops and animal pastures caused J2 mortality greater than 50% after 24 hours.

- Five *Trichoderma* isolates, including a commercial *Trichoderma* strain (EcoT®), caused a significant reduction in the number of galls and egg masses when applied as seed dressing on soybean (P≤0.0001). All the test isolates also significantly increased fresh shoot weights of soybean plants when compared to the inoculated Control (P≤0.0001).

- Isolate C29 caused the greatest reduction of galling and caused the greatest increase in shoot weight.

- Blast analysis revealed that the four selected isolates,C29, C59, C63 and C97, exhibited similar sequences to *T. harzianum*, *T. spirale*, *T. spirale* and *T. virens*,respectively, as listed on the Gen-Bank.

- Isolate C29 was isolated from a tobacco plant root zone, C59 from a sweet pepper plant root zone, C63 from a sheep pasture and C97 from a goat pasture.

Implications

- Only 10% of test *Trichoderma* isolates from the root zone of field crops and animal pastures were found to sporulate well and cause mortality greater than 50%.

- The root zone of field plants and animal pastures harbour a reservoir of biocontrol agents that can be utilised for the control of plant parasitic nematodes.

- Seed treatment with *Trichoderma* isolates offers good prospects for the management of plant parasitic nematodes because of their versatility and ease of production.

Chapter 4: Use of *Trichoderma* and *Bacillus* isolates to protect tomato plants grown in soil infested with *Meloidogyne javanica* (Chitwood)

Major findings

- Seed treatment of tomato with *Trichoderma* and *Bacillus* isolates before transplanting into soil infested with rootknot nematode caused a significant decrease in both gall severity and nematode counts in the soil and roots when compared to the inoculated Control.

- Seed treatment with *Trichoderma* strains C29 and EcoT®+C97 caused the greatest reductions in gall severity of 45% and 40%, respectively.

- Seed treatment of tomato with *Trichoderma* and *Bacillus* isolates before transplanting also caused a significant increase in yield when compared to the inoculated Control.

- The greatest yield increase was observed in nematode infested plots planted to C29 treated seed with an increase of 66%.

Implications

- Seed treatment of tomato with *Trichoderma* and *Bacillus* offers a cost effective alternative to nematicide use.

- *Trichoderma* isolates C29, EcoT® and EcoT®+C97 can be considered for use as bionematicides after evaluation for consistency of results, both spatially and temporally.

- EcoT®is a registered biocontrol agent for the control of other phytopathogens and hence its role for the management of a wide range of pathogens is ideal, especially in Integrated Pest Management. Given that it is already registered for other biocontrol uses, its registration would be relatively quick.

- The yield increase caused by seed treatments with *Trichoderma* isolates, when combined with tolerant and resistant cultivars, may justify the reduction or replacement of nematicides, especially in fields with low initial nematode densities.

Chapter 5: Use of *Trichoderma* and *Bacillus* isolates as seed treatments to protect soybean plants grown in soil infested with *Meloidogyne javanica* (Chitwood)

Major findings

- Seed treatment of soybean with five*Trichoderma* and one *Bacillus* isolate before transplanting into soil infested with rootknot nematode caused a significant decrease in both gall severity and nematode counts in the soil and roots when compared to the inoculated Control.

- Seed treatment with *Trichoderma* strains EcoT®+C97 caused the greatest reductions in gall severity of 56%.

- However, seedtreatment of soybean with all test isolatesdid not cause a significant increase in yield in soybean when compared to the inoculated Control.

Implications

- All the test*Trichoderma* isolates can be considered as candidates for commercialisation as bionematicides on soybeans after evaluation for consistency of results both spatially and temporally.

- EcoT®, a registered biocontrol agent,may give an advantage over the other *Trichoderma* isolates because it has already been found to be compatible with soybean *Rhizobium* spp. and has passed the regulatory hurdles as a biocontrol agent of root diseases.

- The potential of the novel *Trichoderma* isolatesto enhance crop yields needs to be further evaluated in field trials.

Chapter 5: Screening of *Trichoderma* isolates against *Rhizoctonia solani* (Kühn)-*Meloidogyne javanica* (Chitwood) root-rot disease of soybean.

Major findings

- Four test isolates (EcoT®, C29, C59 and C63) were found to be highly inhibitory to *R. solani* mycelial growth*in vitro.*

- Seed treatment of soybean with the four *Trichoderma* strains (EcoT®, C29, C59 and C63) caused a significant increase in seedling survival and shoot mass (P≤0.0001) while reducing the number of galls in the glasshouse when compared to the inoculated Control.
- Seed treatment with Isolate C97, however, did not cause a reduction in galling and seedling survival, and shoot mass was comparable to the inoculated Control.

Implications

- Four test *Trichoderma* isolates (EcoT®, C29, C59 and C63) were effective in suppressing *R. solani* mycelial growth and hence could be used for its control.
- The four test isolates could be used for the management of the *R. solani-M. javanica* disease complex on soybean.
- Extensive field trials needs to be conducted to confirm their efficacy and the return on investment for farmers applying *Trichoderma* to soybean seed.

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