BIOLOGICAL CONTROL OF ROOT-KNOT NEMATODES (*MELOIDOGYNE* SPP.) USING BACTERIAL AND FUNGAL ANTAGONISTS

By

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Thesis Summary

Root-knot nematodes are an important pest of many crops worldwide. Chemical nematicides are the main control methods used to reduce damage caused by nematode pests on crops. However, there are aims of reducing the use of chemical nematicides, resulting in a shift towards the use of biological control, which is an environmentally friendly and safer method of control. Potential antagonists of *Meloidogyne javanica* were isolated from grazing pastures of livestock, and the rhizosphere of tomato plants grown under glasshouse conditions. A total of 94 bacterial and 22 fungal isolates were screened *in vitro* by means of microwell bioassays. Twenty bacterial and eight fungal isolates showed nematicidal activity, causing root-knot nematode second-stage juvenile (J2) mortalities of between 47.0% and 65.4%, and 33.0% and 66.3%, respectively. Five bacterial and three fungal isolates caused J2 mortalities of more than 60%. *In vitro* studies were conducted to evaluate the efficacy of these isolates (*Bacillus* spp., *Hypocrea lixii*-the teleomorph of *Trichoderma harzianum*) and *Trichoderma spirale*, together with two previously isolated biocontrol agents, *Hypocrea lixii* Strain Eco-T® and *Clonostachys rosea,* on the root-knot nematode *M. javanica*. All the bacterial isolates and fungal treatments caused significant levels of J2 mortality of *M. javanica* of between 59.0% to 94.0% after 12, 24 and 48 h. *Bacillus thuringiensis* (Isolate BG25) and *H. lixii* (Isolate Cr5) caused the highest mortality of J2. *B. thuringiensis* (Isolate BG25) and *H. lixii* (Isolate Cr5), when applied as a seed dressing or as a soil drench significantly ($P<0.001$) reduced penetration of *M. javanica* J2into the roots of tomato plants. The two isolates also reduced disease severity and significantly ($P<0.001$) reduced formation of galls, production of egg masses and the number of eggs per root. Growth parameters in terms of shoot length, shoot weight and dry shoot weight were significantly ($P<0.001$) increased by seed dressing and soil drench treatments of all bacterial and fungal isolates. *B*. *thuringiensis* (BG25) and *H. lixii* (Cr5) caused the greatest effect on growth parameters measured on tomato plants under greenhouse conditions.

DECLARATION

I, Gilmore Taenzaniswa Pambuka, 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 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.

Signed: ……..
Gilmore T. Pambuka

Signed: ……..
Prof. Mark D. Laing

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Thesis Introduction

Root-knot nematodes (*Meloidogyne* spp.) have detrimental effects on a wide range of crops worldwide (Sahebani and Hadavi, 2008; Jamshidnejad *et al*., 2013). Infection by*Meloidogyne* species can be diagnosed by the presence of galls on plant roots (Collange *et al*., 2011). They are recognized as major sedentary endoparasites. They disturb the physiology of host plants, reducing yields (Jamshidnejad *et al*., 2013).Four speciesgenerally affect vegetable production worldwide severely: *Meloidogyne arenaria* (Neal) Chitwood, *Meloidogyne javanica* (Treub) Chitwood, *Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogyne hapla* Chitwood (Moens *et al*., 2009). They are predominantly found in the tropical and sub-tropical regions of the world (Kayani *et al*., 2013). Root-knot nematodes have been primarily controlled using chemical nematicides (Zasada *et al*., 2010). However this is expensive, and many nematicides are highly toxic, with negative effects on the environment (Hasabo and Noweer, 2005), humans (Verdoorn, 2012) and mammals (Rehman *et al*., 2009).

Several methods of control have been suggested as alternatives to the use of chemical nematicides (Hussain *et al*., 2011; Radwan *et al*., 2012; Mukhtar *et al*., 2013a). These include biological control of these pests, which is considerably safer and more environmentally friendly (Mukhtar *et al*., 2013b). Several genera of bacteria and fungi are known to kill nematodes. Rhizobacteria and nematophagous fungi are the most studied bacterial and fungal antagonists of nematodes (Lamovsek *et al*., 2013). These organisms have also demonstrated the ability to promote plant growth in most crops, enhancing yield, while at the same time they reduce and suppress populations of *Meloidogyne* species.

The overall objective of the current study was to isolate bacteria and fungi from local soils and the rhizosphere of vegetable crops, identify theisolates that performed superiorly and evaluate their potential as biocontrol agents and as plant growth promoters.

The specific objectives were as follows;

- To review the literature covering the biology, epidemiology, economic importance and control options for root-knot nematodes (*Meloidogyne* spp).

- To isolate and screen potential antagonists against the root-knot nematode *Meloidogyne javanica* from different habitats of the livestock and glasshouse sections at the Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg.
- To evaluate the nematicidal potential of selected fungal and bacterial isolates against *M. javanicain vitro*.
- To evaluate the nematicidal potential of bacterial and fungal isolates in controlling *Meloidogyne* infesting tomato plants under greenhouse conditions.

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CHAPTER ONE

LITERATURE REVIEW

1.1 The Genus *Meloidogyne*

1.1.1 Introduction

The genus *Meloidogyne* (root-knot nematode) represents sedentary endoparasites, representing a small part of allplant parasitic nematodes, but are the most frequently observed and the most damaging species (Elgorban *et al*., 2013). Barkeley in 1885 was the first to notice the existence of root-knot nematodes, when he observed galls on roots of cucumber plants in a glasshouse (Nasr Esfahani, 2009, Moens*et al*., 2009). Based on infections of the roots of *Onobrychis sativa* Lam, Cornu in 1879 first named the root knot nematode as *Angiullula marioni*, which Muller in 1884 later changed to *Heterodera radicicola* (Hunt and Handoo, 2009). In 1887, Goeldi was the first to use the term *Meloidogyne* when he described the species *Meloidogyne exigua* (Moens *et al*., 2009).

Several names were given to root-knot nematodes. They were initially placed in the same genus as cyst nematodes, *Heterodera*, until Chitwood (1949) reclassified them to the genus *Meloidogyne* as proposed by Goeldi, which is used to describe the four most common species: *Meloidogyne javanica* (Treub 1885), *M. incognita* (Kofoid and White, 1919), *M. arenaria* (Neal, 1889) and *M. hapla* (Chitwood, 1949). The occurrence of root-knot nematodes worldwide was summarised by Sasser in 1977, of which 11 of those species were found in Africa (Nasr Esfahani, 2009).

New species of *Meloidogyne* are being discovered over time, with more than 90 having been ascribed to this genus (Moens *et al*., 2009). The current taxonomic classification of *Meloidogyne* by Chitwood (1987) is as follows:

Kingdom: Animalia

Phylum: Nematoda

Class: Secernentea

Subclass: Diplogasteria

Order: Tylenchida

Suborder: Tylenchina

Super family: Tylenchoidea

Family: Heteroderidae

Subfamily: Meloidogyninae

Genus: *Meloidogyne*

1.1.2 Identification

Meloidogyne species are identified based on morphological, biochemical and molecular approaches. Morphological identification is based on the use of electron or light microscope observations involving comparisons of morphological structures, which include perineal patterns, and the morphological characteristics of the females, second-stage juveniles (J2) and males, body length, stylet shape, and the shape of the head and tail (Blok and Powers, 2009).

Biochemical methods are an important tool for identification of *Meloidogyne* species. Two methods are used in biochemical identification, namely protein separation and serological methods (Abrantes *et al*., 2004). Protein characterization is based on the use of one-dimensional and two-dimensional gel electrophoresis and the use of four enzyme patterns (non-specific esterases, malate dehydrogenase, superoxide dismutase and glutamate-oxaloacetate transaminase) (Moens *et al*., 2009). Serological methods include the use of polyclonal and monoclonal antibodies (Blok and Powers, 2009).

Molecular techniques have helped in accurate identification of various *Meloidogyne* species (Blok, 2005). Molecular methods are more reliable than morphological or biochemical methods (Powers *et al*., 2005). Polymerase chain reaction (PCR), molecular markers and deoxyribonucleic acid (DNA) sequencing are the most commonly used for identification (Blok, 2005; Goncalves de Oliveira *et al*., 2011). Most commonly used DNA based identification techniques are based on ribosomal DNA (rDNA), mitochondrial DNA (mtDNA), restriction fragment length polymorphism (RFLPs), micro-satellite DNA (SatDNA) and random amplified polymorphism DNA (RAPD) (Blok and Powers, 2009).

1.1.3 Climatic Distribution

Meloidogyne species are distributed worldwide and their presence is mainly determined by prevailing soil moisture and temperatures. They occur in tropical, subtropical and temperate areas where they cause extensive damage, reducing quality and quantity of vegetable crops (Kayani *et al*., 2012). The most common and widespread *Meloidogyne* species are *M. javanica*, *M. incognita*, *M. arenaria* which are thermophile species and *M. hapla* which is a cryophil species (Moens *et al*., 2009). *M. javanica*, *M. incognita* and *M. arenaria* are found in tropical and sub-tropical areas, with *M. hapla* being found in warm temperate climates (Alcals, 2007; Perry *et al*., 2009). According to Taylor and Sasser (1978), *Meloidogyne* development is suppressed at temperatures higher than 40°C and below 5°C. Thomson and Lear (1961) found that *M. javanica* and *M. incognita* reproduced prolifically at 20, 25 and 30°C, but if reproduction occurred at 35°C, few galls and egg masses appeared.

1.1.4 Pest significance

Meloidogyne cause damage to vegetable crops estimated at 5% in yield loss globally (US$100 billion per annum) (Cetinas and Yarba, 2010a). Their additive interaction with plant pathogenic fungi and bacteria increases yield losses (Rivera and Aballay, 2008). The four most common species of economic importance are *M. javanica, M. incognita*, *M. arenaria* and *M. hapla* (Golzari, 2008). A typical example is *Meloidogyne* on tomato (*Lycopersicon esculentum* L.), which has been shown to be able to cause losses of up to 80% (Sikora and Fernandaz, 2005; Kaskavalci, 2007). In South Africa, *Meloidogyne* species pose a threat to agricultural production, with *M. javanica* and *M. incognita* being the most common in the major tomato producing regions of the country (Kleynhans *et al*., 1996; Coyne *et al*., 2007), accounting for losses of 14% in both agricultural and horticultural crops (ARC, 2014).

1.1.5 Host Range

Meloidogyne attack a wide range of important field and vegetable crops and several common weed species (Sikora and Fernandez, 2005; Sahebani *et al*., 2008). They have a host range, covering approximately 5000 plant species (Bagheri *et al*., 2014). Rapid multiplication of *Meloidogyne* on a susceptible host makes it difficult to prevent

crop damage, even at small initial population levels, because they initially increase rapidly in one growing season (Ehwaetim *et al*., 1998; Shahab and Sharma, 2011). Tomato, potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), lettuce (*Lactuca sativa* L.) and pepper (*Capsicum annuum* L.) are highly susceptible hosts, while tobacco (*Nicotiana tabacum*L.), sugarcane (*Saccharum officinarum* L.), maize (*Zea mays* L.), sugarbeet (*Beta vulgaris* L.), wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum* L.), cassava (*Manihot esculenta* C.), and members of the Umbelliferae and various Poaceae (grasses and weeds) are able to sustain *Meloidogyne* populations. Moderately and slightly susceptible hosts are cabbage (*Brassica oleracea* var. *capitata* L.), cauliflower (*Brassica oleracea* var. *botrytis* L.), rice (*Oryza sativa* L.), and onions (*Allium cepa* L.).

1.1.6 *Meloidogyne* disease cycle

The life cycle (Fig 1.1) of *Meloidogyne* species has six developmental stages: the egg, first-stage juvenile (J1), second-stage juvenile (J2), third-stage juvenile (J3), fourth-stage juvenile (J4) and the adult male or female stage (Agrios, 2005). The cycle starts with the female laying eggs in a gelatinous substance composed of a glycoprotein matrix produced by her. This protects the eggs from predators and harsh environmental conditions, and keeps them intact. Within the egg, the embryo undergoes several stages of development, giving rise to the J1, which remains and moults in the egg developing into a J2, which is the infective stage of *Meloidogyne* species that finally hatches. The J2s move through the soil to find a suitable host. Attraction of J2s toroots is due to several gradients of volatile and non-volatile compounds including amino acids, ions, pH, and temperature and carbon dioxide (Perry *et al*., 2013). They use one of these gradients to find a suitable host, reducing the time the nematodes move without food.

Once the J2s finds roots of a suitable host, they enter the roots by embedding their heads into the developing vascular bundles. Feeding commences on the cells by use of its stylet, secreting saliva into the cells. The larvae then become sedentary and thicken in size, to assume a “sausage” shape. Feeding causes enlargement of the cells surrounding the J2, liquefying the contents of the cell. Under favourable conditions, the J2 stage undergoes a second moult to the J3, which is stouter and lacks a functional stylet. J3 undergoes a third moult and is referred to as the J4. At this stage the male and female can be distinguished. The J4 undergoes a fourth and

final moult, giving rise to an adult nematode. The male leaves the root and enters the soil, while the female remains inside the root. The female continues to swell, increasing in girth and in length and with or without fertilisation by the male, produces eggs inside or outside the root tissue, depending on her relative position. A life cycle is completed in approximately 21 to 25 days, at temperatures of approximately 27℃. Each *Meloidogyne* species generates a different number of completes per year (Agrios, 2005).

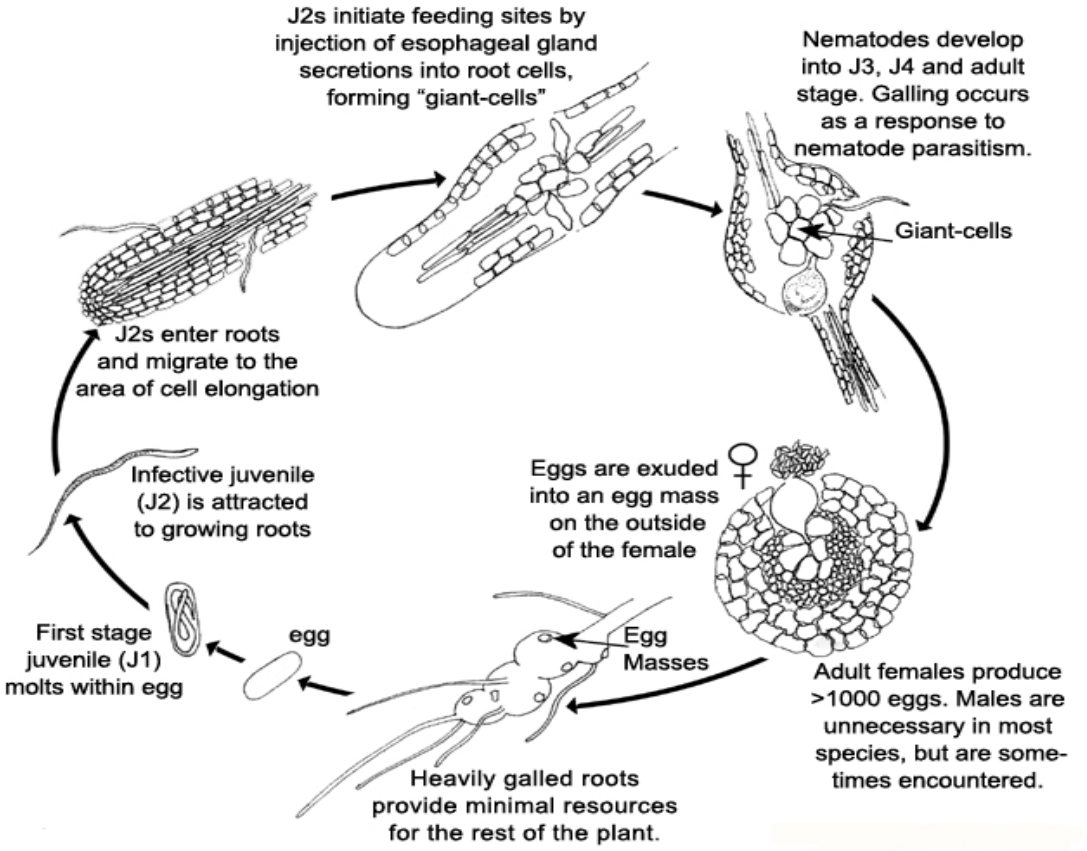

Figure 1.1: The disease cycle of the root knot nematode (Mitkowski and Abawi, 2003)

1.1.7 *Meloidogyne* spp. symptoms

Meloidogyne cause varying degrees of abnormal above ground symptoms. Spatial distribution of root-knot nematodes in the soil causes uneven and irregular growth patterns in the field. Infected plants may appear small and limited clumps or in large patches, or are widely distributed. Damaged plants may exhibit symptoms such as wilting and leaf roll, and mild to severe stunting, depending on disease severity.

Foliage may show symptoms of nutrient deficiency such as chlorosis. In severe cases, plants may die before maturation (Agrios, 2005).

Observing below ground symptoms involves uprooting of the plant or excavation of roots. Infected roots exhibit galls or knots (Bagheri *et al*., 2014). This is due to invasion and feeding by the J2, forming giant cells in the plant vascular bundles, resulting in swelling of the roots (Agrios, 2005). This explains the above ground symptoms that can be noticed because the root infections change the physiology of the plant (Perry *et al*., 2009). Galls vary according to the *Meloidogyne* species, and the susceptibility of the crop and cultivar. When infection increases, roots form massive clumps of tissue, which coalesce, imparting a clubbed appearance to the roots. Infected roots show stunted growth, necrosis (Bagheri *et al*., 2014), and eventually rotting towards the end of the season (Agrios, 2005).

1.2 Management of *Meloidogyne* spp.

Management of *Meloidogyne* is aimed at reducing and eliminating populations within the soil, and protecting agricultural and vegetable crops from damage. Control of *Meloidogyne* species is based on either chemical nematicides or non-chemical methods (Zasada *et al*., 2010). However, nematicide use is being reduced due to environmental problems, and human and animal health concerns, creating pressure for the development of novel non-chemical methods.

1.2.1 Chemical control

Nematicides have been the major management method in controlling *Meloidogyne* in crops. Their formulations and active compounds interfere with the life cycle of *Meloidogyne,* reducing reproduction and hence curtailing nematode populations in the soil. Nematicides are classified into two groups: fumigants and non-fumigants (Zasada *et al*., 2010).

Fumigant nematicides are commonly found as gases, volatile liquids, gels, flowables, spray concentrates or granules. All convert to gases, enabling them to diffuse rapidly through the soil killing the nematodes (Netscher and Sikora, 1990). The broad spectrum of activity of fumigant nematicides also makes them effective in controlling nematode eggs and juveniles (Netscher and Sikora, 1990). The most widely used fumigant nematicides have been methyl bromide, chloropicrin, ethyl dibromide,

metham sodium, methyl iodide and 1, 3-dichloropropene (Table 1.1). Methyl bromide has a broad spectrum activity and has been the premier soil fumigant, effective in managing *Meloidogyne a*nd other nematode species as well as other soil-borne pests (Zasada *et al*., 2010). However, methyl bromide has been removed from the market. Its high toxicity and volatility makes it dangerous to handle and it has negative side effects on the environment. However few alternatives have been found to replace it in most developing countries (Zasada *et al*., 2010).

Non-fumigants can be found in liquid or granular forms and are very soluble in water. Unlike the fumigant nematicides, non-fumigants are not phytotoxic and can be applied at planting by surface or drip irrigation. They are placed in close proximity to the future root zone, and spread through rain water and soil moisture in the soil to the targeted nematodes. However, they are less effective against *Meloidogyne* eggs and rarely kill the juveniles and adults at the recommended doses, but are used to delay infection a few weeks into planting. Non-fumigant nematicides are grouped into carbamates and organophosphates. Examples of carbamates include: oxamyl (Vydate®), carbofuran (Furadan®). Common organophosphates used in controlling *Meloidogyne* are fenamiphos (Nemacur®), cadusafos (Rugby®) and isozofor (Viral®) (Table 1.1).

Table 1.1 Selected fumigants and non-fumigants used to control plant-parasitic nematodes, since ban on use of methyl bromide (Zasada *et al*., 2010)

Chemical name	Common name	Trade name	Manufacturer
Trichloronitromethane	chloropicrin	Chloropicrin	Dow AgroSciences, Indianapolis, IN
Sodium N-Methyldithiocarbamate	metam sodium	Vapam	Amvac Chemical Corporation, Los Angeles, CA
Potassium N-Methyldithiocarbamate	metam sodium	k-Pam	Amvac Chemical Corporation
Tetrahydro-3,5-Dimethyl-2H-1,3,5-Thiadiazine-2-thione	dazomet	Basamid G	Certis, Columbia, MD
1,3-Dichloropropene		Telone II, Telone C-35, Telone C-17 Cordon, Inline	Dow AgroSciences
Methyl iodide	iodomethane	Midas	Arysta LifeScience, Cary NC
Dimethyl disulfide	DMDS	Paladin	Arkema Inc, Philadelphia, PA
Sulfuryl fluoride			
[MethylN'N'-dimethyl-N-[(methyl carbamoyl)oxy]-1-thiooxamimidate]	oxamyl	Vydate	DuPont Agricultural Products, Wilmington, DE
O-Ethyl S-(1-methylpropyl)(2-oxo-3-thiazolidinyl)phosphonothioate)	fosthiazate	Nemathorin	Syngenta International AG, Basel, Switzerland

1.2.3 Use of plant extracts and indigenous medicinal plants

In recent years there has been considerable interest in the use of plant extracts to reduce nematode pest populations. Most plants have naturally occurring compounds which may have a nematicidal effect on *Meloidogyne* (Cetintas and Yarba, 2010b). Entry of nematodes into the roots creates a hypersensitive reaction within the plant that releases nematicidal compounds such as phenols, alkaloids, terpenes and amino acids (Tando *et al*., 1989). According to Umar *et al*. (2010), botanicals such as *Azadirachta*, *Eucalyptus*, *Chrommelina* and *Targetis* have been found to be effective in controlling root-knot nematodes. Plants such as neem (*Azadiriachta indica*), African basil (*Ocimum gratissimum*), bitter leaf (*Vernonia amygdalina*) and moringa (*Moringa oleifera*) have been reported to contain pesticidal properties that inhibit egg-hatching and development of *Meloidogyne* species. Several of these plants can be used in rotation, intercropping with susceptible crops (Kalaiselvam and Devaraj, 2012). Marigold is known to suppress 14 genera of plant-parasitic nematodes, with root-knot nematodes (*Meloidogyne* spp.) the most affected (Suatmadji, 1969). Below is a table outlining the various botanicals and their effects on *Meloidogyne*.

Table 1.2 Some botanicals used in the control of *Meloidogyne*.

Plant Family	Nematicidal components	Plant Scientific Name(s)	Nematode target and antagonistic effect	Parts of plant used	References
Meliaceae	Acetic acid,butyric acid, hexanoic acid, decanoic acid and furfurol	*Melia azedarach*	Reduced *M. incognita* females by 50%	Aqueous solution. All plant parts used	Katooli *et al*., 2010, Cavorski *et al*., 2012
Fabaceae	1,2-Dehydropyrrolizidine alkaloids	*Crotalaria* spp, *Medicago sativa, Ononis natrix*	Reduced populations of *Meloidogyne* spp. *Pratylenchus penetrans* and *Heterodera* spp.	Shoot and roots	Thoden *et al*., 2009, D'Addabbo *et al*., 2010, Leonetti *et al*., 2011
Asteraceae	Polyacetylenes polyethienyls flavonoids	*Tagetes* species (Marigold)	Reduced populations of *Meloidogyne* spp. *Pratylenchus penetrans* and *Heterodera* species.	Flower extracts	El Allagui *et al*., 2007, Marahatta *et al*., 2010, Faizi *et al*., 2011
Brassicaceae	Glucosinolates	*Brassica juncea, B. napus* and *Sinapis alba* (Mustards)	*Pratylenchus penetrans* and *M. incognita*	Seed, Used in powder form.	Zasada *et al*., 2009, D'Addabbo *et al*., 2007
Verbenaceae	p-Hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, and 7-glucoside	*Lantana camara*	High mortality rate in *M. javanica* and *M. incognita*	Leaves. Used as aqueous solution	Ahmad *et al*., 2010
Amaryllidaceae	Sulfides	*Allium sativa*	Reduced root galling and egg masses in *Meloidogyne* spp.	Seed, Used in powder form	Cetintas and Yarba, 2010b
Anacardiaceae		*Pistacia terebiathus*	Reduced *M. incognita*	Leaves. Used as an	Ntalli *et al*., 2011

		populations	aqueous solution.	
Rutaceae	*Ruta chalepensis*	*M. javanica* and *M. incognita* Cause paralysis	Aerial parts	Ntalli *et al*., 2011
Simaroubaceae	*Ailanthus altissima*	Killed *M. javanica*	Wood extract	Caboni *et al*., 2012

1.2.4 Crop rotation

Crop rotation aims at reducing *Meloidogyne* populations to levels which allow following crop(s) to establish and complete early growth before heavy infestation. Rodriguez-Kabana and Canullo (1992) reported that sufficient time should lapse after growing a susceptible host crop to reduce nematode populations to a level that allows the next susceptible crop to grow and yield at an acceptable level. According to Halbrendt and LaMondia (2004), allelopathic plants, trap crops or green manure crops may be included in a rotation. A good rotation should not include a crop susceptible to the main nematode pest more than once in four growing seasons. Atkinson *et al*. (2012) mentioned, that although rotations are a good management tool for plant-parasitic nematode, sometimes they are non-sustainable because they impose non-optimal use of the land and can increase total acreage involved in cropping.

1.2.5 Genetic plant resistance

Nematode resistance is defined as the ability of plant roots to resist penetration by the pest (Steiner, 1925). For farmers, plant resistance is the most useful and cheapest means of nematode control. Generally, resistant genes are more effective on endoparasitic nematodes such as *Meloidogyne*, *Globodera* and *Hirschmanniella* than against ectoparasitic nematode such as *Xiphinema*, *Trichodorus* or migratory endoparasitic nematodes such as *Pratylenchus* and *Ditylenchus* (Barker, 2003). Some resistance genes against sedentary endoparasitic nematodes prevent or greatly reduce nematode population increases in roots.

According to Roberts (1992), resistance to root-knot nematodes has been selected in cowpeas (*Vigna unguiculata* L.), beans (*Phaseolus vulgaris* L.), peas (*Pisiumsativa* L.), soybean (*Glycine max* L.), pepper (*Piper nigrum* L.) and sweet potato (*Ipomoea batatas* L.). Williamson (1998) reported that the *Mi-1* gene for resistance to *Meloidogyne javanica*, *M. arenaria* and *M. incognita* has been used successfully for over 125 years in tomato. According to Castelli *et al*. (2005), most resistance to *Globodera* in potato was based on the dominant *H1* gene.

1.2.6 Organic amendments

Organic amendments for management of *Meloidogyne* have been used in recent years (Bari *et al*., 2004; Oka, 2010). According to Agbenin (2011), poultry manure, cow dung, green manure and crop residues have been widely used by smallholder farmers. Nico *et al*. (2004) reported that cow dung and poultry manure when applied to soils, reduce populations of root-knot nematodes and other plant parasitic nematodes.Organic matter improves both the nutrient and water holding capacities of the soil. This improves plant growth vigour (Ullah *et al.*, 2008) and hence increases tolerance to nematodes (El-Sherif, 2008).

1.2.7 Soil solarisation

Soil solarisation is the heating of soil beneath transparent polyethylene mulch. It is an environmentally safe, non-chemical method to control soil borne pests, pathogens and weeds (Camprubí *et al*., 2007). Literature indicates that to achieve effective pest and pathogen management, soil solarisation should be carried out for at least four weeks (McGovern *et al*., 2002). Soil temperatures in the upper layers of the soil increase under the plastic, killing a variety of plant pathogens, hence make it a useful tool for control of plant parasitic nematodes. Soil solarisation has been adapted in several regions in the world, mainly for seed beds. It has been adopted in areas with relatively cloudless conditions, and hot, in humid climates (Chellemi *et al*., 1997). However, climate and weather affect the efficacy of solarisation (Wang *et al*., 2008) as do soil structure, moisture, temperature, day length and intensity of light.

1.2.8 Biological control

Biological control can be defined as the use of natural or modified microorganisms to target a specific microorganism, to maintain or reduce populations of the target to

levels where they cease to cause a problem and economic loss (Nekouam, 2004). Dunnington in 1951 was the pioneer of biocontrol on nematodes (Lamovsek *et al*., 2013). Thereafter, several biological agents have been tested in recent years with the capacity to control plant-parasitic nematodes (Hallman *et al*., 2009).

According to Agbenin (2011), knowledge of biological interactions at ecosystem, organism, cellular and molecular levels has been the basis for the control of plant-parasitic nematodes, hence making it a more complicated management strategy than physical and chemical methods. Agbenin (2011) also noted that this method is usually more stable, longer lasting and environmentally friendly.

Felde *et al.* (2006) mentioned that biocontrol organisms are useful in practices where nematicides are prohibited, such as in organic farming, and in areas where low nematode densities are recorded over time. However, they do not necessarily reduce the populations of the target below the required economic thresholds.

Numerous microbes are antagonistic to plant-parasitic nematodes, but only a few have been commercialized as biocontrol agents. This is mainly due to the inconsistency of control in the field and a lack of broad spectrum activity compared to chemical pesticides (Meyer and Roberts, 2002). Bacteria, fungi and actinomycetes are microorganisms that have been used to control and reduce populations of *Meloidogyne* species (Lamovsek *et al*., 2013). Each uses different mechanisms to control *Meloidogyne*. The following characteristics should be met for a biocontrol agent to be used in biocontrol strategies: the bio-agent should be (i) host specific, (ii) lethal, (iii) easily manipulated and mass produced in the laboratory, (iv) easily distributed with standard equipment, (v) potential for establishment, (vi) long life, and providing long term control and (vii) environmentally friendly.

Use of bacteria

Several attempts have been made to use bacteria as biocontrol agents (Tian *et al*., 2007). They are the most abundant micro-organisms in the soil and co-exist in the rhizosphere where they may affect the life cycle of *Meloidogyne* as endoparasites or antagonists. *Meloidogyne* juveniles and femalesare affected adversely by *Pasteuria* species, an endoparasite (Gowen *et al*., 2008). *Pasteuria penetrans* is the most common species, which reduces the fecundity in *Meloidogyne* females, reducing the ability of the females to optimally produce eggs, and also affecting the J2 nematode, which is the infective stage in the life cycle of *Meloidogyne* (Davies *et al*., 1991,

Hallman *et al*., 2009). Rhizobacteria are bacteria which are found in the rhizosphere of the host plant. They colonise the roots of the plant and most of them stimulate plant growth. These types of growth-promoting bacteria are called plant-growth promoting rhizobacteria (PGPR). The most important rhizobacteria which adversely affect *Meloidogyne* species are *Pseudomonas fluorescens*, *Bacillus thuringiensis*, *B. subtilis*, *B. sphaericus* and some genera such as *Agrobacterium*, *Alcaligenes*, *Clostridium* and *Streptomyces* (Tian *et al*., 2007; Son *et al*., 2009). *Bacillus firmus* has also showed potential as a biocontrol agent and is available commercially in most countries (Mendoza *et al*., 2008; Terefe *et al*., 2009). Mode of action against *Meloidogyne* varies with the species of bacteria. They reduce gall formation, egg hatching and affect juvenile survival by production of toxins, antibiotics; inducing systematic resistance in plants; and disrupting plant-host recognition (Alcals, 2007). Most commercially available nematicidal biocontrol products using bacteria (Table 1.3) are sold as seed treatments, to ensure that the bacteria colonise the root of the plant before nematodes can compete for entry points.

Use of Fungi

Nematophagous fungi are a group of fungi found free living in the soil, with the capacity to parasitize nematodes at various stages of their life cycles (Yang *et al*., 2007). Nordbring-Hertz *et al.* (2006) stated that they are the most studied antagonistic organisms for root-knot nematodes. They are classified as Zygomycetes, Hyphomycetes and Ascomycetes (Stirling, 1991). In addition, they have distinct morphological adaptions and features for capturing nematodes and using them as a source of nutrients (Nordbring-Hertz *et al*, 2006). Nematophagous fungi can be divided into different groups depending on their mode of infection: nematode trapping, endoparasitic, egg and female parasitic and toxin producing fungi (Liu *et al*., 2009).

Nematode-trapping fungi have specialized trapping organs along their vegetative hyphae and are known to capture motile nematodes (Jansson and Lopez-Llorca, 2001). The trapping nature of the fungi differs between species and is categorized as follows: (i) adhesive nets, (ii) adhesive knobs, (iii) adhesive branches, and (iv) constricting rings (Swe *et al*., 2011). According to Jansson and Lopez-Llorca (2001), nematode-trapping fungi appear to exhibit little host specificity, and live in the soil with varying degrees of saprophytism.

Modes of infection by endoparasites are by the use of spores to infect their nematode host, which are either motile or non-motile. Many spend their vegetative lives inside their nematode host, but reproducingoutside the nematode body. Jansson and Lopez-Llorca (2001) reported that endoparasites have a more restricted hostrange than nematode-trapping fungi. Sedentary stages such as the egg and females in the life cycle of root knot nematodes have been shown to be vulnerable to infection by fungi. Some fungi produce toxins that are antagonistic towards plant parasitic nematodes and exhibit a nematicidal or nematistatic nature (Luo *et al*., 2004). Fungi such as *Pochonia chlamydosporia* and *Paecilomyces lilacinus* affect *Meloidogyne* by parasitising their eggs and females (Lamovsek *et al*., 2013). They penetrate the shell by wrapping around the egg, and releasing proteases that destroy the inside of the egg (Esteves *et al*., 2009). *Trichoderma* and *Aspergillus* spp. are known to have toxic effect on *Meloidogyne* species affecting juveniles and egg-hatching (Goswami and Mittal, 2004; Tripathi *et al*., 2006).

Table 1.3 Commercialised biological control products (Dong and Zhang, 2006; Hallman *et al*., 2009).

Product Name	Microbial origin and mode of antagonist	Nematode target	Product form	Company/country
Abamectin	*Streptomyces avermitilis* Toxin production	*M.incognita, Pratylenchus spp, Radophilus similis*	Powder	Syngeta
Biocon	*Paecilomyces lilacinus* Egg and female parasitism	*Meloidogyne* spp.	Powder	Asiatic Technologies, Manila, Philippines
BioNem-WP Nortica VOTiVO	*Bacillus firmus*	*Meloidogyne* spp. *Heterodera avenae*	Wettable powder; Solution	AgroGreen, Israel; Bayer CropScience, USA
DiTera	*Myrothecium verrucaria* Toxin production	*G. rostochiensis, G. pallida, H. glycines*, *Radophilus* spp, *Meloidogyne* spp	Powder	Valent Biosciences Corporation, Canada
Econem	*Pasteuria penetrans* Females, second stage juveniles	*M. javanica*, *M. incognita*	Solution or Powder	Syngenta; Nematech, Japan
Biostart	*Bacillus* spp. Mixture	*Meloidogyne* spp.	Liquid	Microbial Solutions, S Africa
Deny Blue Circle	*Burkholderia cepacia* Reduction of egg hatch and juvenile mortality	*M. incognita*	Powder or Solution	CCT Corp, USA; Stine Microbial Products, USA;
KlamiC	*Pochonia chlamydosporia* Egg parasitism	Root knot nematodes	Granulate	Cuba
Nemix	*Bacillus* spp. Toxin production	Root knot nematodes	Powder	AgriLife/Chr Hansen, Brazil
Nemout	Unspecified nematode-trapping fungus	*M. javanica,* Reniform species.	Powder	Unspecified
Bioact WG PL Gold	*Purpureocillium lilacinus* Egg and females	*M. javanica*, *M. incognita*	Water dispersible granulate; Wettable Powder	Bayer CropScience, USA ; BASF Worldwide
Miexianning	*Paecilomyces lilacinus* Eggs and female parasitism	Root knot nematodes parasitizing tobacco	Powder	Agricultural Institute, Yunnan Academy of Tobacco Science, Yunnan Province, P.R China
Biotode 1	Living cells of *Bacillus laterosprous*	Root-knot nematodes		Agro-Organics, South Africa

1.3 Summary

Meloidogyne species are an important pest in field and vegetable crops worldwide. Chemical nematicides have been the major control strategy to reduce *Meloidogyne* populations in the soil, to reduce crop damage and yield losses. However, the use of chemicals has been reduced to minimise their effect on the environment, humans and animals. This has shifted the focus to biological control as an alternative. The effect of parasitic fungi and bacteria in reducing populations of *Meloidogyne* on plants and in soils, and increasing plant performance and yield has been studied for many years. Knowledge of interactions of these microorganisms in the rhizosphere gives us a better understanding on how they can be utilized as biological control agents. Many microorganisms in the soil are still to be isolated.

Nematophagous fungi and rhizobacteria have the ability to colonise the developing root system of the plant in the presence of other organisms, which are already competing for nutrients. After colonization they produce and excrete metabolites or chemicals, and produce trapping structures, which inhibit entry or trap nematodes, so that they cannot enter the host root system. These microbes are now being isolated and screened worldwide for commercialization as biopesticides, and a number of biopesticides have been released and are being used by farmers. Most are easily isolated, spore-bearing, have a long shelf life, and their spores are easy to produce in large quantities. Their ability to colonise host root systems quickly makes them easy to apply as a seed treatment or a soil drench. There are still many questions that need to be answered on the mechanisms used by these microbial agents and future microbial agents being isolated and screened against *Meloidogyne* development in soils and on plants.

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CHAPTER TWO

ISOLATION, PRELIMINARY SCREENING AND IDENTIFICATION OF BACTERIA AND FUNGI FOR NEMATODE BIOCONTROL

Abstract

Root-knot nematodes are an important pest of many plants worldwide. Potential antagonists of *Meloidogyne javanica* were isolated from grazing pastures of livestock and the rhizosphere of tomato plants in the glasshouse. A total of 94 bacterial and 22 fungal isolates underwent preliminary screening by means of microwell bioassays with second-stage juveniles of *M. javanica* as the target nematode pest. Twenty bacterial and 8 fungal isolates displayed nematicidal activity of between 47.0 and 65.4% and 33.0 and 66.3% respectively, with only five bacteria (BG25, BG21, BG32, BS29, BS39) and three fungi (Cr6, Cr12 and Cr5) causing J2 mortality of more than 60.0%, and were selected for further studies.

Keywords: Root-knot nematodes; nematophagous fungi; rhizobacteria

2.1 Introduction

Root-knot nematode (*Meloidogyne* species) infestations in field and vegetable crops cause substantial economic losses worldwide (Bagheri *et al*., 2014). Chemical control using nematicides is currently the major control method for root-knot nematodes (Haydock *et al*., 2013). Frequent use of nematicides has an adverse effect on the environment. All nematicides have high mammalian toxicity, which has resulted in the removal of certain nematicides from the pesticide market (Siddiqui and Shaukat, 2003). As a result biological control is becoming an important tool in the control of root-knot nematodes.

The concept of biological control mainly focuses on the use of microbial agents for the control of plant pests and pathogens. Soils are known to contain a diverse array of microbial agents that are capable of parasitizing nematodes (Lamovsek *et al*., 2013). These organisms suppress nematode populations in the soil to levels that cause minimal damage to crops (Javed *et al*., 2012; Khalil*et al*., 2013). Target sites for isolation, and knowledge of microbial interactions in soils, are critical because this

assists in finding effective biocontrol agents, making screening easier (Lamovsek *et al*., 2013). It is relatively easy to isolate potential biological control agents. However, success is dependent on accurate screening, which eliminates non-performing biocontrol agents and selects the promising ones (Chiou and Wu, 2003).

Researchers have used several methods for screening of biocontrol agents for control of root-knot nematodes (Dawar *et al*., 2008; Joo *et al*., 2012; Ann, 2013; Ramezani Moghaddam *et al*., 2014). Although the techniques differ, they all use the same concept of screening where suspensions of the biocontrol agent and the targeted root-knot nematode J2 are mixed into wells or cavity blocks to assess nematicidal activity on such life stages after a certain period of time. The objectives of this study were to isolate and screen potential antagonists against the J2 of root-knot nematode *Meloidogyne javanica* (Treub) Chitwood.

2.2 Materials and Methods

2.2.1 Sample collection

Soil samples were taken from two different sites at the Ukulinga Research Farm (University of KwaZulu-Natal, Pietermaritzburg, South Africa): (a) the livestock section comprising of cattle, sheep and goat pens and grazing pastures; (b) glasshouses with tomato plants. Top soil (1cm) was removed with a sterile spatula. Approximately 20g of soil was collected and placed in plastic bags. The soil samples were stored at 4℃ until further use.

2.2.2 Isolation and culturing of potential biocontrol agents

(a) Fungi

A 1 g sample of soil was suspended in 9 ml of sterile distilled water, from which a 1ml suspension was plated onto Potato Dextrose Agar (PDA). 100 mg/L of streptomycin solution was added to the medium before pouring into petri-plates to prevent bacterial growth. Petri-plates were incubated at 25°C in the dark and monitored daily for one week. Developing fungal colonies were sub-cultured onto PDA plates. Each isolate was incubated for 1-2 wk at 25°C depending on its growth and sporulation rate. Agar blocks (2 x 2 mm) were cut from each isolate and stored in 10ml of double sterilised distilled water.

(b) Bacteria

A sample of 1 g of soil was suspended in 9 ml of sterile distilled water and heat treated for 15 min at 80°C in a rotary water bath. One ml of the suspension was serially from 10^{1}-10^{5} and 0.1 µl of each dilution was spread onto nutrient agar plates and incubated at 28°C in the dark for 72 hr. After incubation, a section of each colony was transferred to tryptone soy agar plates and incubated at 28°C for 3 d. Pure culture of bacterial isolates were then suspended in a sterile 30% glycerol solution and stored at -80°C.

2.2.3 Preparation of nematode inoculum

Local populations of *Meloidogyne javanica* eggs and J2s where reared in vivo in roots of tomato (cv Floradade) and obtained from the University of Potchefstroom, Unit of Environmental Sciences and Management. They were propagated on tomato (*Solanum lycopersicum* L.) cv. Floradade under glasshouse conditions. Nematode inoculum was obtained by extracting *M. javanica* eggs from infected roots. Roots were washed thoroughly under running tap water and then cut into 1-2 cm pieces. Two hundred and fifty ml of 0.5% sodium hypochlorite (NaOCl) solution were added to the root pieces in a 500ml glass bottle and vigorously shaken for 5 min (Hussey and Barker, 1973). The suspension was poured over a series of sieves, 250 µm and 25 µm pore size respectively. The eggs were collected on the 25 µm pore size sieves and washed thoroughly five times with tap water to remove all NaOCl and then collected into a 100 ml beaker. The suspension was placed in a modified Baermann dish and incubated for 5 days (Hooper *et al*., 2005). Hatched J2were obtained by placing the eggs in sterile distilled water for 3-5 d at 28°C in the dark. They were collected and stored in a beaker in tap water at 4°C for further use.

2.2.4 Screening of biocontrol agents

Agar blocks containing actively growing mycelia were transferred to PDA plates and incubated at 28°C for 2 wk. After incubation, mycelium was removed from the plates by scraping the surface of the plate with distilled water added to it and the suspension was passed through a cheese cloth into a conical flask. Spore concentration was then adjusted to 10^{7} conidia.ml^{-1} using a haemocytometer.

The bacterial cultures were inoculated into 50ml tryptone soy broth (TSB) and incubated for 2wk in the dark at 28°C in an orbital shaker (MRC) incubator at

150rpm. Spores were harvested by centrifugation at 10,000rpm for 15min at 4°C. The supernatant was discarded and the pellet was washed once with 0.1M NaCl and twice in sterile cold distilled water. The pellet was suspended in 50ml distilled water. Spore counts were determined using a dilution plating technique and the spore concentration was adjusted to 10^8spores.ml^{-1}.

The activity of the biocontrol agents was screened *in vitro* for their antagonistic effect on *Meloidogyne javanica*J2 by the use of microwell assays. One ml of the fungal and bacterial spore suspension was transferred to a 96-well plate to which 1 ml of a suspension containing 25-30 freshly hatched J2 was added and the plates were incubated at ambient temperature of 23°C. Preliminary screening was performed by determining the reduction of mobile J2 after 12h of incubation. Numbers of dead nematodes were counted using a stereomicroscope (Olympus BX41). Fungal and bacterial strains that caused more than 60% nematode mortality were selected for further study.

2.2.5 Identification of potential fungal and bacterial isolates

Molecular characterisation of the five bacterial isolates that were superior in causisng J2 mortality was conducted through 16S rRNA sequence analysis (Ann, 2013). Molecular characterisation of the best three fungal isolates was conducted through Internal Transcribed Spacer (ITS) analysis (Schoch *et al*., 2012). All characterisation was carried out at Inqaba Biotechnical Industries (www.inqababiotec.co.za). Sequence data obtained from Inqaba were deposited in the National Center for Biotechnology Information (NCBI) and all results were compared with available 16S rDNA and ITS sequences in the Gene Bank database.

2.3 Results

2.3.1 Isolation of bacteria and fungi

A total of 94 bacteria and 22 fungi were isolated and selected on the basis of morphology, colony size and colour. The majority of the bacterial isolates originated from goat pastures (41.1%) followed by sheep pastures (21.3%), tomato rhizosphere (21.3%) and cattle pastures (10.6%). Forty five percentof the fungal isolates originated from the tomato rhizosphere, followed by sheep (22.7%), goat (22.7%) and cattle (9%) pastures.

2.3.2 Preliminary screening of bacterial and fungal isolates

Of the 94 bacterial isolates screened for nematicidal activity against *M. javanica*J2s, 20 isolates caused a reduction in their mobility of ranging between 47.0% and 65.4%.Five isolates, BG25, BG21, BG32, BS29 and BS39, resulted in reductions of J2 mobility of 60% and above (Table 2.1). These five isolates caused a high nematicidal activity and were considered/selected for further studies. Most of the J2 bodies and cuticles were destroyed by the isolates.

Of the 22 fungal isolates screened against *M. javanica*, eight isolates effectively reduced the mobility of juveniles in the microwell assay. Mobility ranged between 38.0% and 66.3%, with only three isolates, Cr12, Cr5 and Cr6, causing a reduction in mobility of 62% and above (Table 2.1). These three isolates showed high levels of anti-nematode activity and were used in subsequent studies. Dead nematode bodies appeared rigid and when probed, did not move or coil.

Table 2.1 Percentage *Meloidogyne javanica* mortality of second-stage juveniles after preliminary screenings bacterial and fungal isolates that were superior in this regard.

Bacteria	% mortality
BG21	64.2
BG32	64.0
BG25	65.4
BS29	60.3
BS39	62.0
Fungi	
Cr6	64.0
Cr5	66.3
Cr12	62.0

2.3.3 Identification of selected isolates

The best 5 bacterial isolates and the best 3 fungal isolates selected from preliminary screening for nematicidal activity were further classified using molecular tools. The bacterial strains were characterised in order to determine specific strains for endospore production. The fungal strains were selected for characterisation primarily due to their enhanced ability to restrict mobility of the J2.

Identification was based upon comparison of the 16S rRNA and ITS regions of the bacterial and fungal isolates, respectively, with already registered sequences in the Gene Bank database (Table 2.2 and Table 2.3).

Table 2.2 Blast and identification details of the selected bacterial isolates as obtained from the Gene Bank Database

Isolate No.	Isolate name	Identified Species	Primer	E-value	% Similarity	Accession Number
1	BG25	*Bacillus thuringiensis*	16S rRNA	0.0	100	KM250110.1
2	BG21	*Bacillus thuringiensis*	16S rRNA	0.0	100	KM250110.1
3	BG32	*Bacillus cereus*	16S rRNA	0.0	99	KC692199.1
4	BS29	*Bacillus cereus*	16S rRNA	0.0	100	JX133203.1
5	BS39	*Bacillus cereus*	16S rRNA	0.0	100	JK935083.1

Table 2.3 Blast and identification details of the selected fungal isolates as obtained from the Gene Bank Database

Isolate No.	Isolate name	Identified Species	Primer	E-value	% Similarity	Accession Number
1	CR12	*Trichoderma spirale*	Universal	0.0	100	AY857246.1
2	CR5	*Hypocrea lixii*	Universal	0.0	100	AY605754.1
3	CR6	*Hypocrea lixii*	Universal	0.0	100	AY605754.1

2.4 Discussion

The study showed that soils from pastures of livestock and the rhizosphere of tomato plants are rich with *Bacillus* species. This confirms findings by Nicholson (2002) who reported that *Bacillus* can be isolated from most agricultural soils and diverse environments including rocks and dust. m\Majority of the fungal isolates in the current study originated from the rhizosphere of tomato plants. This is in agreement with Vargas Gil *et al*. (2009) who showed that soils have a diversity of microbial organisms that act as biocontrol agents against various plant pathogens. Lamovsek *et al*. (2013) mentioned that most of the best known fungi that attack most *Meloidogyne* species are found in the rhizosphere of host plants.

In vitro screening methods that provide rapid, repeatable and reliable results are an important step in the initial screening of potential antagonists for the biocontrol of plant diseases. During the screening process, the biocontrol agents were placed in microwells with J2 of the root-knot nematode. Screening was carried out to find the best five bacterial isolates which possessed a pronounced nematicidal activity. Similar research was conducted by Joo *et al*. (2012), who screened 114 bacterial isolates to identify those with exceptional anti-nematode activity. Ann (2013) also screened 150 potential bacterial strains with nematicidal activity in preliminary teststowards*Meloidogyne incognita* in order to identify the best *Bacillus* strains. The number of selected isolates were reduced to nine, and four *Bacillus* strains (MPB04, MPB93, MPB098, and MPB115) were identified and those which showed more than 50% nematode reduction were selected for further analysis.

Twenty-two fungal isolates were screened for nematicidal activity against *M. javanica*. Only three were found to have nematicidal activity of over 60%. Dong *et al*. (2006) carried out preliminary screening of several fungal isolates over a period of time. They found that 15 fungal isolates that caused 50% mortality of the pine wood nematode (*Bursaphelenchus xylophilus*) after screening of 181 isolated fungi. Nematodes were considered dead if they gave no response to physical stimuli such as probing with a needle.

The screening procedure conducted in this study identified potential biocontrol agents and for this reason any antagonists which showed 60% mortality (or higher) were selected for further studies to their potential as biocontrol agents against *Meloidogyne javanicain vivo*.

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CHAPTER THREE

BIOLOGICAL CONTROL OF *MELOIDOGYNE JAVANICAIN VITRO* BY BACTERIAL AND FUNGAL ISOLATES

Abstract

Biological control of plant-parasitic nematodes is becoming increasingly important due to a shift towards reducing the use of nematicides as a means of control. *In vitro* studies were conducted to evaluate the efficacy of previously screened isolates of *Bacillus* spp., *Hypocrealixii* (teleomorph stage of *Trichoderma harzianum*), *Trichoderma spirale*, Eco-T® (a commercial formulation of *Trichoderma harzianum*) and *Clonostachys rosea*against a root-knot nematode *Meloidogyne javanica*. All the bacterial isolates significantly ($P < 0.001$) increased second-stage juvenile (J2) mortality of *M. javanica*, by between 60.0 and 94.0% after 48 h. *Bacillus thuringiensis* (Strain BG25), after 12, 24 and 48 h, caused the greatest mortality of J2 of 68.0, 79.3 and 94.0%, respectively. The fungal antagonists also caused significant ($P < 0.001$) increases in the mortality of *M. javanica* J2 of between 59.3 and 93.9%. Isolates of *Hypocrea lixii* (Cr5 and Cr6) caused high mortality after 12 h (79.3 and 67.9, respectively). Overall, *H. lixii* (Cr5) caused the highest mortality of juveniles after 12, 24 and 48 h of 79.3, 87.98 and 93.9%, respectively.

3. 1 Introduction

Root-knot nematodes (*Meloidogyne* spp.) are recognized as major sedentary endoparasitic nematodes, affecting a wide range of crops worldwide (Sahebani and Hadavi, 2008). They are predominantly found in tropical and sub-tropical regions (Kayani *et al*., 2013). The life cycle of root-knot nematode has four juvenile stages and moults (Agrios, 2005). The most infective stage is the second-stage juvenile (J2), which hatch from eggs in the soil, laid by the mature female in a gelatinous substance. The J2 penetrates susceptible roots to start the infection cycle.

Chemical nematicides has been widely used to control root-knot nematodes. However, due to effects of the chemicals on the environment humans and animals, other alternatives are being explored to control root-knot disease (Kiewnick and Sikora, 2006). As a result biological control is increasingly becoming an alternative to

control root-knot nematodes (Radwan *et al*., 2012; Mukhtar *et al*., 2013). Various microbial agents have been found to kill root-knot nematodes. These micro-organisms live in the soil with the nematodes. They reduce or suppress the population of nematodes in the soil (Bent *et al*., 2008). Both fungi and bacteria have the ability to control root-knot nematodes (Khan *et al*., 2008).

Antagonistic fungi have been shown to produce toxic substances and secondary metabolites or enzymes (Regaieg *et al*., 2010), and many exhibit catching devices such as constricting and non-constricting rings, sticky branches, networks, knobs and adhesive spores, which kill the nematodes (Mukhtar and Pervaz, 2003). *Trichoderma* species are well known filamentous fungi that can kill root-knot nematodes (Spiegel and Chet, 1998). Sharon *et al.* (2007) showed that conidia of *Trichoderma* can attach to the nematode cuticle and to the egg shell, and then parasitize them. *Clonostachys rosea* (Schroers) is a mitosporic fungus. It is widely known for its parasitism of fungi (Li *etal*., 2002), but has been shown to be able to parasitize and digest on plant parasitic nematodes (Zhao *et al*., 2005).

Different bacterial antagonists have shown promise in controlling *Meloidogyne* spp. (Giannakou *et al*.,2004; Lamovsek *et al*., 2013). Rhizobacteria are the best studied bacterial antagonists on plant-parasitic nematodes. In this group *Bacillus* species have the potential to control *Meloidogyne* spp. (Hashem and Abo-Elyous, 2011). Two species which are commonly used against *Meloidogyne* are *B. thuringiensis* and *B. cereus* (Wei *et al*., 2003)

Therefore, the aim of the experiments below was to evaluate the nematicidal potential of selected fungal and bacterial isolates against *M.javanica* (Treub) Chitwood *in vitro*.

3.2 Materials and Methods

3.2.1 Preparation of biocontrol agents

A total of 5 pathogenic bacteria (BG21, BG32, BG25, BS29, and BS39) and 3 fungi (Cr6, Cr12, Cr5) were isolated from pasture soils from the livestock section and glasshouses at the University of KwaZulu-Natal Research Farm, Ukulinga. *Trichoderma* (Eco-T®) and *Clonostachys rosea* (Schroers) were grown and formulated by Plant Health Products (Pty) Ltd, Nottingham Road, South Africa.*C.*

rosea showed nematicidal activity on animal nematodes and has potential to be used as a biocontrol on plant-parasitic nematodes. They did not constitute as controls in the experiments.

Fungal Isolates

Eco-T® (*T*. *harzianum*) and an isolate of *Clonostachys rosea* each contained approximately 10^8 conidia.g^{-1}. The mycelia and conidia of selected wild-type fungal isolates were produced by growing them on potato dextrose agar (PDA) for 2 wk in Petri dishes. Mycelia and conidia were recovered by carefully scraping and washing the surface of the agar covered with mycelia, with sterile distilled water, into a beaker. The conidial suspension was filtered through a double layer of cheese cloth into a sterilized beaker to remove mycelia. The concentration of conidia was determined by a haemocytometer and adjusted to a concentration of 10^6 conidia.ml^{-1}.

Bacterial isolates

Bacterial cells and spores were obtained from scraping frozen cultures stored at -80˚C. Spores were streaked on Tryptone Soy Agar (TSA) and incubated at ambient temperature for 24 h in the dark. The cells and spores were harvested and placed into 250ml Erlenmeyer flasks containing 40 ml of Tryptone Soy Broth (TSB). The isolates were grown in the TSB at 28˚C for 72 h in an orbital shaker at 150rpm. After incubation, the cultures were centrifuged at 10 000rpm at 4˚C for 15 min (Beckman Coulter Avanti J-26XPI). The supernatant was removed and the pellet was washed once in 0.1 M NaCl and twice in cold distilled water. The pellet was suspended in sterile and distilled water (10ml), the number of endospores was determined through dilution plating, and this number was adjusted to a concentration of 10^8 endospores.ml^{-1}.

3.2.2 Hatching of *Meloidogyne javanica* juveniles

Local populations of *Meloidogyne javanica* eggs and second stage juveniles (J2s) where reared *in vivo* in roots of tomato (cv. Floradade) and extracted from the roots at the Environmental Sciences and Management Unit, University of Potchefstroom. They were propagated on tomato (*Solanum lycopersicum* L.) cv. Floradade under glasshouse conditions of 25°C±5°C at the Controlled Environment Facilities (CEF) at

the University of Kwazulu Natal. Nematode inoculum was obtained by extracting *M. javanica* eggs from infected roots. Roots were washed thoroughly under running tap water and then cut into 1-2 cm pieces. Thereafter, 250 ml of 0.5% sodium hypochlorite (NaOCl) solution were added to the root pieces in a 500ml glass bottle and shaken vigorously for 5 min (Hussy and Barker, 1973). The suspension was poured over a series of sieves, 250 µm and 25 µm pore size sieves respectively. The eggs were collected on the 25 µm sieve and washed thoroughly with tap water to remove NaOCl and then collected into a 100 ml beaker. The suspension was placed in a modified Baermann dish and incubated at 25°C for 7-10 d (Hooper *et al*., 2005). Hatched second stage juveniles of *M. javanica* were obtained by placing the eggs in sterile distilled water for 3-5 d at 28°C. The inoculum was collected and stored at 4°C in a beaker with distilled water for further use in the experiment. The number of juveniles was estimated in 1ml aliquots of the inoculum suspension.

3.2.3 Effect of *Bacillus* and fungal antagonists on J2 mortality of *M. javanica in vitro*

A microwell bioassay was carried out to determine the activity of the bacterial and fungal isolates against J2s. Using a pipette, a 1 ml sample of the nematode suspension containing approximately 60 J2 was placed into each well of a 96-well plate containing 1ml of each biocontrol agent. Wells which received the same number of J2 with 2 ml of distilled water added to them served as controls. A total of five replications for each treatment were carried out at ambient temperature. Nematode mortality in each well for each treatment was counted after 12, 24 and 48 h under a stereoscopic microscope (Zeiss Scope.A1 AX10). A juvenile was classified as dead if its body was straight and did not exhibit any movement after prodding (Choi *et al*., 2007). Percentage mortality was calculated as:

$$\frac{\text{Mean of dead number of J2 in treatment}}{\text{Total number of J2 in treatment}} \times 100$$

3.2.4 Statistical analysis

Treatments were arranged in a Completely Randomized Design and replicated five times with three sampling periods/times each. Experiment was repeated twice and data was combined. Data was analysed using ANOVA Genstat® Release 16.1. Treatment mean separation was done using Duncan's Multiple Range Test at 5% level of significance.

3.3 Results

3.3.1. Effect of *Bacillus*and fungal antagonists on J2 mortality of *M. javanica*

All bacterial isolates significantly increased % mortality compared to the control ($P<0.001$) (Table 3.3). Isolate BG25 showed the highest level of nematicidal activity against *M. javanica*, although all the bacteria showed pronounced nematicidal effects on the J2 after 48 h. For the *in vitro* assay, the effect of BG25 and BG21 was not significantly different after 12 and 24 h with mortality of 68.0 and 79.3%, and 67.3 and 75.3%, respectively. After 48 h, % mortality caused by Strain BG25 reached 94.0%, with BG21 at 86.7%. BS39 caused less % mortality at 12, 24 and 48 h of 60.0%, 64.7% and 70.7%, respectively. BS29 also caused less % mortality at 12, 24 and 48 h of 60.0%, 66.7% and 71.3%, respectively.

Table 3.1 Mean mortality of second-stage juvenile (J2) of *Meloidogyne javanica* caused by bacterial isolates after 12, 24 and 48 h.

Isolate species	Bacterial Treatments	Mortality of J2 after 12 hours (%)	Mortality of J2 after 24 hours (%)	Mortality of J2 after 48 hours (%)
	Control (Sterilised water)	0.7**d**	1.3**c**	1.9**e**
Bacillus thuringiensis	**BG25**	68.0**a**	79.3**a**	94.0**a**
Bacillus cereus	**BG32**	66.0**ab**	69.3**b**	78.7**c**
Bacillus cereus	**BS29**	60.0**c**	66.7**b**	71.3**d**
Bacillus thuringiensis	**BG21**	67.3**a**	75.3**a**	86.7**b**
Bacillus cereus	**BS39**	60.0**b**	64.7**b**	70.7**d**
	LSD	5.74	5.55	5.52
	F pr.	< 0.001	< 0.001	< 0.001
	CV (%)	8.1	7.1	6.2

Means followed by the same letter in rows are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level.

The fungal isolates caused significant adverse effects on the mortality of J2*M. javanica*. In general, all treatments caused more juvenile mortality than the Control (Table 3.4). After 12 h, Cr5 was the best isolate that killed many of the J2 followed by Cr6, *Clonostachys rosea*, Eco-T® and Cr12 (*Trichoderma spirale*) with mortality rates of 79.3, 67.9, 64.6, 60.6 and 59.3 % respectively. With increased exposure time, Cr5 caused the greatest mortality of 87.9% and 93.9% after 24 to 48 h, respectively. Eco-T® caused the lowest mortality rate after 24 and 48 h (64.6% and 69.3%, respectively), although at 12 h of exposure it caused a higher mortality than Cr12.

Table 3.2 Mean % of mortality of second-stage juvenile (J2) of *Meloidogynejavanica* caused by fungal isolates after 12, 24 and 48 h.

Isolate species	Fungal Treatments	Mortality of J2 after 12 hours (%)	Mortality of J2 after 24 hours (%)	Mortality of J2 after 48 hours (%)
	Control	0.2**d**	1.3**d**	1.9**e**
Trichoderma harzianum	**Eco-T®**	60.7**c**	64.7**c**	69.3**d**
Clonostachys rosea	***C. rosea***	64.7**bc**	68.0**c**	76.0**c**
Hypocrea lixii	**CR 6**	67.9**b**	78.7**b**	81.4**b**
Trichoderma spirale	**CR 12**	59.3**c**	67.9**c**	72.6**cd**
Hypocrea lixii	**CR 5**	79.3**a**	87.9**a**	93.9**a**
	LSD	5.105	4.848	3.936
	F pr.	< 0.001	< 0.001	< 0.001
	CV (%)	7.0	6.0	4.5

Means followed by the same letter in rows are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level.

3.4 Discussion

Considerable attention has been directed to the development of antagonistic microorganisms to manage plant-parasitic nematodes. Each microbe has its own mechanism that is used to parasitize nematodes. In this study, the nematicidal

activity of five *Bacillus* isolates and five fungal isolates were evaluated against *M. javanica*J2s in the laboratory.The bacterial and fungalisolates significantly increased mortality of J2s *in vitro* compared to the control.

Cell culture of the *Bacillus* isolates (*B. thuringiensis* and *B. cereus*), especiallystrains of BG25 and BG21 showed remarkable nematicidal activity, killing 94.0 and 86.7% of *M. javanica* juveniles within 48 h, respectively. These results are consistent with the work done by Dawar *et al*. (2008) and Ramezani Moghaddam *et al*. (2014) that showed a significant increase in mortality of *M. javanica* J2s over an increased exposure time with bacterial antagonists. In another study, Ashoub and Amara (2010) reported that *Bacillus thuringiensis* bv.$_2$ and *B. thuringiensis* bv.$_3$, caused 90.67% and 93.67% mortality of *M. incognita invitro,* respectively, after 48 hours of exposure to the bacteria. The cuticle of nematodes is rigid and is made up of proteins and chitins. In experiments by the latter authors,the cuticle and body of most of the nematodes were destroyed. This may be due to the proteolytic activity of the *Bacillus* strains (Ann, 2013). According to Mendoza *et al*. (2008) and Haung *et al*. (2010), the nematicidal activity of *Bacillus* isolates may be due to secondary nematicidal metabolites that are produced by *Bacillus* species. These metabolites affect the vitality of the J2 stage of *M. javanica*, directly killing of the nematode. This confirms that *Bacillus* species have the potential to control *Meloidogyne* species (Radwan *et al*., 2012).

Nematophagous fungi are known to have nematicidal effects on plant parasitic nematodes (Sharma and Pandey, 2009). Although the *Trichoderma* isolates and Eco-T® used in this study showed increased mortality, the isolates demonstrated their ability to kill *M*. *javanica*J2*in vitro*. These findings are in agreement with those of Jegathambigai*et al*. (2011) who found that *in vitro*, some isolates of *H. lixii* were effective in causing nematode mortality. Further research conducted by Golzari *et al*. (2011) showed that at 10^5 fungal conidia ml^{-1}, two strains of *Trichoderma harzianum* (T1 and T2) had apronounced effect on mortality of J2 juveniles of *M. javanica* (83.87% and 80.80%, respectively) after 48 h. Yang *et al*. (2010) had similar results when they worked on various *Trichoderma* species and their nematicidal activity on *Panagrellus redivivus* (Linnaeus) Goody and *Caenorhabditis elegans* (Maupas). Elgorban *et al*. (2013) also showed that the culture filtrates of *H. lixii* can cause significant mortality to nematodes.

The nematicidal activity of *Trichoderma* isolates in this study may have been due to direct parasitism on the of *M*. *javanica* J2due to production of proteolytic or chitinolytic enzymes (Naserinasab *et al*., 2011). This may explain why this strain of *H. lixii* isolate Cr5 caused the greatest mortality due to its high proteolytic activity on the J2, while *T. spirale* isolate Cr12 and Eco-T® caused lower levels of mortality due to less proteolytic activity (Sharon *et al*., 2007). Sharon *et al*. (2001) also demonstrated this mechanism of control by *Trichoderma harzianum* (T-203) on *M. javanica* under *in vitro* conditions.

Clonostachys rosea (Schroers)is a potential biocontrol agent. It has been studied for the control of various fungal plant pathogens (Xue, 2003). *In vitroC. rosea* showed its ability to kill *M. javanica*J2 . Limited studies have been conducted to show its effect on *Meloidogynein vitro* but work done by Zhang *et al*. (2008) showed the effect of *C. rosea* conidia and its potential to control nematodes. Dong *et al*. (2004) also showed the nematicidal effect of *C. rosea* on various nematodes, specifically *C.elegans*, *P.redivivus* and *B.xylophilus* (Steiner and Buhrer).

In conclusion, the *Bacillus* isolates and fungal antagonists all exerted a nematicidal effect on the J2 root-knot nematode, *Meloidogyne javanica*. This provides a positive step towards finding alternatives to chemical nematicides for the control of root-knot nematodes.

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CHAPTER FOUR

EFFICACY OF SELECTED *BACILLUS* AND FUNGAL ISOLATES ON *MELOIDOGYNE* ON TOMATO UNDER GREENHOUSE CONDITIONS

Abstract

Application of bacterial and fungal biocontrol agents as a seed dressing or a soil drench was examined for the reduction of *Meloidogyne javanica* (Treub) Chitwood on tomato. *Bacillus thuringiensis* Isolate BG25 and *Hypocrea lixii* Isolate Cr5, applied as a seed dressing or a soil drench, significantly ($P < 0.001$) reduced penetration of *M*. *javanica* second-stage juveniles (J2) into the roots of tomato plants (11.0 and 17.0; 6.0 and 9.0 juveniles per root, respectively). This also had an effect on root-knot disease severity. BG25 applied as a seed dressing and as a soil drench significantly reduced the formation of galls, production of egg masses and number of eggs per root (1.6 and 2.3; 59.3 and 68.0; 19.2 and 23.7, respectively). Complete suppression of disease severity of *M*. *javanica* was observed with*H*. *lixii* (Cr5) as a seed dressing and soil drench, with a reduced gall index (1.0 and 1.3), egg mass (42.0 and 50.3) and number of eggs per root (10.0 and 12.2). The effect of biocontrol agents on growth parameters was also studied. Growth in terms of shoot length, shoot weight and dry weight were significantly ($P < 0.001$) increased by the seed dressings and the soil drenches by all bacterial isolates and the fungal isolates as compared to the control. *B*. *thuringiensis* (BG25) and *H*. *lixii* (Cr5) had the strongest effects on growth parameters on tomato. In terms of reduction ofJ2 penetration and disease severity by the biocontrol agents, seed dressing is an efficient method of application.

4.1 Introduction

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop grown worldwide (Saravanapriya and Sivakumar, 2005). This crop is grown for its edible fruit (Jones, 1999; Saravanapriya and Sivakumar, 2005). In South Africa, tomato is the second most important fresh vegetable food source after potato (*Solanum tuberosum* L.), playing an important role in human nutrition (Department of Agriculture, Forestry and Fisheries, 2014).

Tomato is highly susceptible to root-knot nematode damage by *Meloidogyne* spp. (Khan *et al*., 2005). Root-knot nematodes reduce yield and quality in tomato production, and can cause losses of more than 30% (Sikora and Fernandez, 2005, Karajeh, 2008). They produce galls on the roots which block the efficient uptake of water and nutrients, resulting in chlorosis, stunted growth and in severe cases, eventual death of the plant (Agrios, 2005). Symptoms are similar to, and can easily be mistaken as nutritional deficiency.

Root-knot nematodes have been primarily controlled using chemical nematicides (Zasada *et al*., 2010). However this is expensive and usually has a negative effect on the environment (Hasabo and Noweer, 2005), humansand mammals (Rehman *et al*., 2009). As a result, biological control is becoming an important alternative method for control of root-knot nematodes (Mukhtar *et al*., 2013).

The concept of biological control of root-knot nematodes revolves around on the use of microbial agents. Previous studies have focused on the use of parasitic fungi and bacteria (Lamovsek *et al*., 2013). These microbial agents may also promote plant growth and suppress nematode pest populations in the soil and around the rhizosphere of the plant (Tian *et al*., 2007). The efficacy of biocontrol agents is dependent on various abiotic, biotic factors and time of application (Dabbat and Sikora, 2007). Most researchers use seed treatments (Dawar *et al*., 2008b, Padgham and Sikora, 2007; Tariq and Dawar, 2010) and soil drenching or soil incorporation of such agents (Dabbat, 2007; Mendoza, 2008) to introduce the nematicidal fungi and bacteria into the soil.

The aim of this study was to evaluate the nematicidal potential of 5 bacterial and fungal isolates to control *Meloidogyne javanica* infesting tomato plants under greenhouse conditions.

4.2 Materials and Methods

4.2.1 Preparation of nematode inoculum

Local populations of *Meloidogyne javanica* eggs and second stage juveniles (J2s) where reared in vivo in roots of tomato (cv. Florrdade) at the Unit of Environmental Sciences and Management, University of Potchefstroom to obtain nematodes for application in experiments, *M. javanica* (Treub) populations were multiplied and

maintained on tomato plants in a glasshouse at 25°C±5°C at the University of KwaZulu-Natal (Agriculture Campus). Roots infested with *M. javanica* were collected from the glasshouse.Nematode inoculum was obtained by extracting *M. javanica* eggs from infected roots. Roots were washed thoroughly under running tap water and then cut into 1-2 cm pieces. Thereafter, 250 ml of 0.5% sodium hypochlorite (NaOCl) solution was added to the root pieces in a 500ml glass bottle and shaken vigorously for 5 min (Hussy and Barker, 1973). The suspension was poured over a series of sieves, 250 µm and 25 µm pore size sieves. The eggs were collected on the 25 µm sieve and washed thoroughly with water to remove the NaOCl and then collected into a 100 ml beaker. The suspension was placed in a modified Baermann dish and incubated at 25°C for 7-10 d (Hooper *et al*., 2005). Hatched J2 stage juveniles of *M. javanica* were obtained by placing the eggs in sterile distilled water for 3-5 days at 28°C. The inoculum was collected and stored at 4°C in a beaker with distilled water for further use in the experiment. The number of juveniles was estimated in 1ml aliquots of the inoculum suspension.

4.2.2 Preparation of biological control inoculum

4.2.2.1 Bacteria

Five bacterial isolates that were highly suppressive against *M. javanica in vitro* (Chapters 2, Table 2.1) were chosen for testing under glasshouse conditions. Bacterial cultures were obtained from scraping cultures stored at -80°C. These cultures were streaked onto Tryptone Soy Agar (TSA) and incubated for 4 d at 28°C. The bacterial cultures were inoculated into 50 ml tryptone soy broth (TSB) and incubated for 2 wk at 28°C in an orbital shaker incubator at 150 rpm. Spores were harvested by centrifugation at 10,000 rpm for 15 min at 4°C (Beckman Coulter Avanti J-26XPI). The supernatant was discarded and the pellet was washed once with 0.1M NaCl and twice in cold sterile distilled water. The pellet was suspended in 50 ml distilled water. Spore counts were done using a dilution plating technique and the spore concentration was adjusted to 10^8 spores per.ml^{-1}

4.2.2.2 Fungi

Trichoderma harzianum (Eco-T®) and *Clonostachys rosea* (Schroers) were grown and formulated by Plant Health Products (Pty) Ltd (Nottingham Road, South Africa). Each contained approximately 10^8 spores.g^{-1}. Three wild-type fungal isolates that

were highly suppressive against *M. javanica* were also chosen for testing under glasshouse conditions. Agar blocks containing actively growing mycelia were transferred to potato dextrose agar (PDA) plates and incubated at 28°C for 2 wk. After incubation, the mycelia were removed from the plates by scraping the surface of the plate with a metal rod in distilled water and the suspension passed through a cheese cloth. The conidial suspension was transferred into a conical flask. The concentration of the conidia was then adjusted to 10^6 conidia.ml^{-1} using a haemocytometer.

4.2.3 Inoculation of seed with suspension spores of selected fungal and bacterial isolates

Tomato seeds were disinfected by soaking them in 2% sodium hypochlorite for 2 min, then they were rinsed 5 times with sterile distilled water. Each isolate was separately mixed with a 2% (w/v) carboxymethyl cellulose (CMC) sticker suspension. Seed inoculation was done by soaking the seed in each slurry suspension of bacteria and fungi, and allowing a contact period of 2 h to enhance adhesion of spores onto the seed. Control seeds were soaked in 2% CMC in sterile distilled water. The seed were removed from the suspensions and air-dried on a laminar flow bench for 12-18 h after which the seeds were planted in planting trays in sterile potting media (Yobo *et al*., 2010).

4.2.4 Drenching of tomato plants with spore suspension

At the transplanting stage, 3 wk old tomato seedlings were transplanted into pots (12.5 cm) filled with a sterile potting mix. Immediately after transplanting, a 15 ml aqueous spore suspension (10^6 spore/ml) of each bacterial and fungal isolate was inoculated by watering into holes made around the roots of each tomato plant, shaking the flasks occasionally to re-suspend the spores in solution. The plants were maintained in the glasshouse at 25°C±5°C.

4.2.5 Inoculation of tomato plants with *M. javanica* second-stage juveniles

Experiments were carried out using tomato (cv. Floradade, susceptible to *Meloidogyne*) grown in a glasshouse. Temperatures were maintained at 25°C±5°C. Two holes were made at the base of each tomato plant around the root area. Approximately 1000 freshly hatched nematode J2 were inoculated into these holes. The plants were not watered for 48 h after inoculation to allow the J2 to penetrate the

roots. Tomato plants were uprooted from the pots to determine nematode penetration at 7 days post-inoculation. The roots were stained with 1% acid fuchsine solution (Adam *et al*., 2014). Each root was rinsed thoroughly and soaked in 5.25% NaOCl for 4 minutes. The roots were soaked for 15 minwith sterile distilled water and dipped in 1% acid fuchsine solution for 30 sec and excess acid fuchsine was removed by washing the roots in tap water. For the J2 counts, the roots were cut into small pieces and mixed thoroughly. A 1 g sample of the roots was placed per microscope slide and the number of J2 in the roots was counted at 20 x magnification under a stereomicroscope (Zeiss Scope. AX10).

Separate plants were kept for 50 days in the greenhouse after bacteria, fungal and nematode inoculation. Fresh shoot and shoot length were measured (Al-Fattah, 2007). Above-ground/ aerial parts of plants were then dried at 60°C for 72 h and dry weight was recorded. The roots were washed under running tap water and were stained in Phloxine B for 20 min. Excessive stain was removed by washing the roots in tap water to facilitate egg mass counting. Root gall severity was assessed on a 0-8 rating scale in which, 0= free from galls; 1= trace, less than 5; 2= 6-25 galls; 3= 26-100; 4= moderate, galls 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 (Dautlon and Nasbam, 1969). The roots were cut in 1-2cm pieces and transferred into a glass bottle half filled with 0.5% sodium hypochlorite solution. Roots were vigorously shaken for 5 min and the suspension was washed with tap water and passed through 250 µm and 25 µm aperture sieves. Eggs were collected and transferred into a glass beaker. A 1ml suspension of the eggs was placed on a counting slide and numbers of eggs per ml were counted at 20x magnification using a stereomicroscope (Zeiss Scope AX10).

4.2.6 Statistical Analysis

Treatments were arranged in a Completely Randomized Design. Seven treatments were replicated five times. The experiment was repeated twice and the data was combined.Data were subjected to analysis of variance (ANOVA) using Genstat 16th edition. Duncan's multiple range test was used to compare treatment means at a 5% level of significance.

4.3 Results

4.3.1 Effect of bacterial and fungal isolates on J2 penetration

Seven days after inoculation of *M. javanica* juveniles into pots planted with tomato treated with the antagonists, the number of J2s in each root was counted (Fig 4.1). All *Bacillus* isolates significantly reduced the number of J2s that had entered the root compared to the control ($P < 0.001$) (Table 4.1). Seed dressing and soil drenching of plants with BG25 resulted in the lowest number of J2 penetrations into the roots, (11.0 juveniles and 17.0 juveniles, respectively). Isolates BG21 and BG32 performed reasonably well at reducing nematode penetration into tomato roots. Bacterial treatments with the antagonist, BS39, did not result in a satisfactory reduction in J2 penetration. Overall, seed dressing for all *Bacillus* isolates reduced the number of J2 per gram of root better than the soil drenching treatment.

Table 4.1: Effect of *Bacillus* isolates on *M. javanica* second -stage juvenile (J2) penetration (7 days after inoculation).

Isolate species	Treatments	Number of J2 penetrated per gram of root (7 D.A.I)	
		Seed Dressing	Soil Drenching
	No RKN	0.0**f**	0.0**f**
	RKN	52.0**a**	50.**a**
Bacillus thuringiensis	**BG25**	11.0**e**	17.0**e**
Bacillus cereus	**BG32**	15.0**d**	19.0**d**
Bacillus cereus	**BS29**	21.0**c**	23.0**c**
Bacillus thuringiensis	**BG21**	16.0**d**	19.0**d**
Bacillus cereus	**BS39**	24.0**b**	25.0**b**
	LSD	1.525	1.307
	F pr.	< 0.001	< 0.001
	CV (%)	6.5	5.1

Means followed by the same letter are not significantly different at $P<0.05$; means were compared using Duncan's multiple range test at a 5% level. RKN= Root-knot nematode.

Fungal isolates where found to significantly reduce the number of J2 entering the roots compared to the controls (Table 4.2). Seed dressing for all fungal treatments

resulted in fewer J2s in the roots compared to soil drenching. *Hypocrea lixii* isolate CR5 (seed dressing) significantly ($P < 0.001$) reduced the number of J2s in the roots (6.0 J2), with its soil drench also significantly ($P < 0.001$) reducing the number of juveniles penetrating the roots (9.0 J2) compared to the control with *M*. *javanica* only (49.0 J2).

Table 4.2 Effect of fungal isolates on *M. javanica* second-stage juvenile (J2) penetration (7 days after inoculation).

Isolate species	Treatments	Number of juvenile penetrated per gram of root (7 D.A.I)	
		Seed Dressing	Soil Drenching
	No RKN	0.0**f**	0.0**e**
	RKN	48.0**a**	49.0**a**
Trichoderma harzianum	**Eco-T®**	15.0**b**	12.0**c**
Clonostachys rosea	***C. rosea***	17.0**b**	16.0**b**
Hypocrea lixii	**Cr6**	9.0**d**	12.0**c**
Trichoderma spirale	**Cr12**	12.0**c**	13.0**c**
Hypocrea lixii	**Cr5**	6.0**e**	9.0**d**
	LSD	2.552	2.277
	F pr.	< 0.001	< 0.001
	CV (%)	14.2	12.2

Means followed by the same letter are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level. RKN= Root-knot nematode.

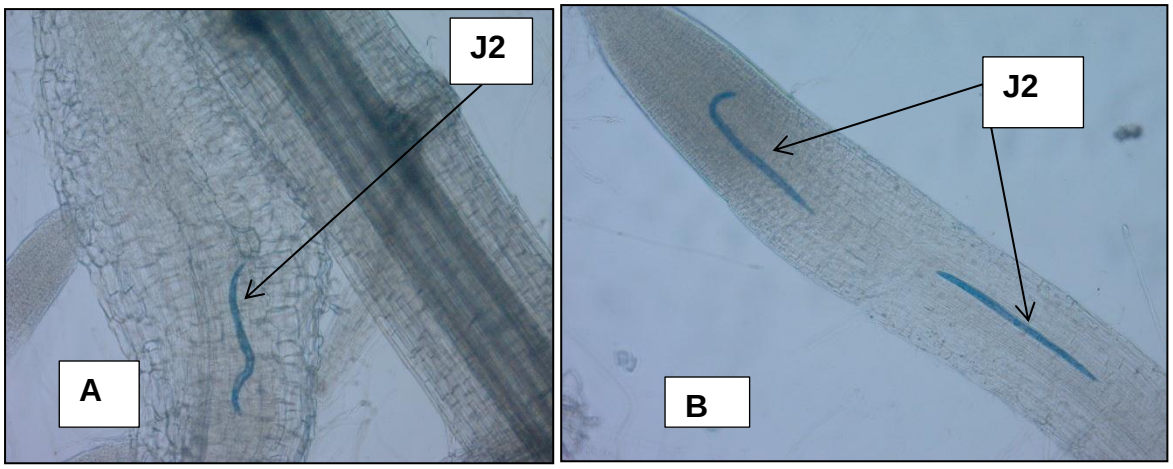

Figure 4.1 Roots stained with acid fuchsine to show penetrated second stage juveniles (J2s), (A) Seed dress Cr5, (B) Seed dress BG25 (Photos: G. Pambuka, UKZN)

4.3.2 Effect of bacterial and fungal isolates on *M. javanica* reproduction and disease severity under glasshouse conditions

In the glasshouse experiments, application of *Bacillus* isolates significantly reduced the galling index, egg masses and number of eggs produced by *M*. *javanica* when

compared to the controls (P < 0.001) (Table 4.3). Control plants that were inoculated with *M. javanica* had the highest gall index (4.3 and 4.5), egg masses (127.8 and 131) and number of eggs (42.5 and 43.5). Lower gall indexes and egg masses resulted from treatment with BG25 for both seed dressing and soil drenching (1.6 and 2.3; 59.3 and 68.0, respectively). BG25 also had an effect on the number of eggs per root for both seed dressing and soil drench (19.2 and 23.7, respectively). Overall, BG25 applied as a seed dressing significantly reduced gall index, number of egg masses and number of eggs produced compared to its soil drench. BG21 also performed exceptionally well in reducing the number of eggs in the roots, with both seed dressing and soil drenching (21.6 and 24.3, respectively).

Table 4.3 Effect of *Bacillus* isolates on reproduction and disease severity of *M. javanica* on tomato plants.

Isolate species	Treatments	Seed Dressing			Soil Drench		
		Gall Index	Egg masses	Number of eggs (x1000)	Gall Index	Egg masses	Number of eggs (x1000)
	No RKN	0.0**e**	0.0**f**	0.0 **f**	0.0**e**	0.0 **e**	0.0 **d**
	RKN	4.3**a**	127.8**a**	42.5**a**	4.5**a**	131**a**	43.8**a**
Bacillus thuringiensis	**BG25**	1.6**d**	59.3**e**	19.2**e**	2.3**d**	68 **d**	23.7**c**
Bacillus cereus	**BG32**	2.5**c**	67.2**d**	24.5**c**	2.7**bd**	72 **c**	25.3**c**
Bacillus cereus	**BS29**	3.1**b**	71.5**c**	32.9**b**	3.3**b**	78 **b**	33.6**b**
Bacillus thuringiensis	**BG21**	2.3**c**	66.5**d**	21.6**d**	2.5**d**	67 **d**	33.6**b**
Bacillus cereus	**BS39**	3.1**b**	77.0**b**	33.3**b**	3.3**bc**	81 **b**	35.1**b**
	LSD	0.583	3.030	1.810	0.635	3.410	2.242
	F pr.	<0.001	< 0.001	< 0.001	<0.001	< 0.001	< 0.001
	CV (%)	20.2	3.8	6.2	20.2	4.1	7.2

Means followed by the same letter are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level. RKN= Root-knot nematode.

The fungal treatments had a significant effect on disease severity on tomato plants compared to the control (P<0.001) (Table 4.4). The one control (RKN) had the highest root gall index, egg mass counts and number of eggs in the roots (4.2 and 4.3; 128.6 and 131.7; 42 and 44.7, respectively). Both seed dressing and soil drench with the isolate Cr5 resulted in the lowest gall index (1.0 and 1.3, respectively). This isolate also had an effect on the number of egg masses and number of eggs present on and in the roots. The effect of the isolates on disease severity was evident as a result of both Cr5 and Cr6 inoculation. Seed dressing proved to be more effective in reducing disease severity.

Table 4.4: Effect of fungal treatments on reproduction and disease severity of *M. javanica* on tomato.

Isolate species	Treatments	Seed Dressing			Soil Drench		
		Gall Index	Egg masses	Number of eggs (x1000)	Gall Index	Egg masses	Number of eggs (x1000)
	No RKN	0.0**d**	0.0**f**	0.0**g**	0.0**c**	0.0**g**	0.0**d**
	RKN	4.2**a**	128.6**a**	42.0**a**	4.3**a**	131.7**a**	44.7**a**
Trichoderma harzianum	**Eco-T®**	2.0**b**	72.3**c**	19.0**c**	1.7**b**	74.7**d**	17.0**b**
Clonostachys rosea	***C. rosea***	2.0**b**	79.8**b**	21.0**b**	1.7**b**	81.8**c**	18.2**b**
Hypocrea lixii	**Cr6**	1.3**bc**	64.6**d**	13.0**e**	1.5**b**	66.0**e**	13.7**c**
Trichoderma spirale	**Cr12**	1.5**bc**	70.3**c**	16.0**d**	1.8**b**	88.0**b**	18.5**b**
Hypocrea lixii	**Cr5**	1.0**c**	42.0**e**	10.0**f**	1.3**b**	50.3**f**	12.2**c**
	LSD	0.698	3.222	1.925	0.577	2.487	1.738
	F pr.	<0.001	< 0.001	< 0.001	<0.001	< 0.001	< 0.001
	CV (%)	34.5	4.2	9.4	27.8	3.0	8.3

Means followed by the same letter are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level. RKN= Root-knot nematode.

4.3.3 Effect of bacterial and fungal isolates on growth parameters of tomato plants under glasshouse conditions

The effect of *Bacillus* treatments on plant growth was determined at the end of the experiment. Seed treatment with all bacterial antagonists significantly increased shoot length, shoot weight and dry weight in comparison to the controls (P<0.001) (Table 4.5). Seed and soil drench treatments with BG25 caused a significant enhancement in growth parameters.

Table 4.5: Effect of *Bacillus* isolates on growth parameters of tomato 50 days after being infected with *Meloidogyne javanica* second-stage juveniles (J2).

Isolate species	Treatments	Seed Dressing			Soil Drench		
		Shoot Length (cm)	Shoot Fresh Mass (g)	Shoot Dry mass (g)	Shoot Length (cm)	Shoot Fresh Mass (g)	Shoot Dry mass (g)
	No RKN	29.0**d**	22.1**c**	2.9**e**	27.9**b**	21.1**d**	2.6**d**
	RKN	23.0**e**	16.4**d**	1.6**f**	22.9**c**	16.9**e**	2.0**e**
Bacillus thuringiensis	**BG25**	37.2**a**	34.8**a**	5.1**a**	36.0**a**	33.3**a**	4.7**a**
Bacillus cereus	**BG32**	34.7**b**	32.6**a**	4.1**c**	33.8**a**	30.5**b**	3.9**b**
Bacillus cereus	**BS29**	31.3**c**	28.3**b**	3.2**d**	30.0**b**	27.6**c**	3.0**c**
Bacillus thuringiensis	**BG21**	34.7**b**	33.7**a**	4.7**b**	33.8**a**	31.8**ab**	4.6**a**
Bacillus cereus	**BS39**	29.8**cd**	22.6**c**	3.4**d**	28.8**b**	22.0**d**	3.2**c**
	LSD	2.035	3.314	0.271	2.812	2.263	0.307
	F pr.	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	CV (%)	5.5	10.3	6.5	7.8	7.3	7.6

Means followed by the same letter are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level. RKN= Root-knot nematode.

The data in Table 4.6 confirms that the application of fungal treatments caused increased plant growth parameters with both seed dressing and soil drenching. Shoot length, fresh shoot weight, dry shoot weight of the control plants inoculated with *M. javanica* were significantly lower than plants treated with the fungi. Application of Cr5 as a seed dressing and soil drench significantly increased shoot length, fresh shoot weight and dry shoot weight compared to the controls (40.2 and 38.8; 35.8 and 34.2; 5.6 and 4.9, respectively).

Table 4.6: Effect of fungal treatments on growth parameters of tomato 50 days after being infected with *Meloidogyne javanica* second-stage juveniles (J2).

Isolate species	Treatments	Seed Dressing			Soil Drench		
		Shoot Length (cm)	Shoot Fresh Mass (g)	Shoot Dry mass (g)	Shoot Length (cm)	Shoot Fresh Mass (g)	Shoot Dry mass (g)
	No RKN	29.5**c**	20.8**d**	2.2**d**	27.7**c**	21.9**d**	2.2**e**
	RKN	20.5**d**	14.2**e**	1.4**e**	19.3**d**	12.5**e**	1.3**f**
Trichoderma harzianum	**Eco-T®**	36.8**b**	31.7**b**	4.3**b**	38.3**a**	33.6**a**	4.2**b**
Clonostachys rosea	***C. rosea***	30.6**c**	24.3**c**	3.4**c**	29.5**c**	22.8**cd**	3.2**d**
Hypocrea lixii	**Cr6**	36.7**b**	31.6**b**	4.5**b**	33.7**b**	30.8**b**	4.2**b**
Trichoderma spirale	**Cr12**	35.1**b**	25.4**c**	4.3**b**	34.1**b**	23.8**c**	4.1**c**
Hypocrea lixii	**Cr5**	40.2**a**	35.8**a**	5.6**a**	38.9**a**	34.2**a**	4.9**a**
	LSD	2.850	2.096	0.247	3.028	1.687	0.172
	F pr.	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	CV (%)	7.4	6.8	5.7	8.1	5.6	4.3

Means followed by the same letter are not significantly different at P<0.05; means were compared using Duncan's multiple range test at a 5% level. RKN= Root-knot nematode.

4.4 Discussion

4.4.1 Effect of bacterial and fungal isolates on J2 penetration

One week after inoculating with *M. javanica*, the number of juveniles in the roots was counted. Isolate BG25 (*B. thuringiensis*) applied as a seed dressing significantly reduced *M. javanica* J2 penetration into tomato roots. This shows that early inoculation of the seed with the bacteria reduced nematode penetration. This is in accordance with work done by Adam *et al*. (2014) who showed repellence of *M. incognita* J2s by antagonistic bacterial strains on tomato roots. Other research conducted by Padgham and Sikora (2007) showed that a strain of *B.megaterium* reduced J2 penetration of the rice nematode, *M.graminicola*, on rice into roots. Although the study did not look at the mode of action of the bacteria isolates, it has been hypothesized that repellence may be due to the bacteria altering chemical or physical properties of root exudates, or the production of metabolic compounds that

reduce the number of J2 entering the roots, interfering with nematode-to-root attraction (Sikora *et al*., 2007 and Li *et al*., 2005).

With the fungal treatments, penetration of *M*. *javanica* was significantly reduced by *H. lixii* isolate Cr5 applied as a seed dressing or a soil drench, when compared to the other treatments and the controls, especially, seed dressing. This suggests that when *H. lixii* is applied early, it has enough time to establish on the roots and in the rhizosphere of tomato plants. Similar work done by Le (2010) showed that *Fusarium moniliforme* isolate Fe14 significantly reduced J2 penetration of *M*. *graminicola* into the rice root. Many studies have shown that *Trichoderma* species produce nematicidal compounds which directly affect penetration of nematodes into roots and make them less attractive for penetration by nematodes (Jambhidriejab*et al*., 2013).

4.4.2 Effect of bacterial and fungal isolates on *M. javanica*reproduction and disease severity under glasshouse conditions

The effect of five bacterial isolates on disease severity was observed in glasshouse experiments. The results showed significant differences in the reduction of disease severity compared to the control. Application of BG25 significantly reduced root-knot nematode reproduction and disease, with both seed dressing and soil drenching. Chen *et al*. (2006) noted that some rhizobacteria (*Bacillus* spp.) produce lipopeptides, surfactins, bacillomycin D and fengycins, which are secondary metabolites with pathogen suppressive activity. However, Isolate BG25 applied as a seed dressing, reduced the gall index, number of eggs and egg masses of *M. javanica* significantly under glasshouse conditions compared to its drench. Application of the bacterial and fungal agents asseed dressing was more effective than soil drenches at reducing disease severity on tomato, showing its potential as an economic way of introducing bacterial antagonists (Varma, 2007). The results are in accordance with work done by Tariq and Dawar (2010), who also showed that seed treatment was more effective in reducing root-knot nematode (*M. javanica*) than soil drenching treatment on okra (*Abelmoschus esculentus* L.)and mung bean (*Vigna radiata* (L) Wilczek). Similar work by Dawar *et al*. (2008a) also showed the same effect of several *Bacillus* spp. on *M. javanica* disease severity when applied as seed dressing on cowpea (*Vigna unguiculata* (L.)Walp). Previous studies by Adam*et al*. (2014) showed that tomato seeds treated with bacteria reduced the number of

eggs and egg masses of *M.incognita* (Kofoid & White) Chitwood under pot conditions.

The present results for the fungal treatments showed that Isolates Cr5 and Cr6 applied both as seed dressings and soil drenches, significantly reduced *M*. *javanica* populations and disease severity on tomato plants when compared to the control (nematodes only) and the uninoculated (without nematodes) controls. However, seed treatment with *Hypocrea lixii* isolate Cr5 imposed excellent control of *M*. *javanica* on tomato. Seed dressing is a better option for introducing the biocontrol agent because this facilitates early protection from infection as the seedling grows. Research has been done on the ability of *Trichoderma* species to act as a biocontrol agent (Sharon *et al*., 2007). Dawar *et al*. (2008b) observed maximum suppression of disease incidence when *T. harzianum* was applied as a seed treatment. They also hypothesized that *Trichoderma* spp. have the ability to induce roots to repel root-knot nematodes by production of metabolic compounds. This could be the reason for the reduction in expression of disease and reproduction of *M*. *javanica* by *H. lixii* when applied to tomato seeds and as a drench because the isolates of *H. lixii* may also produce inhibitory compounds. Dabbat *et al*. (2006) suggested that *Trichoderma* should be applied early for good establishment of the fungus in the rhizosphere for good nematode control. Similar work done by Mascarin *et al*. (2012) showed that seed treatment with *Trichoderma harzianum* worked well on the biocontrol of *M.incognita* on cucumber (*Cucumis sativus* L.).

4.4.3 Effect of bacterial and fungal isolates on growth parameters of tomato plants under glasshouse conditions

Bacteria were introduced into the soil as a seed dressing or as a soil drench to facilitate early colonization of the roots. Results show that the test isolates, particularly BG25, BG21 and BG32, promoted plant growth with both the seed dressing and soil drenching treatments. BG25 caused the highest increase in growth. This is in accordance with work done by Almaghrabi *et al*. (2013) who showed that inoculation with six strains of plant growth promoting rhizobacteria (PGPR) increased plant growth parameters on tomato. A similar report was made by Tariq and Dawar (2010) where *Bacillus* isolates used as seed dress and soil drench onto okra significantly increased plant growth parameters. It has also been shown

that when isolates of *B. thuringiensis* are applied as seed dressing and soil drenches, they can cause a significant increase in shoot length and shoot weight (Sheikh *et al*., 2006). The exact mechanism in which PGPR stimulate plant growth is not clear, although two mechanisms, direct or indirect promotion, have been hypothesized to be the reason for enhanced plant growth (Beneduzi *et al*., 2012). Direct promotion of plant growth involves several mechanisms such as the production of phytohormones, activation of phosphate solubilization and promotion of mineral nutrient uptake (Xie *et al*., 1996; Jeonn *et al*., 2003; Egamberdiyeva, 2005 and Esitken *et al*., 2010). PGPRs indirectly promote plant growth by decreasing the competition by phytopathogenic microorganisms by production of antibiotics, enzymes or metabolic compounds (Weller *et al*., 2002; Lucy *et al*., 2004).

The fungal treatments for both seed dressing and soil drench had a positive effect on plant growth. *Hypocrea lixii* isolate Cr5 had a significant effect on growth parameters with both seed dressing and soil drenching. Dawar *et al*. (2008b) found that isolates of *Trichoderma* spp. applied as a seed treatment on sunflower (*Helianthus* L.) and okra significantly increased growth parameters. Elgorban *et al*. (2013) also reported that isolates of *H. lixii*, applied as a soil drench at conidial concentration of 10^6 cfu.ml^{-1} increased fresh shoot weight and dry weight of tomato plants. *Trichoderma* enhancement of growth has been known for many years (Adams *et al*., 2007). Several mechanisms have been suggested through which *Trichoderma* species promote growth (Harman *et al*., 2004). Among these is production of growth-regulating metabolites and phytohormones (Benitez *et al*., 2004). The ability of *Trichoderma* to rapidly colonise the roots results in effective competition for nutrients with pathogens (Benitez *et al*., 2004). This indirect mechanism reduces the effect of the pathogen on the host plant, thereby increasing plant growth. This may explain the results in this study showing the effect of *H. lixii* on the growth of tomato.

4.5 Conclusion

The results of the present study show the potential of seed dressing and soil drench as a way of introducing fungal and bacterial antagonists into the soil. However, seed dressing proved to be more effective in the reduction of root-knot nematode infection on tomato, and as growth promoters, than the soil drench treatment. Seed treatment

is regarded as the most economical method of application because this provides early protection from infection and it requires small amounts thereby reducing the cost of application of the antagonists into the soil (Elzein *et al*., 2006). Future research could investigate the modes of action of the bacterial and fungal antagonists identified here.

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Thesis Overview

The impact of root-knot nematodes (*Meloidogyne* spp) on crops has been widely researched (Trudgill and Blok, 2001; Sahebani and Hadavi, 2008; Jamshidnejab*et al*., 2013). Chemical nematicides have been used as the main startegy to control these pests. However, concerns of the effects of nematicides on the environment, and the associated health hazards to humans and animals has prompted research on alternative forms of control (Elgorban *et al*., 2013). Biocontrol agents are relatively safe to use and environmentally friendly, and research on the use of microbial agents as nematicides has been well documented (Lamovsek *et al*., 2013). Over the years, several biocontrol products have been developed and released on the market (Hallman *et al*., 2009). Previous biocontrol studies have focused on the use of parasitic fungi and bacteria (Lamovsek *et al*., 2013). As a bonus to crop production, many of the biocontrol bacteria and fungi have also been shown to influence plant growth and yields through several mechanisms such as production of phytohormones, activation of phosphate solubilisation and promotion of mineral nutrient uptake (Benitez *et al*., 2004; Esitken *et al*., 2010).

In the current study, isolations of bacteria and fungi were done at the Ukulinga Research Farm, UKZN, Pietermaritzburg from samples taken from grazing pastures and pens of the Livestock Section, and from artificial media in a tomato (*Lycopersicon esculentum* L.) greenhouse. The isolates were screened for their potential nematicidal activity on *Meloidogyne javanica* (Treub) Chitwood. The five bacteria and three fungi that were superior in causing J2 mortality of more than 60% were selected for further research. The bacterial isolates were then identified using 16sRNA sequence analysis and fungi using Internal Transcribed Spacer (ITS) analysis. The bacteria were identified as *Bacillus thuringiensis* and *B. cereus,* and the fungi as *Hypocrea lixii* (*Trichoderma harzianum* Rifai) and *Trichoderma spirale* (Bisset). Soil samples from the goat pastures and the rhizosphere of tomato plants were a good source for biological control agents which were used in this study.

In vitro studies were conducted to determine the nematicidal activity of the superior isolated bacteria and fungi, together with (Eco-T®, a commercial biocontrol agent (BCA) of *T. harzianum*), and an isolate of *Clonostachys rosea* with known nematicidal activity. They were screened on second-stage juveniles (J2) of *M. javanica* at 12, 24 and 48 h intervals. The *Bacillus* isolates and the fungal isolates

caused mortality of *M. javanica* after 48 h. Isolate BG25 (*B. thuringiensis*) and Isolate Cr5 (*H. lixii)* caused mortality of over 90% after 48 hours. In these experiments, the cuticle and body of most of the nematodes were destroyed and they were considered dead if they gave no response to physical stimuli such as probing with a needle.

The bacterial and fungal isolates used during *in vitro* studies were also investigated *in vivo* for their effects in promoting the growth of tomato plants, as well as their potential to control *M. javanica* on tomato under glasshouse conditions. Two methods of application were used for the biocontrol agents: seed dressing and soil drenching. Both forms of application of the biocontrol agents reduced*M. javanica* reproduction and disease severity on roots of tomato. However, seed dressing with *B. thuringiensis* (Isolate BG25) and *H. lixii* (Isolate Cr5) performed in the best at reducing gall formation, number of egg masses on the roots, and number of eggs in the egg masses. It was also established that when these two isolates where applied as seed treatments they had a greater impact on the growth of tomato plants. Seed dressing with the biocontrol agents increased shoot length, shoot weight, and dry shoot weight of tomato.

To summerize, the goals set at the beginning of the study have been achieved.

Future Studies

- This study produced promising bacterial and fungal isolates that have the potential to be used against plant *Meloidogyne* spp. They have proven to be effective under laboratory and glasshouse conditions. Therefore it is necessary to conduct field trials to further evaluate the efficacy of the biocontrol agents under field conditions.
- The effect of the biocontrol isolates on the J2 of *M. javanica* was demonstrated during *in vitro* studies. The mode of action and efficacy of the biocontrol agents involved in killing *M. javanica* needs to be determined for each of its life stages. In addition, the effect of the isolates on eggs needs to be determined.
- Microbial organisms interact with each other in the rhizosphere. Further studies focused on microbial interactions in the plant rhizosphere needs to be

evaluated. Furthermore, studies on increasing their activity in the rhizosphere need to be determined.

- The biocontrol agents should also be tested on a wide range of dicotyledonous and monocotyledonous crops to evaluate their effect on growth parameters and nematode control.
- The best biocontrol agents should be tested on various plant-parasitic nematodes to see if they can be used as broad spectrum nematode control agents.

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