# Biological control of *Meloidogyne incognita* race 2 on soybean (*Glycine max* (L.) Merrill) by means of plant growth-promoting rhizobacteria

By

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DECLARATION

I, the undersigned, declare that the MSc thesis entitled: “Biological control of *Meloidogyne incognita* race 2 on soybean (*Glycine max* (L.) Merrill) by means of plant growth-promoting rhizobacteria” submitted to the University of Pretoria is my own original work and it has not formed previously the basis for the award of any degree.

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**Summary**

Plant growth-promoting rhizobacteria (PGPR) are bacteria that colonize the rhizosphere and/or roots in the presence of other soil microflora and have growth promotion activity and/or biocontrol activity. Plant growth-promoting rhizobacteria can be integrated into modern agriculture. The main objective of the current study was to identify strains of PGPR for use as biocontrol agents against *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 on soybean and to study the mechanisms of control involved.

A seedling bioassay was used to screen PGPR (from the PGPR collection of the University of Pretoria) for biocontrol of *M. incognita* on soybean seedlings. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens* strain N04 reduced the number of *M. incognita* galls per plant by 30 % or more during two seedling bioassays.

The selected strains were tested in greenhouse trials. *S*train T19 proved to be the most consistent-performing biocontrol agent among the selected strains. The strain (applied as a Perlite powder seed treatment) reduced the number of *M. incognita* egg masses on roots of soybean plants significantly. Strains T19 and T22, applied as Perlite powder seed treatments, increased dry shoot mass and leaf area of soybean plants in experiments to test plant growth enhancement.

The ability of selected rhizobacteria to produce nematode-suppressive metabolites was assessed with *in vitro* assays. Strains T19, T22 and N04 appear to produce metabolites (in broth culture) that reduce motility as well as hatching of *M. incognita* second-stage juveniles. A split-root assay was attempted. Strain T22 induced resistance against *M. incognita* in soybean in one of two split-root experiments.

*Lysinibacillus sphaericus* strain T19 may be useful for control of *M. incognita* on soybean within an integrated pest management system. Strains T19 and T22 may also be useful as biofertilisers and could allow application of fertilisers at reduced rates.

# CHAPTER 1

# General Introduction

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## 1.1 Background

The yields from cereals and soybean may have to increase at a higher rate, in future, to meet the projected needs of the human population. Increased yields per hectare, reduced food wasting and reduced use of animal products may be needed in future to protect food security (Ray *et al.* 2013). In different regions in Africa, cereal and soybean yields have stagnated due to water shortages, soil degradation, low inputs, lack of appropriate cultivars and socio-economic problems (Ray *et al.* 2012). Root-knot nematodes (*Meloidogyne* Göldi, 1887) are another important cause of yield losses experienced for various crops in Africa, exacerbating the effects of water shortages, poor soil fertility and root rot diseases (Onkendi *et al.* 2014).The southern root-knot nematode *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 is one of the most important root-knot nematode species in soybean-producing regions (Wrather *et al.* 1997; Wrather *et al.* 2010).

Most soybean cultivars planted in South Africa are susceptible to *M. incognita*, and soybean is typically included in crop rotations that facilitate *M. incognita* reproduction. Root-knot nematodes are widespread in South Africa in different soil types and may reduce soybean yields by 25% to 70%. No synthetic, post-plant nematicides have been registered for use on soybeans in South Africa, because nematicide application was found to be too costly (Fourie *et al.* 2015). Several nematicides have been deregistered due to safety risks and environmental damage. Certain nematicides sometimes have phytotoxic effects, even if applied correctly (Jones *et al.* 2017). Long-term use of soil nematicides can also result in changes to the bacterial population, so that the nematicides are degraded rapidly (Sturza & Kimpinski, 1999). Application of a nematicide to soil is therefore only justified if the number of nematodes is above an economic threshold, so that the grower gains a significant increase in profit. Use of economic thresholds for root-knot nematodes on soybean is, however, complicated by the interactions between crop genotype, nematode genotype and environmental conditions (Fourie *et al.* 2015). Improved nematode control could be obtained by combining different management strategies, including the application of plant growth-promoting rhizobacteria (PGPR) (Barker & Koenning, 1998).

Plant growth-promoting rhizobacteria may protect crops against certain abiotic or biotic stresses and may increase yield through various mechanisms. Several PGPR strains are suitable for use on field crops and have both growth promotion and biocontrol effects. Multi-purpose products containing these strains have advantages above certain other biocontrol products (Dimpka *et al.* 2009; Kloepper *et al.* 2004). Bionematicides containing PGPR could therefore be beneficial to soybean growers in South Africa. Hassen (2007) isolated PGPR from virgin grasslands in South Africa. These PGPR have been the subject of continued research at the University of Pretoria and should be screened for activity against root-knot nematodes.

### 1.2 Research objectives

The main objective of the current study was to identify strains of PGPR for use as biocontrol agents against *M. incognita* on soybean and to study the mechanisms of control involved.

Specific objectives:

1. To screen selected rhizobacterium strains for compatibility with the nodule-forming bacterium *Bradyrhizobium japonicum.*
2. To screen selected rhizobacterium strains for suppression of *M. incognita* in roots of soybean seedlings.
3. To determine the effects of selected rhizobacterium strains on gall development caused by *M. incognita* infection of soybean roots.
4. To determine the effects of selected rhizobacterium strains on soybean growth in the greenhouse.
5. To elucidate the mechanisms of *M. incognita* control of selected strains of PGPR.

### 1.3 Feasibility and impact

The study has a high degree of feasibility. Several research groups have succeeded in demonstrating nematode-suppressing activity of rhizobacteria. Some of these rhizobacteria (including *Bacillus* spp., *Burkholderia cepacia*, *Paenibacillus macerans* and *Pseudomonas fluorescens*) have been successfully commercialised (Li *et al.* 2015). The impact of this study is thus expected to be significant. Studies on rhizobacteria may not only lead to development of commercial products but may also reveal new details of the molecular interactions between plants, soil fauna and bacteria (Li *et al.* 2015).

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# CHAPTER 2

# Literature review

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## 2.1 Soybean production and utilization

The genus *Glycine* most likely evolved from a perennial ancestor in Southeast Asia. Modern soybean (*Glycine max* (L.) Merr.) originated through domestication of the wild annual soybean (*Glycine soja* Sieb. and Zucc.) during the Shang dynasty (1766 to 1125 BCE) (Hymowitz, 2008). Soybean cultivation spread to other parts of Asia via different routes, with products of the crop as well as seeds being first introduced to Europe during the 18th century (Hymowitz, 2008). During the 20th century, soybean production expanded within Argentina, Brazil and the United States. This expansion was facilitated by plant breeders, who developed cultivars suitable for these regions. Argentina, Brazil and the United States are currently the top three exporters of soybean, soybean oil and soybean meal (Goldsmith, 2008). Soybean is rotated with other crops in some areas. In southern parts of the United States, soybean may be rotated with winter wheat (Herschman & Bachi, 1995). In South Africa, soybean is included in different crop rotation systems which may include maize, potatoes and sunflower (Riekert, 1996). Maize plants are often able to tolerate a certain level of nematode damage, mainly due to root compensation taking place throughout the growing season (Riekert, 1996), while allowing root-knot nematodes to reproduce, leading to yield losses on other crops (Dias *et al.* 2016).

Soybean has numerous uses. In China, soybean meal was used as a fertilizer before synthetic ammonium fertilisers became available (Schmitz *et al.* 2008). Soybean oil is used to manufacture certain inks and lubricants, while soy protein is used to manufacture paper coatings (Schmitz *et al.* 2008). Most soybean seeds are processed to produce meal and oil, with 98% of the meal used as livestock feed and 95% of the oil used for food purposes (Hartman *et al.* 2011). Soybean is the most important source of vegetable oil and lecithin in the world and is the best source of vegetable protein for monogastric animals. Soybeans also contain various vitamins (O'Brien, 2008; Stein *et al.* 2008). Several studies have concluded that regular consumption of soy foods reduce the incidence of certain diseases, but further studies are needed to verify these claims (Hill *et al.* 2008).

In the United States, soybean is graded by measuring the percentage damaged seeds, the percentage foreign contaminants, the percentage seeds with an incorrect colour and the moisture content. Environmental conditions affect the percentage protein and oil in seeds, but usually do not affect protein quality significantly. Seed-infecting fungi can reduce soybean quality and may produce mycotoxins, although the risk posed by mycotoxins on soybean is lower than on certain other field crops (Paulsen, 2008). Certain buyers offer higher prices for soybean with specific characteristics (Paulsen, 2008).

Soy protein-based foods were successfully commercialised in South Africa during the 20th century (Odendal, 1965). Most soybean oil and meal used in South Africa is imported (Dlamini *et al.* 2014). Efforts to initiate large-scale soybean cultivation during the 20th century were largely unsuccessful, but soybean production has expanded since 2004. This expansion has been aided by increased soybean processing capacity and the introduction of glyphosate-resistant cultivars (Dlamini *et al.* 2014; Fourie *et al.* 2015). The Mpumalanga province is currently the most important soybean-producing region in South Africa. Fertiliser is only used on 40% of soybean fields, and farm-saved, uncertified seed accounted for 85% of seed planted in the 2007/2008 season (Dlamini *et al.* 2014).

Important challenges facing soybean growers include pests, diseases and abiotic factors. Worldwide, the soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) is considered the most important biotic cause of yield loss in certain countries (Hartman *et al.* 2011; Wrather *et al.* 2010). However, *H. glycines* has not been introduced to South African fields yet. Several *Meloidogyne* spp. are also listed as major pests of soybean and known to affect soybean production in South Africa, with *M. incognita* being one of the most important. Root lesion nematodes (*Pratylenchus* Filip'ev, 1936) also affect soybean production but are generally less important than root-knot nematodes (Fourie *et al.* 2015).

Important challenges facing soybean industries include the presence of trans-fatty acids in partially hydrogenated oil-containing products, and the presence of phytates in soybean meal. Trans-fatty acids pose a threat to human health. Certain government bodies have implemented measures to restrict the trans-fatty acid content of food. Enzyme-catalysed interesterification processes may allow products such as margarine to be produced from soybean oil without any harmful trans-fatty acid content (Pande & Akoh, 2012). Phytates act as anti-nutritional factors. The phytate content of soybean products can be reduced using low-phytate cultivars or use of enzyme treatments (Stein *et al.* 2008).

## 2.2 Root-knot nematodes

Root-knot nematodes were first discovered in 1855, only two years after the first book about plant-pathogenic fungi was published (Karssen, 2002a). Göldi (1887) studied root-knot nematodes feeding on coffee tree roots and described the genus *Meloidogyne.* Subsequent authors placed root-knot nematodes in other genera, but Chitwood (1949) reinstated the genus.

The genus *Meloidogyne* contains more than 80 species. The most economically important root-knot nematodes in tropical and subtropical regions are the obligate apomictic species (such as *M. incognita*) (Lunt, 2008). These species appear to have originated relatively recently through hybridization between two ancestral root-knot nematode species. *Meloidogyne incognita* has one of the widest host ranges of any plant-parasitic nematodes (Lunt, 2008). Different *M. incognita* pathotypes occur. Populations of *M. incognita* may differ in host range and in susceptibility to the hyperparasite *Pasteuria penetrans*. The obligate apomictic species tend to have higher rates of reproduction than other root-knot nematodes and tend to cause more damage to crops (Trudgill & Blok, 2001). Root-knot nematodes appear to have acquired genes through horizontal gene transfer with bacteria or plants (Bellafiore & Briggs, 2010).

Morphological and morphometric characteristics have traditionally been used to identify *Meloidogyne* spp. Diagnosticians are faced by various challenges when using traditional microscopy-based methods for nematode identification. Different species of root-knot nematodes may sometimes produce perineal patterns like those of *M. incognita* (Onkendi *et al.* 2014). Molecular biology may offer alternatives to these traditional methods (Onkendi *et al.* 2014). Zijlstra *et al.* (2000) used the sequence-derived amplified region-polymerase chain reaction (SCAR-PCR) to identify nematodes. Next-generation technology such as the droplet digital polymerase chain reaction (ddPCR) and loop-mediated isothermal amplification (LAMP) allow improved detection of specific nematode species (Amoah *et al.* 2017).

Root-knot nematodes impair the uptake of water and nutrients by plant roots, and may cause reduced nitrogen fixation, reduced plant growth, reduced leaf chlorophyll content and early senescence (Sikora *et al.* 2005). The above-ground symptoms of nematode damage are like those of other crop disorders, including drought stress and nitrogen deficiency. (Castillo *et al.* 2008). Nematodes move relatively slowly in the field (compared to other pests) and occur in heterogeneous, aggregated patterns in fields (Hughes, 1996; Trudgill & Block, 2001). Root-knot nematodes can spread to new areas within plant material, water, soil and agricultural wastes (Hugo & Malan, 2010; Trudgill & Block, 2001).

Various biotic and abiotic factors affect the amount of yield loss caused by nematodes. High temperatures and fungal infections can cause the nematode resistance of a cultivar to become ineffective, while nematode infections can result in the same phenomenon regarding resistance against fungal diseases. Fungal strains that are normally non-pathogenic on a crop may act as opportunistic pathogens on nematode-infected plants (Prot, J.-C., 1993). *Meloidogyne incognita* may increase the transpiration rate of plants and predispose the plant to pollution injury (Khan & Khan, 1997).

Rasmann *et al.* (2011) reviewed the effects of root-knot nematodes on ecological succession. Early-stage plants (e.g. first in ecological succession) are likely to be exposed to lower numbers of phytonematodes and are likely to have nematode tolerance traits. Nematode-tolerant plants can compensate for a certain level of damage to roots and other below ground parts. Plants found later in ecological succession are likely to be exposed to higher phytonematode numbers and are more likely to have nematode resistance traits (such as chemical defenses). Plants in natural ecosystems also defend themselves by forming associations with microorganisms.

Chakraborty *et al.* (2012) and Pritchard *et al.* (2011) reviewed literature on the potential effects of climate change and rising carbon dioxide levels on plant diseases. Climate change may cause the areas in which crops are planted and the areas in which certain diseases occur, to shift. Plants may produce more secondary metabolites and the activity of certain plant growth-promoting microorganisms may be increased. The effects of climate change and rising carbon dioxide levels on root-knot nematodes is therefore difficult to predict. Yield losses will likely become even more difficult to predict than in the present. Root-knot nematodes may develop and reproduce faster in large areas. Pathogens and pests may also evolve faster.

### 2.3 Root-knot nematode life cycle

The life cycle of *M. incognita* can be divided into an exophytic phase and an endophytic phase. Several stages in the nematode life cycle can be targeted to reduce nematode reproduction (Dietrich & Sommer, 2009; Karssen, 2002b). The exophytic phase includes the eggs and the second-stage juveniles (J2). The endophytic phase includes the late J2 stage (after a feeding site has been initiated and J2 became swollen), the J3 and J4 stages (which only exist for short periods of time, do not have stylets, and do not feed) and the mature females. The mature female produces a gelatinous egg mass in which eggs are deposited. Environmental conditions, host physiology and the number of nematodes feeding in the roots (or other below ground parts) of a host affect the development and reproduction of the nematode after feeding

site initiation (Dietrich & Sommer, 2009; Karssen, 2002b). The mature males that form in certain cases could be considered an exophytic stage. Males are normally rare, but an increase in the number of males occurs if conditions are unfavourable for the nematode. A temperature of 25 – 32 °C is optimal for reproduction of *M. incognita* (Taylor & Sasser, 1978).

Root-knot nematode J2 migrate to host roots and other below plant parts through soil water by means of chemotaxis. Certain chemicals (such as the exudates from suitable hosts) act as nematode attractants, while certain other chemicals (including exudates from certain poor hosts) act as repellents. The J2 juveniles moves in random directions in the absence of attractants and may deplete their lipid reserves with time if a suitable host is not infected (Reynolds *et al.* 2011).

The J2 usually enter roots at the root elongation zone, and secrete enzymes that degrade the middle lamellae, allowing intercellular migration (Kyndt *et al.* 2012). Juveniles migrate towards the root apex, turn around and then migrate toward the vascular cylinder, where feeding sites are initiated. The J2 display cyclic behaviour: periods of head movement and stylet thrusting followed by periods of stylet-tip protrusion and median bulb pumping. The J2 displays this behaviour during migration. The late, swollen J2 stages and the mature females also display this cyclic behaviour (Wyss & Grundler, 1992; Miyashita *et al.* 2014).

A feeding site is initiated by injecting a group of procambium cells with oesophageal gland secretions. The secretions include effectors that reprogram the gene regulation of the host, as well as plant cell wall-degrading enzymes. The procambium cells undergo nuclear division and polyploidisation without completing cell division, thereby forming giant cells. Tissue around the nematode grows to form a gall. Gall formation disrupts the vascular system and tends to reduce root length, root branching and root hair formation. Giant cells synthesize substances (including sugars and proteins) that are utilised by the feeding root-knot nematode J2 or female (Kyndt *et al.* 2012; Taylor & Sasser, 1978).

The bodies of the J2 nematode become swollen as it develops. Hence, the late J2, third-stage (J3), fourth-stage juveniles (J4) and females become sedentary as the nematode grows. The head of the nematode remains mobile, allowing the J2 and females only to periodically feed on the different giant cells and inject these cells with effectors (Miyashita *et al.* 2014). Certain nematode effectors suppress the defences of the host (Kyndt *et al.* 2012). Usually, the female breaks through the surface of the gall when egg mass formation occurs. On certain highly susceptible hosts, however, nematodes may complete more than one life cycle within large galls. Egg masses may be hidden from natural enemies (such egg mass-parasitsing fungi) in these large galls (Kerry, 2001).

## 2.4 Suppressive soils and biological control

Certain soil organisms reduce the damage caused by phytonematodes and other root pathogens. In theory, two types of suppressive soils occur in nature: soils with increased activity of general soil organisms, and soils with increased activity of specific types of organisms (Cook & Baker, 1983). Biological control is the exploitation of antagonistic organisms to reduce the damage caused by weeds, pathogens or pests (Butt *et al.* 2001). Inundative biological control is the use of mass-produced antagonistic organisms with the expectation of short-term control. Inoculative biological control is the release of an organism with the expectation of long-term control (Cory & Franklin, 2012).

The terms 'biofertiliser' and 'biopesticide' have been used in different ways by different authors. Malusá & Vassilev (2014) suggest that the term 'biofertiliser' should only be used for a formulated product containing growth-promoting microorganisms, and not for the microorganisms themselves. These authors also suggest that a fermentation product that does not contain living cells of the microorganism should also not be referred to as 'biofertilisers'. In this dissertation, the term 'bionematicide' shall be used to refer to a nematode-suppressing product containing a living biocontrol agent.

Different types of organisms (including oomycetes) have been shown to play a role in suppressive soils, but only some of these organisms are suitable for mass production (Cumagun & Moosavi, 2015; Graff & Madelin, 1989). Biocontrol agents that have been commercialised for control of root-knot nematodes include endoparasitic bacteria (such as *Pasteuria* spp.), egg-parasitic fungi (such as *Purpureocillium lilacinus* and *Pochonia chlamydosporia*), and PGPR (Li *et al.* 2015). Other potential biocontrol agents include endoparasitic fungi, predacious (trap-forming) fungi and plant growth-promoting fungi (Gray, 1987; Martinez-Medina *et al.* 2017).

*Pasteuria* spp. are generally more host-specific than other nematophagous microorganisms (Gray, 1987; Luc *et al.* 2010). Biocontrol agents with narrow host ranges such as *Pasteuria* spp. are generally able to persist in the environment for long periods but may kill their hosts relatively slowly. *Pasteuria penetrans* endospores only germinate after the root-knot nematode has established a feeding site. *Pasteuria* spp. generally kill their hosts during the mature stage of the host life cycle and tend to cause gigantism in their hosts, thereby allowing large numbers of spores to be produced. These spores are released when the infected roots decay. Broad spectrum biocontrol agents often do not persist in the environment for long periods due to competition with other organisms but can be effective as inundative biocontrol agents (Cory & Franklin, 2012; Davies, 2009).

Certain authors regard the egg-parasitic ascomycetes as highly promising biocontrol agents. The egg-parasitic ascomycetes are generally able to colonize the rhizosphere and can be mass-produced relatively easily. Certain strains of *P. chlamydosporia* may persist in the soil for three years or more after being applied once (Kerry, 2001). These fungi can suppress phytonematodes through different mechanisms (Jacobs *et al.* 2003; Kiewnick & Sikora, 2006).

*Esteya vermicola* J. Y. Liou, J. Y. Shih & Tzean is an example of an endoparasitic fungus. The fungus has promising activity against the pinewood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934), Nickle, 1970 but can infect nematodes in different genera. The fungus grows within pine trees as a saprophyte and uses chemical mimicry to attract the pinewood nematode. Media have been developed for mass-production of the fungus, but further research and development is needed before the fungus can be commercialised (Chu *et al.* 2015).

Endophytes have been studied as potential biocontrol agents. Systemic endophytes are those microorganisms that are transmitted vertically to the progeny of the plant. Transient endophytes are affected by environmental conditions and competing microorganisms to a greater degree than systemic endophytes (Wani *et al.* 2015). Certain strains of *Neotyphodium* spp. can colonise grasses as systemic endophytes and are promising biocontrol agents for protection of turf grasses (Cumagun & Moosavi, 2015).

Predacious fungi can be grouped into spontaneous trap formers and non-spontaneous trap formers. Spontaneous trap formers are weak saprophytes that form traps soon after spore germination. Spontaneous trap-forming fungi undergo typical predator-prey population fluctuations. Non-spontaneous trap formers only produce traps in the presence of nematodes and may use these traps as defences against fungivorous nematodes (Gray, 1987). An example of a non-spontaneous trap-forming fungus is *Arthrobotrys oligospora* Fresenius. Some endophytic strains of *A. oligospora* are promising for control of root-knot nematodes. These strains have different modes of action against phytonematodes and can also promote plant growth and improve fruit quality (Singh *et al.* 2013). The hypothesis that certain fungi use traps as defences against fungivorous nematodes is supported by studies on the Basidiomycota. Fungi in the genus *Sphaerobolus* encapsulate and paralyse fungivorous nematodes after the nematodes feed on specialised structures, but the fungus does not feed on the encapsulated nematodes (Tanney & Hutchison, 2011). Certain basidiomycetes form nematode-paralysing droplets on specialised structures. Some of these fungi rarely feed on the paralysed nematodes (Tanney, 2011). These findings lend further support to the hypothesis that certain soil organisms spend energy on defence against nematodes.

The different types of biocontrol agents are not always neatly delineated by taxonomic groupings. The genus *Hohenbuehelia* includes endoparasites, spontaneous trap formers, species that produce both traps and adhesive spores and one species that produces nematode-paralysing droplets (Koziak *et al.* 2007).

### 2.5 Sustainable nematode management

Barker & Koenning (1998) reviewed sustainable nematode management practices. Sustainable agriculture should be profitable while ensuring food safety and enhancing agricultural ecosystem productivity. An integrated pest management system should include data-driven decision making and different management tools (including chemical and non-chemical control). Numerous nematode control tactics have been developed. Some of these tactics are not feasible for field crops, or only have moderate levels of efficacy.

Nematode monitoring and soil mapping could be used to reduce the costs of nematode control. Haygood *et al.* (2013) found that the amount of fumigant nematicide needed in cotton fields could be reduced by using soil mapping techniques to delineate high risk zones. Hughes (1996) discussed models incorporating heterogeneous pest distributions. Mathematical models suggest that a moderate reduction in nematode reproduction could significantly reduce the percentage of plants that become infected by the nematode, but only if the nematode population is below a certain level. Cost-effective management tools with moderate levels of efficacy could be useful in low risk zones

Highly effective nematode control can be obtained by combining different control tactics (Barker & Koenning, 1998). Biological control can be used in combination with crop rotation systems that include resistant cultivars (Kerry, 2001). Mixtures of PGPR have been successfully commercialised (Meyer, 2003). Several synergistic combinations of entomopathogenic fungi and chemical insecticides have also been discovered (Inglis *et al.* 2001). Combinations of biocontrol agents and low concentrations of chemical nematicides could also have synergistic effects. Roy (1982) found that sub-lethal doses of the organophosphate nematicide ethoprophos increase the susceptibility of *M. incognita* to the facultative endoparasitic fungus *Catenaria anguillulae* Sorokin. Improved nematode control could also be obtained by combining different active ingredients. Nikoletta *et al.* (2016) discovered synergistic mixtures of chemical nematicides.

Selected types of nematode control methods will be discussed in greater detail in the sections that follow. The role of microorganisms will be included in each section.

### 2.5.1 Cultural control

Numerous cultural practices can be used to control nematodes within horticulture. Certain of these control methods are unsuitable for use on field crops such as soybean (Barker & Koenning, 1998). Crop rotations should create a host-free period during which a nematode pest such as *M. incognita* does not reproduce. Root-knot nematodes decrease in number after a host-free period of 2 to 3 years, but shorter crop rotations may be viable if other nematode control methods are also included in the system (Katsvairo *et al.* 2006; Kerry, 2001). Cultural control may be ineffective if weeds are not managed, because of the wide host range of *M. incognita* (Barker & Koenning, 1998).

In some regions, the legumes *Crotalaria spectabilis* Roth and *Crotalaria ochroleuca* G. Don are included in rotations with soybean to control root-knot nematodes (Dias *et al.* 2016). *Crotalaria* spp. can be used as cover crops or green manures, and are poor hosts for various phytonematodes (Oka, 2010). Use of *Crotalaria* spp. is accompanied by certain challenges. *Crotalaria spectabilis* is more poisonous than other *Crotalaria* spp. Growers should be cautioned that small numbers of *C. spectabilis* seeds can contaminate livestock feed, harm animals and render dairy products unsafe (Guerra *et al.* 1999; Mosjidis & Wang, 2011).

Crop rotations including perennial grasses have also been used successfully to control phytonematodes on soybean and improve soil properties (Katsvairo *et al.* 2006; Weaver *et al.* 1995). Certain cultivars of weeping lovegrass (*Eragrostis curvula* (Schrad.) Nees) and guinea grass (*Panicum maximum* Jacq.) are poor hosts of certain root-knot nematode species. Certain growers in South Africa use these grasses in crop rotations, and farmers can also use these grasses to feed livestock (Katsvairo *et al.* 2006). Cultural practices that improve soil properties could improve the tolerance of crops to nematode damage, because nematodes impair the water and nutrient uptake by roots (McIntyre, 2000).

Microorganisms and soil fauna may play an important role in the nematode-suppressive effects of certain cultural control methods. Including perennial grasses in crop rotations could (in theory) increase the number and diversity of microbivorous nematodes in the soil (Katsvairo *et al.* 2006). Increasing the organic matter content of soil as well as the number of microbivorous nematodes could increase the numbers of endoparasitic fungi, spontaneous trap-forming fungi and other natural enemies of nematodes (Gray, 1987). Tillage and fertilisation influence the rate at which plant material is broken down as well as the number of microbivorous nematodes in the soil (Liphadzi *et al.* 2005).

Mycorrhizal fungi may reduce the infection of roots by phytonematodes as well as increase fertiliser use efficiency and ameliorate abiotic stress (Vos *et al.* 2011). Mycorrhizal fungi can also improve the nutritional value of food (Hart *et al.* 2015). Allen *et al.* (2001), Ellis (1998), Ridge & Theodorou, (1972) and Thompson (1987) discussed the effects of agricultural practices on mycorrhizal fungi and the importance of conservation of mycorrhizal fungi. Certain cultural practices such as flooding, bare fallowing and the inclusion of non-mycorrhizal plants (e.g. crucifers) in crop rotations can reduce the numbers of mycorrhizal fungi in soil. Excessive levels of phosphorus in fertilisers can also inhibit the formation of mycorrhizal associations. Loss of mycorrhizal fungi has been linked to reduced yields of cereals, legumes, sunflower and linseed in certain soils, such as clay soils northern Australia. These fungi can currently only be mass-produced using living plant hosts, precluding the inoculation of field crops with mycorrhizal fungi.

Growers can conserve and promote mycorrhizal activity in the soil through use of appropriate cultural practices (Ellis, 1998; Thompson, 1987). The flavonoid chemical formononetin (Myconate® (VAMTech L.L.C., Lansing, MI, USA)) can also be used to stimulate the sporulation of mycorrhizal fungi in the soil (Davies *et al.* 2005). Formononetin has been successfully used to increase the yield of various crops such as potato and may be useful as part of an integrated pest management system (Davies *et al.* 2005).

**2.5.2 Resistant cultivars**

A crop rotation system should ideally include different cultivars or crops with different resistance genes, to prevent resistance-breaking nematode populations from developing (Hartman *et al.* 2011). Resistant cultivars are the cheapest nematode control option for growers (Carpentieri-Pípolo*et al.* 2005). In Brazil, root-knot nematode resistant soybean cultivars have 10-15% higher yields than susceptible cultivars (Carpentieri-Pípolo*et al.* 2005). Efforts are underway to breed soybean cultivars suitable for different regions in South Africa with resistance to both *M. incognita* and *M. javanica* (Treub, 1885) Chitwood, 1949 (Fourie *et al.* 2015). Multi-locus resistance against *M. incognita* was found in cultivar LS 5995. *Meloidogyne incognita* J2 can migrate inside the roots of LS 5995, but formation of feeding sites is impaired, and necrosis may occur near the giant cells. This cultivar also has a higher level of tolerance for nematode damage than susceptible cultivars (Fourie *et al.* 2015).

Studies on the metabolomics of plants could help to elucidate the mechanism through which certain plants resist nematodes. Carpentieri-Pípolo *et al.* (2005) found no significant differences between the amounts of metabolites found in a resistant and a susceptible

soybean cultivar before being infected by *M. incognita* J2 but found higher concentrations of certain isoflavonoids in the resistant cultivar after the plants were parasitized by this root-knot nematode species. Similar results had been reported by other authors with insect-resistant soybean cultivars. The authors concluded that the isoflavonoid concentration of soybeans could be used in future studies as an indication of cultivar resistance or induced resistance.

Dias *et al.* (2016) found that *M. incognita* had reproduction factors larger than 1 on certain Brazilian soybean cultivars that were previously regarded as resistant against *M. incognita.* These soybean cultivars had significantly lower reproduction factors than other soybean cultivars and therefore had partial resistance. The authors concluded that a different *M. incognita* genotype than the genotype used in previous studies caused this result. Similarly, scientists in South Africa found that a *M. incognita*-resistant cultivar from the United States was susceptible to South African genotypes of *M. incognita* (Fourie *et al.* 2015).

Dominant resistance genes have been discovered that could be incorporated into certain crops through breeding. An example of such a gene, found in a wild relative of peanut, has successfully been incorporated into certain peanut cultivars (Bendezu & Starr, 2003). Second-stage juveniles of *Meloidogyne arenaria* Neal, 1889 (Chitwood, 1949) tend to leave the roots after penetration of plants with this gene. Those J2 that succeed in establishing feeding sites develop more slowly than nematodes in susceptible plants (Bendezu & Starr, 2003).

Many studies suggest that genetically modified crops are safe and beneficial to humanity (Mampuys & Brom, 2015). Genetic modification has been used to produce nematode-resistant cultivars. Ibrahim *et al.* (2011) showed that development of *M. incognita* and gall formation on soybean can be significantly reduced by means of post-transcriptional gene silencing. Huang *et al.* (2006) found that silencing the *16D10* effector gene in *Arabidopsis thaliana* (L.) Heynh significantly reduced reproduction of four different root-knot nematode species by 69–93%, thus offering a broader spectrum of resistance than numerous natural resistance genes. Gene silencing does not necessarily reduce reproduction factors to below 1, but silencing more than one effector gene has additive, adverse effects on nematode reproduction (Ibrahim *et al.* 2011).

Certain crystal toxins produced by strains of *Bacillus thuringinsiensis* are toxic to bacterivorous nematodes. These crystal toxins can be used against *M. incognita* by genetically modifying the plant to produce these toxins inside root cells, so that the nematodes ingest these toxins, and nematode development is prevented (Li *et al.* 2008).

Lilley *et al.* (2011) demonstrated that plants can be genetically modified so that cells in the root cap and root elongation zone produce peptides that inhibit acetylcholine esterase activity,

thereby repelling cyst nematode J2. This study found that reproduction of *Globodera pallida* (Stone, 1973) Behrens, 1975 in potato roots could be reduced by 95% using a nematode-repelling transgene. According to the authors, this resistance transgene has the advantage of reducing the number of cyst nematodes that penetrate and damage the root system early in the season, while some other resistance genes allow penetration.

### 2.5.3 Chemical control

The chemical pesticide industry is in a state of continuing development. Certain of the earlier organochlorine pesticides have been withdrawn from the market and replaced with the organophosphate and carbamate pesticides that are presently used (Niggli, 2007). Safer fumigants have been developed, such as dazomet (Basamid®, BASF Corp., Germany) (Konkler *et al.* 2017). Synthetic non-fumigant nematicides with reduced risks (compared to older pesticides) have been developed. Examples of next-generation nematicides include abamectin (Avicta® 500FS; Syngenta, Basel, Switserland) and fluensulfone (Nimitz® (Adama Ag Solutions, Raleigh, North Carolina). Abamectin can be used as a seed treatment for early-season control of nematodes and is economically viable on certain field crops. Abamectin acts by antagonising the γ-aminobutyric acid receptors of invertebrates (Cabrera *et al.* 2013; Copping & Duke, 2007). Fluensulfone is a synthetic, soil-applied nematicide with some systemic activity that appears to inhibit a nematode-specific biochemical pathway. Spirotetramat is applied to the foliage of the crop and is translocated to the roots (Jones *et al.* 2017). Spirotetramat prevents egg mass formation, but does not significantly affect nematodes in the soil, and acts by inhibiting an enzyme involved in fatty acid biosynthesis in invertebrates (Jones *et al.* 2017; Lümmen *et al.* 2014).

Microorganisms can act as sources of nematode-suppressing chemicals or lead compounds. Abamectin is produced by *Streptomyces avermitilis* and was discovered by testing broth cultures against a vertebrate-parasitic nematode (Cabrera *et al.* 2013; Copping & Duke, 2007). Emodepside is a semi-synthetic derivative of octadepsipeptide PF1022A. Octadepsipeptide PF1022A is produced by *Rosellinia* spp. PF1022, a fungus isolated from a leaf of *Camellia japonica* L. This active ingredient is used to control vertebrate-parasitic nematodes. Emodepside acts upon potassium channels in the nervous systems of nematodes and is effective against nematodes that are resistant to other chemicals (Buxton *et al.* 2011).

DiTera® (Valent BioSciences LLC, Libertyville, Illnois) is a nematicide that is produced by growing the fungus *Myrothecium verrucaria* (Albertini and Schwein) Ditmar isolate AARC-0255 in broth culture and then sterilising the culture (Copping & Duke, 2007). The individual

compounds found in DiTera® do not paraslyse nematodes at the concentrations applied, and only the product (which consists of numerous compounds) paralyses nematodes. DiTera® can be applied before or after planting (Copping & Duke, 2007).

Numerous articles have been published on botanical products with nematode-suppressing activity (Oka, 2010). Research on botanical products is complicated by restrictive bioprospecting legislation in certain countries such as South Africa (Crouch *et al.* 2008). Nikoletta *et al.* (2016) found that (*E,E*)-2,4-decadienal and (*E*)-2-decenal, secondary metabolites found in *Ailanthus altissima* (P. Mill), had suppressive effects on various stages of the life stages of *M.incognita*. This study is remarkable because the toxins had synergistic effects when combined with other nematicides such as furfuraldehyde. Little information is available on synergistic interactions between nematicides.

Furfuraldehyde has been registered for use as a nematicide under different trade names, including CropGuard® (Illovo Sugar Limited, Durban, South Africa) and MultiGuard Protect® (Agriguard Company, LLC, Cranford, New Jersey). This chemical is produced from agricultural residues such as sugarcane bagasse (Crow & Luc, 2014). Use of furfuraldehyde as a nematicide is complicated by the high levels of phytotoxicity of the chemical (Walker, 2007). Use of low doses (application rates) of furfuraldehyde in conjunction with other chemicals, as suggested by Nikoletta *et al.* (2016), could be a viable nematode option in future.

Chemicals that prime plant defences could (in theory) be used to reduce infection by phytonematodes within an integrated management system aimed at conservation of beneficial organisms. These chemicals include DL-β-aminobutyric acid (BABA), silicon, and salicylic acid (SA) analogues such as benzo(1,2,3)thiadazole-7-carbothionic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (Cohen *et al.* 2016; Molinari *et al.* 2016; Silva *et al.* 2010). These chemicals often do not reduce nematode reproduction factors to below 1 and are only effective on certain crops (Molinari *et al.* 2016; Silva *et al.* 2010). DL-β-aminobutyric acid occurs naturally in certain plants but is also synthesised industrially. This chemical reduces penetration of plants by nematodes and appears to prime a jasmonic acid-independent defensive pathway (Cohen *et al.* 2016). Silicon may not only prime plant defences but may also form complexes with other substances in plant cell walls, thereby improving the mechanical strength of plants (Silva *et al.* 2010).

Bacterivorous nematodes have shorter life-cycles than sedentary endoparasitic nematodes, enabling these nematodes to recover faster than the phytonematodes after a nematicide application. Microbivorous nematodes are also less sensitive to certain toxins than phytonematodes (Barker & Koenning, 1998; Sturza & Kimpinski, 1999). In future, growers may therefore be able to use nematicides or transgenic plants as part of integrated management

systems to suppress phytonematodes, while conserving the activity of certain microbivorous nematodes and nematophagous organisms.

### 2.6 Plant growth-promoting rhizobacteria

Kloepper *et al.* (1992a) described PGPR as bacteria that colonize the rhizosphere and/or roots in the presence of other soil microflora and have growth promotion activity and/or biocontrol activity. The culturable plant growth-promoting rhizobacteria (bacteria found in soil associated with roots) include strains from different bacterial phyla, including the Proteobacteria, Firmicutes and Actinobacteria (Cumagun & Moosavi, 2015; Garrity *et al.* 2004, Krechel *et al.* 2002). Strains from other phyla, such as the phylum Cyanobacteria, may also be useful for promotion of plant growth (Prasanna *et al.* 2015).

Rhizobacterium strains differ in their ability to survive in the root zone and on the plant, but some strains can survive for long periods and may even colonize the following crop planted in the soil (McSpadden Gardener, 2004). A single rhizobacterium strain may colonize the roots of different plants. Rhizobacteria form microcolonies on different parts of root surfaces, allowing the bacteria to utilise root exudates. Certain rhizobacteria may also enter the root interior (endorhiza) (Fan *et al.* 2012; Hallmann *et al.* 2001). The rhizosphere bacteria and endophytic bacteria may form a continuum, with some rhizosphere bacteria occurring as endophytes in the plant. Rhizosphere bacteria tend to have larger genomes, enabling these organisms to colonise diverse environments, while non-culturable endophytic bacteria tend to have smaller genomes (Farrar *et al.* 2014).

Rhizobacteria may make use of natural openings to enter plants and may use cell-wall degrading enzymes during endophytic colonisation (Siddiqui & Shaukat, 2003a). Certain rhizobacteria may then move through the apoplast in the roots and may enter the root stele when secondary root formation occurs, allowing PGPR to colonise above-ground plant organs (Kloepper *et al.* 1992b). The internal tissues of the plant may be colonised in a discontinuous manner, comparable to the discontinuous microcolonies found on root surfaces (Hallmann *et al.* 2001). Certain actinobacteria grow on and inside roots as extended mycelial colonies, in contrast to other rhizobacteria that grow as microcolonies (Tokala *et al.* 2002). Endophytic bacteria could in theory be less sensitive to environmental conditions than epiphytic bacteria. Several studies have shown, however, that plant defences can prevent certain beneficial endophytes from colonising the plant interior. Some cultivars of a crop may be permissive to an endophytic bacterium, while other cultivars may initiate a defence response against the endophyte (Farrar *et al.* 2014).

The PGPR include root-associated bacteria that fix atmospheric nitrogen. Nitrogen-fixing PGPR can be divided into bacteria that form specialised structures on certain plants, creating low-oxygen microenvironments optimal for nitrogen fixation, and the bacteria that do not form specialised structures (Farrar *et al.* 2014). Bacteria that form specialised structures include members of the genus *Frankia* (class Actinobacteria), members of the order Rhizobiales (class Alphaproteobacteria), members of the Betaproteobacteria and members of the phylum Cyanobacteria. *Frankia* spp. form nitrogen-fixing nodules on certain plants in the orders Fagales, Rosales and Cucurbitales, while the proteobacteria form nodules on legumes (Angus & Hirsch, 2010; Santi *et al.* 2013). Cycads form specialised coralloid roots that are colonised by nitrogen-fixing cyanobacteria (Santi *et al.* 2013). Some of the plant genes required for formation of endomycorrhizal associations are also required for formation of nitrogen-fixing nodules (Ercolin & Reinhardt, 2011; Santi *et al.* 2013).

The formation of nitrogen-fixing nodules is a complex, host-specific process, but nodule-forming bacteria are able to colonize the roots of different plants and promote the growth of these plants. *Bradyrhizobium* spp. can also form nitrogen-fixing biofilms on the hyphae of different fungi in the soil (Antoun *et al.* 1998; Frey-Klett *et al.* 2007). Rhizobia can also form nitrogen-fixing nodules on plants other than *Parasponia* spp. (members of the Rosales) (Santi *et al.* 2013). Examples of nitrogen-fixing bacteria that do not form specialised structures on hosts include *Azospirillum* spp. and *Gluconacetobacter diazotrophicus*. These bacteria generally fix less nitrogen than nodule-forming bacteria, but some strains can provide a significant fraction of the nitrogen required by cereal crops (Malik *et al.* 1997; Vargas *et al.* 2014).

Various scientists studied root nodules during the 19th century, with M. W. Beijerink being the first scientist to successfully culture rhizobia *in vitro* (Young & Haukka, 1996). Rhizobacteria that do not form nodules were first utilised in the 20th century. Kodiak® (Gustafson, Inc, Plano, Texas), containing *Bacillus subtilis* GB03, was one of the first successful biological products used to protect field crops from soil-borne pathogens. The product was widely promoted by the cotton industry in the United States and is used within an integrated management system in conjunction with fungicides (Brannen & Kenney, 1997; Emmert & Handelsman, 1999).

PGPR have been commercialised for a variety of different uses, including promotion of plant growth, the control of bacteria, fungi and nematodes on plants, and the control of bacterial pathogens of mushrooms (Li *et al.* 2015; Nakkeeran *et al.* 2005). PGPR can be mass-produced using broth or solid-state cultures. Carrier materials (such as minerals or organic substances) may be used to improve the shelf life and survival of PGPR. Biopesticides containing PGPR can be added to soil or growth media or used to treat different types of

planting material. Rhizobacteria can also be sprayed on the foliage of plants or used within postharvest treatments to prevent different diseases (Nakkeeran *et al.* 2005). The application of PGPR is not limited to agriculture and horticulture. PGPR may be useful in the revegetation of ecosystems previously subjected to desertification (Herrera *et al.* 1993).

The selection of PGPR strains for research and development is motivated by different considerations. A great deal of research on PGPR has focused on specific species of PGPR. Endospore-forming bacteria are not necessarily more effective than other biocontrol agents, but most PGPR that have been commercialised are endospore formers because of the long shelf life offered by products containing endospores (Kloepper *et al.* 2004; Ojiambo & Scherm, 2006).

Costa *et al.* (2014) isolated 2211 different strains of rhizobacteria from various soils and plants and tested various growth promotion activities of the bacteria. The authors reported that certain bacterial genera were found more frequently than other genera in the root interior, but also found that soil properties affected the type of bacteria occurring in the root interior. The study lends support to the hypothesis that most bacteria occurring in the root interior are transient endophytes, but the study also suggests that specific groups of bacteria are promising targets for bio-prospectors. Certain strains of *Burkholderia cepacia* have been commercialised for biocontrol of phytonematodes (Li *et al.* 2015). The study of Costa *et al.* (2014) suggests that plant growth-promoting strains of *Burkholderia* spp. are promising for use in soils with low fertility, where these strains are mostly found in the rhizosphere rather than the root interior.

An example of a bionematicide that has been commercialised successfully is BioYield® (Gustafson LLC, Plano, Texas). The product contains *Paenobacillus macerans* and *Bacillus amyloliquefaciens* within a chitosan carrier. The strains of PGPR in the product have different modes of action. Chitosan is a deacetylated derivative of chitin and may contribute to the nematode-suppressive effect of the product by inducing resistance against phytonematodes and by increasing the activity of egg-parasitic microorganisms (Burkett-Cadena *et al.* 2008; Silva *et al.* 2014). BioYield® can be used to protect strawberry, sweet pepper and tomato plants against root-knot nematodes and other root pathogens. The product was developed through cooperation between private sector, university and government researchers (Meyer, 2003). Examples of studies on the biocontrol of phytonematodes on legumes using PGPR are shown in Table 2.1. Table 2.2 contains examples of studies on biocontrol of phytonematodes using PGPR on crops other than legumes.

**Table 2.1 – Examples of studies on nematode control on legumes using plant growth-promoting rhizobacteria (PGPR)**

| Bacterial strain(s) | Nematode species | Effect of PGPR on nematode | Reference |
|---|---|---|---|
| *Bacillus pumilus* and *Pseudomonas alcaligenes* (combined with mycorrhizal fungus) | *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood | Reduction in the number of galls and eggs per chickpea root system (and reduction of *Macrophomina phaseolina* root rot) in autoclaved soil | Akhtar & Siddiqui (2008) |
| *Bacillus* sp. | *M. incognita* | Reduction in the number of eggs per soybean root system in fumigated soil | Nunes *et al.* (2010) |
| *Bacillus subtilis* | *M. incognita* and *M. javanica* (Treub, 1885) Chitwood, 1949 | Reduction in the number of eggs and second-stage juveniles (J2) per soybean root system | Araujo *et al.* (2012) |
| *Pseudomonas fluorescens* strain CHA0 | *M. javanica* | Reduction in the nematode population on mung bean and soybean roots (micronutrients increased effectiveness) | Siddiqui *et al.* (2004). |
| *Pseudomonas fluorescens* strain Wood1R | *M. incognita* | Reduction in the number of eggs per soybean root system in pasteurised soil | Timper *et al.* (2009) |
| *Pseudomonas* spp. | *M. incognita* | Reduction in galling and the number of eggs and J2 on pea root systems | Siddiqui *et al.* (2009) |
| *Pseudomonas* spp. strains P29 and P80, and *Bacillus cereus* strain B1 | *Heterodera trifolii* Goffart, 1932 | Reduction in the fecundity of females and increase in proportion of distorted females | Kempster *et al.* (2001) |

| | | | |
|---|---|---|---|
| *Sinorhizobium fredii* strain Sneb183 | *Heterodera glycines* Ichinohe, 1952 | Reduction in the number of cysts and J2 on soybean roots, reduction of J2 penetration and induction of resistance | Tian *et al.* (2014) |

**Table 2.2 – Examples of studies on nematode control on crops other than legumes using plant growth-promoting rhizobacteria (PGPR)**

| **Bacterial strain(s)** | **Nematode species** | **Effect of PGPR on nematode** | **Reference** |
|---|---|---|---|
| *Bacillus firmus* strain GB-126 | *Rotylenchulus reniformis* Linford & Oliveira, 1940 | Reduction in the number of vermiform nematodes, females and eggs on cotton (application of *B. firmus* also increased the numbers of free-living nematodes) | Castillo *et al.* (2013) |
| *Paenibacillus polymyxa* strains GBR-462 and GBR-508, as well as *Paenibacillus lentimorbus* strain GBR-158 | *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood | Reduction in the number of root-knot nematode galls on tomato roots | Son *et al.* (2009) |
| *Pseudomonas fluorescens* | *Heterodera schachtii* Schmidt, 1871 | Reduction in the percentage of nematode J2 that penetrated sugar beetroots | Oostendorp & Sikora (1989) |
| *Pseudomonas spp.* and *Bacillus spp.* | *Radopholus similis* (Cobb, 1893) Thorne, 1949 | Reduction in the number of nematodes (mixed stages) on banana plants | Chaves *et al.* (2009) |

## 2.7 Modes of action of PGPR

The modes of action of PGPR against phytonematodes are not fully understood (Li *et al.* 2015; Tian *et al.* 2007). Currently, scientists believe that several strains of rhizobacteria suppress nematodes though induction of resistance, production of nematode-suppressive compounds and/or impairment of host-finding behaviour. Rhizobacteria may compete against phytonematodes for space or resources in the plant. The modes of action of PGPR against nematodes can be compared to those of certain plant growth-promoting fungi (Li *et al.* 2015; Tian *et al.* 2007).

The tendency of a bacterium to colonize the root interior may affect the biocontrol activities of the bacterium according to certain studies. Bacteria that mostly colonize the root cortex may be more likely to control nematodes through induced resistance. Endophytes found in the outer root tissues may be more likely to express other anti-nematode activities (Hallmann *et al.* 2001). Bacteria found in the rhizosphere may be more likely to suppress nematodes and root rot fungi than endophytic bacteria (Krechel *et al.* 2002). The different modes of action of PGPR will be reviewed in the sections that follow.

### 2.7.1 Nematode-suppressive toxins

Numerous articles about PGPR suggest that production of toxins is an important mode of action of numerous strains of PGPR. Different strains of bacteria have been shown to produce toxins that act upon different life stages of nematodes (at certain concentrations or doses). Most of the studies on the effect of bacterial toxins on nematodes have made use of *in vitro* assays (Mendoza *et al.* 2008; Meyer *et al.* 2009; Zeng *et al.* 2015; Jansen-Girgan *et al.* 2016). The metabolic pathways of plants and microorganisms may interact, leading to production of novel bioactive molecules (Wani *et al.* 2015).

The definition of an antibiotic and the functions of bacterial antibiotics in the soil is a topic of ongoing debate, but bacterial antibiotics could be present in high concentrations in certain microenvironments (Raaijmakers & Mazzola, 2012). Measuring the amount of antibiotics produced by rhizobacteria in the rhizosphere directly can be technically challenging (Siddiqui & Shaukat, 2003b). Some studies have measured the concentrations of antibiotics produced by *Bacillus subtilis*, *Burkholderia cepacia* and *Pseudomonas* spp. directly in the soil and on plants and linked these antibiotics to suppression of pathogenic fungi (Raaijmakers & Mazzola, 2012). According to Sikora *et al.* (2007), there have been no studies linking direct measurements of bacterial toxins in soil to suppression of phytonematodes, although some

stains of *Bacillus firmus* and other PGPR may produce toxins that suppress phytonematodes at low concentrations. Kobayashi (2015) found that production of lipopeptide antibiotics by *B. subtilis* was upregulated by exposure to methyl salicylate (a plant hormone released in response to certain plant stresses). This study is remarkable and lends support to the hypothesis that plants may recruit rhizobacteria to assist in the defending the roots against soil-borne pathogens. Certain strains of *B. subtilis* produce metabolites that reduce hatching and motility of *M. incognita* J2 (Kavitha *et al.* 2012), but most literature on biocontrol using *B. subtilis* focuses on biocontrol of fungi.

Identification of nematode-suppressive toxins produced by PGPR is complicated by the tendency of PGPR to produce mixtures of bioactive molecules that could be compared to the mixture of compounds found in DiTera®, discussed in Section 2.5.3. Meyer *et al.* (2009) found that the nematode-paralysing activity of *Pseudomonas fluorescens* CHA0 culture filtrates was likely due to the combined effect of hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and other metabolites. Diacetylphloroglucinol can reduce *M. incognita* J2 hatching at certain concentrations (Meyer *et al.* 2009) but can also induce resistance in plants against biotrophic oomycete pathogens (Iavicoli *et al.* 2003). Diacetylphloroglucinol, at concentrations found in the rhizosphere, can increase root length and lateral root formation by stimulating an auxin-dependent signal pathway (Brazelton *et al.* 2008). Diacetylphloroglucinol is produced by enzymes from the polyketide synthesis pathway and the fatty acid synthesis pathway. Production of DAPG in cultures is affected by temperature and by the type of sugars in the medium (Bender *et al.* 1999).

Raaijmakers & Mazzola (2012) reviewed the functions of antibiotics produced by beneficial bacteria. These authors suggest that bacteria may use different antibiotics as multi-purpose tools and discuss numerous examples of antibiotics that inhibit or kill other organisms at high concentrations, but have different functions at sub-lethal concentrations, including repelling of bacterivorous fauna. The study of Nandi *et al.* (2015) supports this hypothesis. *Pseudomonas chlororaphis* PA23 is a promising biocontrol agent for the protection of canola plants against the fungus *Sclerotinia sclerotiorum* (Lib.) De Bary. This strain produces different metabolites including pyrrolnitrin, HCN and phenazine derivatives. Phenazine antibiotics plays a role in biofilm formation in this strain (Selin *et al.* 2010) but can paralyse the bacterivorous nematode *Caenorhabditis elegans* (Maupas, 1900) Dougherty, 1955 at higher concentrations (Cezairliyan *et al.* 2013). Nandi *et al.* (2015) showed that HCN and pyrrolnitrin paralyse *C. elegans* at higher concentrations and repel the nematode at lower concentrations. The antibiotics also had other sub-lethal effects on *C. elegans* at lower concentrations. Pyrrolnitrin, HCN and phenazine reduced egg production in *C. elegans* at lower concentrations, and

pyrrolnitrin caused juveniles to take longer to hatch. Future research should focus on elucidating the sub-lethal effects of toxins produced by PGPR.

### 2.7.2 Hydrolytic enzymes and nematophagous activity

Different strains of rhizobacteria have been shown to produce extracellular hydrolytic enzymes that degrade nematode egg shells or cuticles, often in conjunction with nematode-suppressive toxins (Siddiqui *et al*. 2005). *Pseudomonas fluorescens* CHA0 produces an extracellular protease that degrades *M. incognita* J2 and contributes to the *in vitro* activity of this strain against this life stage of this nematode species. Mutants of CHA0 that do not produce the protease had diminished *in vitro* activity against *M. incognita* J2 and eggs and were less effective than the wild-type strain at reducing reproduction of this root-knot nematode species in pot trials (Siddiqui *et al*. 2005).

Lee *et al.* (2013) isolated extracellular proteases from *Lysobacter capsici* YS1215 that degrade *M. incognita* J2. The bacterium was isolated from a chitin-amended soil and had anti-fungal and nematode-suppressive activities. Isolated proteases may degrade J2 relatively slowly: these authors found that 75% of the J2 were degraded after 5 days at 25 °C. Hydrolytic enzymes may be effective in conjunction with other metabolites produced by PGPR and other modes of action expressed by PGPR.

Certain PGPR could act as opportunistic nematophagous organisms, but little information is available on such bacteria. *Streptomyces albireticuli* (previously classified as *Streptoverticillium albireticuli* (Garrity *et al.* 2004)) can produce toxins that immobilise nematodes. This bacterium can colonise nematodes and grow inside the nematodes in a manner comparable to the nematophagous activity of certain fungi, but only after the nematodes have been immobilised by the toxins. *S. albireticuli* can be cultured on different types of media and has antagonistic activities toward soil-borne fungal and oomycete pathogens (Park *et al.* 2002).

### 2.7.3 Priming of plant defences

Different microorganisms, including PGPR and plant growth-promoting fungi, can prime plant defences against phytonematodes, causing improved defence responses upon challenge with pathogens and pests such as *M. incognita.* Most information regarding induced resistance in plants is derived from studies on above-ground pests and pathogens, and relatively little information is available on priming of plant defences against root pathogens (Martínez-Medina *et al.* 2017).

Differences appear to exist between the mechanisms underlying the induction of resistance by plant growth-promoting microorganisms. Martínez-Medina *et al.* (2017) found that the endophytic fungus *Trichoderma harzianum* Rifai strain T-78 reduced or delayed penetration of tomato roots by *M. incognita* J2 through priming of SA-dependent defence responses. The fungus also reduced development and reproduction of the nematode through priming of jasmonic acid-dependent defence responses. Siddiqui & Shaukat (2004), by contrast, found that *P. fluorescens* CHA0 reduced penetration of tomato roots by *M. javanica* J2 through priming of SA-independent defence responses. *Rhizobium etli* strain G12 reduces penetration of tomato roots by *M. incognita* J2, and inhibited development of *M. incognita* in the plant after penetration (Martinuz *et al.* 2013). Strain G12 does not cause increased levels of certain pathogenesis-related proteins in the plant (Reitz *et al.* 2001), possibly indicating that this strain also primes SA-independent defence responses (Van Loon *et al.* 1998).

More information is needed on the priming of plant defences by endospore-forming bacteria (Kloepper *et al.* 2004). *Bacillus* spp. may produce different substances (including lipopeptides and volatile compounds) that can play roles in priming of plant defences. Lipopeptide antibiotics produced by *Bacillus* spp. are highly heterogeneous, and several strains of *Bacillus* spp. can induce resistance against phytonematodes (Kloepper *et al.* 2004; Ongena & Jacques, 2007).

#### 2.7.4 Other modes of action of PGPR

Bacteria could compete with nematodes for nutrients in an indirect manner by inducing changes in the transport of sugars and other substances in the plant (Martinuz *et al.* 2013; Rasmussen *et al.* 2007). The transport of sugars and nitrogenous substances in plant tissues could have significant effects on endophytic organisms, including the endophytic stages of sedentary nematodes (Martinuz *et al.* 2013; Rasmussen *et al.* 2007).

Certain bacteria can form biofilms on the heads of bacterivorous nematodes without parasitizing the nematodes, causing the nematodes to starve (Tan & Darby, 2004). No literature has been published on this phenomenon with rhizobacteria or phytonematodes. The ability of bacteria to colonise nematodes and cause them to starve can be compared to the ability of fungi in the genus *Sphaerobolus* to encapsulate nematodes, discussed in Section 2.4.

Bacteria could also increase the tolerance of plants to certain levels of nematode damage by promoting root growth (Sikora *et al.* 2007). PGPR can promote plant growth through various

mechanisms, including phytohormone production and improvement of plant nutrient acquisition. Phytohormones produced by PGPR may include auxins, cytokinins, gibberellins and abscisic acid (Velivelli *et al.* 2014). Bacteria may aid plants in nutrient acquisition by oxidising sulphur, fixing atmospheric nitrogen or producing different molecules and enzymes that increase the bioavailability of soil nutrients (Velivelli *et al.* 2014). Bacteria may produce higher amounts of certain organic acids under nutrient-limited conditions, leading to increased mineral dissolution rates (Ullman *et al.* 1996). Certain PGPR (including strains of *B. subtilis* and *Streptomyces lydicus*) may enhance the formation and activity of nitrogen-fixing nodules (Tokala *et al.* 2002). Certain rhizobacteria (including members of the genera *Bradyrhizobium*, *Paenibacillus*, *Pseudomonas* and *Streptomyces*) may act as mycorrhiza helper bacteria. These bacteria may promote the growth of mycorrhizal fungi or may suppress fungi that compete against the mycorrhizal fungi. The mycorrhizal fungi in turn enhance the survival of the rhizobacteria (Frey-Klett *et al.* 2007).

Abiotic stress tends to exacerbate the yield losses caused by phytonematodes, as discussed in Section 2.2. PGPR can improve the germination and growth of crops under abiotic stress conditions (such drought, high temperatures and high soil salinity). The mechanisms through which PGPR ameliorate abiotic stress appear to be complex and are not fully understood (Dimpka *et al.* 2009). *Gluconacetobacter diazotrophicus* PAL5 significantly improves the survival of sugarcane plants under drought stress. Vargas *et al.* (2014) found that this bacterium suppresses certain drought stress responses in the plant but causes upregulation of several genes associated with the abscisic acid-dependent pathway. The effects of PGPR on biotic and abiotic stresses of crops may be linked (in certain cases) by biochemical pathways. *Paenibacillus polymyxa*, for example, can induce resistance to both drought stress and soft rot bacteria by means of a SA-dependent pathway (Timmusk & Wagener, 1999). Certain bacteria may promote plant growth and ameliorate abiotic stresses by reducing plant ethylene levels (Glick, 2014).

### 2.8 Challenges and opportunities for research on PGPR

Registration of biopesticides is cheaper and faster than registration of chemical pesticides in certain countries, but the cost of registration may still be prohibitive because of the small markets at which certain biopesticides are aimed (Butt *et al.* 2001). A biocontrol agent with a broad spectrum of activity may therefore be more economically viable than a narrow-spectrum biocontrol agent. Required registration packages tend to differ between countries (Butt *et al.* 2001). Legislation regarding biofertilisers also differs between countries. Some countries lack

legal definitions for biofertilisers, while some other countries have incomplete or incorrect legal definitions for biofertilisers (Malusá & Vassilev, 2014).

Scheepmaker *et al.* (2012) provide detailed information on the evaluation of environmental safety of biopesticides in Canada, the European Union and the United States. The risks of a biopesticide (intended for seed treatment) to non-target rhizosphere organisms, aquatic organisms and seed-eating animals should be assessed. The effects on above-ground organisms should also be assessed if the bacterium occurs as an endophyte in the plant. In Canada and the United States, a tiered approach is allowed during which the maximum hazard concentration is tested on non-target organisms. If no adverse effects are observed at this maximum concentration, no other concentrations need to be tested. Scheepmaker *et al.* (2012) also emphasize that more information is needed on metabolites produced by microorganisms. The European Food Safety Authority Panel on Biological Hazards does not grant Qualified Presumption of Safety (QPS) status to microorganisms that produce bioactive secondary metabolites with possible antibiotic activity (Hald & Baggesen, 2014). Literature reviewed in Section 2.7.1 of this dissertation suggests that a bioactive molecule that acts as an antibiotic at high concentrations may have different functions at the low concentrations found in the rhizosphere (Raaijmakers & Mazzola, 2012). Improved methods are therefore needed to measure the concentrations of secondary metabolites in the rhizosphere.

Plant growth-promoting rhizobacteria may produce variable results in field trials. The shelf life of products containing PGPR may also be limited. Growers only use certain products (such as seeds, inoculants and certain agrochemicals) at certain times in the year, so that stocks of products containing PGPR that remain unsold at the end of a period of peak demand may no longer be usable when the demand for the product peaks again. Both these challenges (variable effectiveness and limited shelf life) could possibly be solved through the development of improved formulations. Another strategy to improve the effectiveness of PGPR is to increase the amount of inoculum added to the soil, for example by increasing the concentration of bacteria in seed treatments (Arora *et al.* 2011).

Information on the factors affecting bacterial activity in the soil could be exploited to improve the effectiveness of PGPR. Soil nutrient levels may affect the recruitment of PGPR by plants. Costa *et al.* (2014) concluded that phytohormone-producing bacteria are more abundant in the rhizosphere, and more likely to occur as endophytes, in nutrient-rich soil. Bacteria that improve mineral acquisition, by contrast, are more prevalent in the rhizosphere in nutrient-poor soils. Costa *et al.* (2014) also found that certain strains of PGPR are able to produce phytohormones and improve nutrient acquisition and may be effective in soils with different levels of fertility. Bacterial endospores germinate in response to the presence of certain

nutrients, and different *Bacillus* spp. may respond to different mixtures of nutrients. Application of these germination-stimulating nutrients could improve the effectiveness of biopesticides containing endospores (Crane *et al.* 2014). Certain signal molecules, including different lipo-chitooligosaccharides and flavonoids, can be used to stimulate the activity of nodule-forming bacteria. These signal molecules improve the consistency and performance of the product and allows soybeans to be planted at lower temperatures. Optimize® (Novozymes BioAg, Milwaukee, Wisconsin) is a biofertiliser containing *B. japonicum* and lipo-chitooligosaccharides (Leggett *et al.* 2017; Novák *et al.* 2002). Certain flavonoids can also stimulate colonisation of non-legume plants by PGPR (Gough *et al.* 1997) and could improve the performance and consistency of biopesticides.

A successful biopesticide should be compatible with fungicides (Brannen & Kenney, 1997; Emmert & Handelsman, 1999). Rhizobacteria differ in compatibility with seed-applied fungicides, such as thiram (Zablotowicz *et al.* 1992). Numerous fungicides inhibit nodule formation by *Bradyrhizobium japonicum* when applied as seed treatments, leading to significantly reduced yields (Campo *et al.* 2009). PGPR with improved fungicide tolerance could be obtained through directional selection. The bacterium of interest could be repeatedly sub-cultured with the fungicide of interest at a concentration that allows some growth of the bacterium (Shapiro-Ilan *et al.* 2002).

Certain endophytes found in crops, including *Rhizobium* spp., cannot currently be cultured using commonly used laboratory media. Thomas & Soly (2009) found that some of these bacteria could be cultured by soaking crushed plant tissue in diluted nutrient broth and then culturing the bacteria using host tissue extract. Little information is available regarding the biotechnological potential of these bacteria. Some actinobacteria that are currently difficult to culture, such as *Saccharopolyspora* spp., could also be promising targets for bioprospecting (Bérdy, 2005).

### 2.9 Conclusion

Numerous studies have demonstrated the potential of PGPR for use in integrated management systems, but the modes of action through which PGPR suppress phytonematodes are not fully understood. Further research is needed to clarify the ecological role of antibiotic production and the amounts of antibiotics produced in the rhizosphere. Study of PGPR may lead to the development of new chemical nematicides or resistant cultivars.

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## CHAPTER 3

## Screening of plant growth-promoting rhizobacteria for biocontrol of *Meloidogyne incognita*

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### 3.1 Abstract

This chapter describes the screening of selected rhizobacteria for *in vitro* compatibility with the nodule-forming bacterium *Bradyrhizobium japonicum* and for biocontrol of *Meloidogyne incognita* on soybean. A dual culture assay was used to screen rhizobacteria for compatibility with *B. japonicum.* Twenty-six of 33 screened isolates were found to be compatible with *B. japonicum.* A seedling bioassay (in a sand-vermiculite mixture as plant growth medium) was used to screen selected rhizobacteria for biocontrol of *M. incognita* on soybean. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens* strain N04 reduced the number of *M. incognita* galls per plant by 31%, 38% and 32%, respectively, during the first round of screening, and by 67%, 32% and 44%, respectively, during the second round. These strains would subsequently be tested in greenhouse trials to determine their efficacy for control of *M. incognita* on soybean and enhancement of soybean growth.

### 3.2 Introduction

Soybean has been the fastest-growing field crop in South Africa in the period of 2007 to 2017, but soybean growers in South Africa are faced by several challenges (Anonymous, 2018). The production of soybean carries more risk than the production of maize or sunflower, due to the relative sensitivity of soybean to adverse environmental conditions and the volatile climatic conditions in South Africa (Anonymous, 2018). Soybean production in South Africa is also currently less profitable than soybean production in Argentina, Brazil and the United States (Anonymous, 2018).

The carbon footprints of agricultural systems (in terms of $CO_2$ emitted per kg product, and in terms of $CO_2$ emitted per hectare) can be reduced by planting legumes (associated with nitrogen-fixing rhizobia) as rotational- or cover crops (Gan *et al.* 2011). Different bacteria, including *Bradyrhizobium* spp., *Mesorhizobium* spp. and *Sinorhizobium* spp., can form nitrogen-fixing nodules on soybean roots (Rodriguez-Navarro *et al.* 2011). Different species of "wild" rhizobia that occur in soils outside Asia can form nodules on soybean roots, but these

bacteria vary in their efficacy. Most soybean growers in Argentina apply rhizobial inoculants, and appear to benefit from this practise, while only 15% of soybean growers in the United States apply inoculants (Leggett *et al.* 2017). Application of biofertilisers containing *Bradyrhizobium japonicum* is recommended by agronomists in South Africa. Field trials have demonstrated that application of *B. japonicum* in South Africa significantly increases yields (Bloem *et al.* 2009).

Root-knot nematodes (*Meloidogyne* Göldi, 1887) impair the water and nutrient uptake of crops and impair nodule formation on legumes. Certain plant growth-promoting rhizobacteria (PGPR) are suitable for control of phytonematodes that parasitize field crops (as part of sustainable management systems). The mechanisms through which PGPR suppress phytonematodes are not fully understood. Some PGPR have synergistic effects with nodule-forming bacteria and may cause increased *B. japonicum* numbers and leghemoglobin contents in nodules (Khan *et al.* 2016; Wilson & Jackson, 2013). Histick® N/T (Becker-Underwood Inc., Ames, Iowa) is an example of a registered biological product that contains both *Bradyrhizobium japonicum* and *Bacillus subtilis* (Schmidt *et al.* 2015).

PGPR can be screened for compatibility with *B. japonicum* using different dual culture assays (Khan *et al.* 2016; Xiao *et al.* 2002). Similarly, different assays can also be used to screen PGPR for biocontrol activity against phytonematodes. *In vitro* screening is useful for specific types of biocontrol agents and specific modes of action. Seedling bioassays with conditions conducive to phytonematode activity allow for the detection of biocontrol agents with plant-mediated effects such as induced resistance. Use of seedling bioassays may eliminate strains that are unable to colonise the roots of the crop of interest (Knudsen *et al.* 1997). Bacterial strains were previously screened for reduction of galls caused by *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 on soybean using a pot trial. Padgham & Sikora (2007) similarly used a seedling bioassay to screen rhizobacteria for reduction of galls caused by *Meloiodgyne graminicola* (Golden & Birchfield, 1965) on rice.

### 3.3 Aims

The first aim of this study was to screen selected rhizobacteria within the PGPR collection of the University of Pretoria (Hassen, 2007; Pretorius, 2012) for compatibility with *B. japonicum* strain WB 74, a strain recommended for application on soybean seeds in South Africa (Bloem *et al.* 2009). The second aim was to screen selected rhizobacteria for control of *M. incognita* on soybean. Promising strains identified in this section would be tested further within this dissertation.

## 3.4 Materials and methods

### 3.4.1 Biological materials

Soybean seeds (cv. LS6248R) were obtained from Mrs Annelie De Beer (Agricultural Research Council – Grain Crops, Potchefstroom: ARC-GCI). This cultivar is highly susceptible to *M. incognita* (Marais *et al.* 2017). The seeds were stored at room temperature in paper bags.

Plant growth-promoting rhizobacterial isolates were obtained from the PGPR culture collection of the Department of Microbiology and Plant Pathology (now the Department of Plant Sciences). The bacteria were stored in 80% glycerol at -20 °C and revived when necessary by streaking on nutrient agar (NA) (BIOLAB Inc., Budapest, Hungary) using the quadrant streak method. The NA plates were then incubated at 37 °C for 1 day. Broth cultures were produced by inoculating 100 mℓ aliquots of sterilised nutrient broth (BIOLAB Inc., Budapest) with bacteria obtained from single colonies. The Erlenmeyer flasks containing the nutrient broth were incubated at 37 °C for 48 hours on a rotary shaker (200 rpm).

*Bradyrhizobium japonicum* strain WB 74 was obtained from the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI), now the ARC - Plant Health Protection (ARC-PHP). The bacterium was cultured using methods described by Sadowsky & Graham (2006). *Bradyrhizobium japonicum* was cultured on yeast extract mannitol (YEM) agar (10 g of mannitol, 0.2g of $MgSO_4{\bullet}7H_2O$, 0.1g of NaCl, 0.5g of $K_2HPO_4$, 0.2 g of $CaCl_2{\bullet}2H_2O$, 0.01g of $FeCl_3$, 1 g of yeast extract, 20 g of bacteriological agar and 1 ℓ of demineralised water). The pH of the medium was adjusted to 6.8 using aqueous HCl prior to the addition of the agar. The YEM agar was amended (after being autoclaved) with bromothymol blue (BTB) to produce a BTB concentration of 25 mg/ℓ medium. *Bradyrhizobium japonicum* produced mucoid colonies ± 2 mm in diameter after 10 days of incubation at 28 °C. YEM agar with BTB is green in colour at room temperature, but blue haloes form around *B. japonicum* colonies (Sadowsky & Graham, 2006). The bacterial culture was stored in 80% glycerol at -20 °C. Broth cultures were produced when needed by inoculating 100 mℓ aliquots of YEM broth (10 g of mannitol, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of NaCl, 0.5 g of $K_2HPO_4$, 0.2 g of $CaCl_2{\cdot}2H_2O$, 0.01g of $FeCl_3$, 1 g of yeast extract and 1 ℓ of demineralised water, adjusted to a pH of 6.8) with bacteria from single colonies. The Erlenmeyer flasks containing the nutrient broth were incubated at 29 °C for 7 days on a rotary shaker (150 rpm).

*Meloidogyne incognita* was provided by Prof. Hendrika Fourie (Unit for Environmental Sciences and Management, North West University, South Africa). Tomato (*Solanum lycopersicum* L.) cv. Moneymaker seeds were surface-sterilised with 3.5% sodium hypochlorite for 30 seconds and allowed to germinate in 2 ℓ pots filled with sterilised vermiculite (coarse vermiculite from Hygrotech SA (Pty) Ltd.) in a greenhouse. Tomato cv. Moneymaker is an ideal host for reproduction of *M. incognita* (Wright *et al.* 1980). Minimum and maximum temperatures were recorded with a minimum-maximum thermometer. Three-week-old seedlings were transplanted to pots filled with potting substrate, comprising 1 part wettened sand (0.95 – 1.1 mm coarse filter sand, Silica Quartz (Pty) Ltd) and 1 part wettened vermiculite (by volume). Plant growth medium had been autoclaved at 121 °C for 1 hour and allowed to cool to room temperature before use. Each tomato plant was inoculated with 1000 *M. incognita* second-stage juveniles (J2). At least 8 weeks after inoculation (when *M. incognita* J2 were required) tomato roots from the source plants in the greenhouse were washed and cut into 1cm pieces. Eggs and J2 were extracted using the adapted method of Riekert (1995). The tomato root pieces were shaken in 1% sodium hypochlorite for 4 minutes. The resulting suspension was then washed through a series of stacked sieves (1000 µm, 75 µm, 25 µm and 10 µm) connected to a vacuum pump. Suction was applied to enhance the passing of the suspension through the sieves. Eggs and J2 were then rinsed from the 10 µm sieve onto the 25 µm sieve, which was incubated in sterile water in a plastic container at 25 °C. Second-stage juveniles were collected daily by pouring the suspension containing the juveniles onto the 10 µm sieve and rinsing the juveniles into a glass beaker. Juveniles that hatched during the first day of incubation were not used and those older than 3 days were never used for experiments. Second stage juveniles were counted before use. The nematodes were kept in suspension (in sterilised tap water) using a magnetic stirrer while six 20 µℓ subsamples were transferred to a counting dish by means of a pipette. Second-stage juveniles were then counted using a stereomicroscope (32 × magnification) and the mean number of J2 per mℓ was calculated.

### 3.4.2 Confirmation of identity of *B. japonicum* using 16S rDNA sequencing

*B. japonicum* from single colonies on YEM agar were suspended in sterile water. The Quick-gDNA™ Miniprep kit (Zymo Research Corp., Irvine, California) was used to extract genomic DNA from bacteria. The first step of genomic DNA extraction was to mix 800 µℓ of genomic lysis buffer with 200 µℓ of bacterial suspension. The mixture was allowed to stand at room temperature for 5 minutes. The lysed bacterial cells were transferred to a Zymo-Spin™ column in a collection tube and centrifuged at 10000*g* for 1 min. The spin column containing the crude

DNA was then transferred to a new collection tube, and a 200 µℓ aliquot of DNA pre-wash buffer was added to the column. This column was then again centrifuged at 10000 *g* for 1 min, after which the DNA was washed with 500 µℓ of g-DNA wash buffer. The DNA was incubated with 100 µℓ of DNA elution buffer for 5 min at room temperature before being centrifuged at 10000 *g* for 1 min. Purified bacterial DNA was stored at -20 °C until the polymerase chain reaction (PCR) step could be performed.

PCR reaction mixtures consisted of 15 µℓ of DNA with 10 µℓ of master mix. The master mix had been prepared by mixing 24.6 µℓ of water, 30 µℓ of My Biotaq™ reaction buffer (Bioline GmbH, London), 1.8 µℓ of each of the primer solutions (universal primers 1492 and 27) and 1.8 µℓ of My Biotaq™ DNA polymerase solution (5U/µℓ). The PCR step consisted of 35 cycles (2 min at 95 °C, 30 s at 94 °C, 45 seconds at 58 °C and 8.5 min at 72 °C) followed by maintenance at 4 °C. The amplified DNA was separated from other DNA molecules using agarose gel electrophoresis. Bands containing the amplified DNA were cut out and frozen at -20 °C until the DNA could be recovered using a Zymoclean™ DNA recovery kit. The agarose gel fragments were incubated with agarose-dissolving buffer at 50 °C until the gel fragments were dissolved. The mixture was added to a Zymo-Spin™ column and the DNA was washed and purified. The amplified DNA was sequenced and analysed by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria) using a 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, California). Sequences were compared to sequences in the National Center for Biotechnology Information (NCBI) nucleotide database using the BLASTN 2.2.28+ program.

### 3.4.3 *In vitro* screening of rhizobacteria for compatibility with *B. japonicum*

Two dual culture assays were conducted. In one assay, the ability of rhizobacteria (from the PGPR culture collection at the University of Pretoria) to inhibit growth of *B. japonicum* was tested. In the second assay, the ability of *B. japonicum* to inhibit growth of rhizobacteria was tested.

The ability of rhizobacteria to inhibit *B. japonicum* growth was tested by spreading 100 µℓ aliquots of *B. japonicum* cell suspensions on YEM agar plates (without BTB) using sterile swabs. *B. japonicum* cell suspensions had been prepared by centrifuging *B. japonicum* broth cultures for 10 min at 6000 *g* and suspending the cells in an equal volume of ¼-strength Ringer's solution. The agar plates were allowed to absorb excess moisture for at least 10 min before filter discs were placed on the agar. Filter discs (5 mm in diameter, cut from Whatman® grade 1 qualitative filter paper) were sterilised and dipped in the respective rhizobacterium broth cultures. Control discs were dipped in sterile nutrient broth. The filter discs were then

dabbed against sterilised paper towels to remove excess liquid. Two filter discs containing nutrient broth were placed on each *B.* japonicum-seeded plate, while one control disc was placed on each plate. The dual cultures were incubated at 28 °C for 5 days, and then checked for inhibition zones around the filter discs. Plates were opened and viewed under a bright light so that inhibition zones were clearly visible.

The ability of *B. japonicum* to inhibit rhizobacterium growth was tested by spreading 100 µℓ aliquots of the respective rhizobacterium cell suspensions on NA plates using sterile swabs. Rhizobacterium cell suspensions had been prepared in a similar manner as *B. japonicum* cell suspensions, and the plates were also allowed to stand for 10 min. Filter discs were dipped in *B. japonicum* broth cultures, while control discs were dipped in sterile YEM broth. Filter discs were placed on the rhizobacterium-seeded NA plates. The plates were then incubated upside down at 28 °C for 2 days and checked for inhibition zones around the filter discs.

Three plates were prepared for each *B. japonicum*-rhizobacterium combination. The diameters of inhibition zones were measured, but the data was treated as qualitative data.

### 3.4.4 Small-scale pilot experiment to assess growth media for seedling bioassay

A two-factor completely randomised design was used for this experiment. The two factors were growth medium and treatment: inoculated with *M. incognita* and uninoculated. Three replicates were prepared for each growth medium-treatment combination.

Plastic pots (450 $cm^3$ capacity) and saucers were rinsed with hot water, allowed to dry and then sterilised with 1 % NaOCl overnight. Pots were then filled with either soil, sand (0.95 – 1.1 mm coarse filter sand, Silica Quartz (Pty) Ltd) or a mixture of one part sand and one part coarse vermiculite (from Hygrotech SA (Pty) Ltd.) (by volume). The soil (obtained from the Unit for Environmental Sciences and Management ,North West University, South Africa) had been tyndallised by wetting the soil to field capacity and placing the soil in a stainless steel container. This container was closed with foil and the soil was pasteurised three times at 85 °C for 6 h. Each pasteurisation step was followed by cooling period of 18 h. The sand and sand-vermiculite mixture had been autoclaved for 1 hour at 121 °C. Soybean seeds (cv. LS6248R) were surface-sterilised for 5 min in 3.5 % NaOCl and washed 5 times with sterilised tap water. Three seeds were planted in each pot at a depth of 1 cm. Pots were maintained in a greenhouse with uniform irrigation (no fertiliser was applied). The seedlings were thinned to one per pot 6 days after planting. Minimum and maximum temperatures were recorded daily. Plants were inoculated with 2000 *M. incognita* J2 8 days after planting (at the emergence of

the first true leaves). Roots of seedlings were exposed (until the uppermost lateral root of each seedling had been reached) using sterilised plastic spoons. The nematode inoculum was kept in suspension using a magnetic stirrer while a 3 mL aliquot containing 2000 J2 was transferred to the root system of each seedling by means of a pipette. Roots were then covered with plant growth medium. Control plants were treated with sterilised water only, containing no J2. After 10 days, plants were harvested. Roots were excised, washed and placed in plastic containers with ± 200 mℓ tap water. Nematode galls were counted using a commercial magnifying glass (magnification 2 ×). Shoots and roots of plants were washed, blotted dry and weighed while fresh.

The data was subjected to analysis of variance (ANOVA). Means were separated using Tukey's studentized range test ($P \leq 0.05$). The GLM procedure (SAS® University Edition, version university.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1) was used.

### 3.4.5 Screening of rhizobacteria for suppression of *M. incognita* (seedling bioassay)

Bacterial strains to be tested were divided into 6 groups. Strains were screened for preventative biocontrol activity using the seedling bioassay tested in Section 3.4.4. Each seed was planted in the centre of a 450 mℓ pot filled with sand-vermiculite mixture, at a depth of 1 cm. Seeds were treated with rhizobacteria or blank ¼-strength Ringer's solution at planting, and 6 replicates were prepared for each treatment. The bacteria were applied by pipetting 10 mℓ of bacterial suspension onto each seed before the seed was covered with sand-vermiculite mixture. The bacterial concentration in the suspension had been adjusted to ca. $10^8$ cells mℓ$^{-1}$ using a haemocytometer.

Plants were inoculated with 2000 *M. incognita* J2, 8 days after planting, and were assessed 17 days after planting. The seedling bioassay was repeated with those bacteria that reduced the number of galls per plant by 30 % (compared to controls treated with blank ¼-strength Ringer's solution). Eight replicates were prepared per treatment during the second round of screening. Plants were exposed to approximately 14 h of light and 10 h of darkness each day. Minimum and maximum temperatures in the greenhouse were recorded using a minimum-maximum thermometer. The air-conditioning system in the greenhouse had been set to 25 °C, but temperatures deviated from this level.

Before analysis, data were tested for normality using the Shapiro-Wilkinson test ($\alpha$=0.01). Stem leaf plots, box plots and normal probability plots were also assessed. The UNIVARIATE procedure (SAS® University Edition, version university.cny.sas.com@sas:university-

3p.2/3p.2.f23fd5825fb4-1-1) was used. The data was then subjected to an analysis of variance (ANOVA) and treatments were compared to controls using one-tailed Dunnett tests ($P \leq 0.05$). Gall numbers were subjected to square root transformation (if necessary) to ensure homogeneity of variances. Outliers were not excluded from analysis in this study. The GLM procedure (SAS® University Edition, version university.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1) was used.

## 3.5 Results

### 3.5.1 Confirmation of identity of *B. japonicum* using 16S rDNA sequencing

Sequencing of a part of the 16S rDNA gene of the bacterial strain obtained from the ARC-PPRI yielded a readable sequence of 671 base pairs. The sequence was similar to that of other partial *B. japonicum* 16S rDNA sequences within the NCBI nucleotide database, including a sequence from *B. japonicum* strain DSM 30131 (accession NR_119191). Hence the identity of the *B. japonicum* strain used in this study was confirmed.

### 3.5.2 *In vitro* screening of rhizobacteria for compatibility with *B. japonicum*

Thirty-three bacterial isolates were screened for compatibility with *B. japonicum* strain WB 74. Seven of the strains are provisionally regarded as incompatible with *B. japonicum* (Table 3.1), until a pot trial is conducted to confirm the incompatibility of the strains with *B. japonicum*. Some of the incompatible strains (S5, S7 and T29) were included in the seedling bioassay to screen bacteria for biocontrol of *M. incognita* on soybean. Strain T29 is able to enhance the growth of maize and wheat (Pretorius, 2012; Rudolph *et al.* 2015), while strain S7 is able to enhance the growth of maize (Rudolph *et al.* 2015).

### 3.5.3 Small-scale pilot experiment to assess growth media for seedling bioassay

Only the sand and the sand-vermiculite mixture were assessed as growth media, because only two seeds planted in the tyndallised soil germinated normally. A soil phytotoxicity test confirmed that tyndallisation caused a reduction in seed germination, compared to untreated soil (results not shown).

Nematode inoculation in sand caused a significant reduction in fresh shoot mass of soybean seedlings, while nematode inoculation in the sand-vermiculite mixture did not cause a significant reduction in fresh shoot mass (Table 3.2). Nematode-inoculated plants in sand had significantly lower fresh root mass than nematode-inoculated plants in the sand-vermiculite mixture. No significant difference was, however, found between the root mass of uninoculated plants in sand and the root mass of uninoculated plants in the sand-vermiculite mixture. The growth media did not have a significant effect on *M. incognita* gall numbers. The sand-vermiculite mixture was selected for the seedling bioassay, because the root mass and gall numbers of plants grown in the sand-vermiculite mixture were less variable than the root mass and gall numbers of plants grown in sand.

### 3.5.4 Screening of rhizobacteria for suppression of *M. incognita* using seedling bioassay

*Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas sp.* strain N04 reduced the number of *M. incognita* galls per plant by 31 %, 38 % and 3 2%, respectively (compared to the control group) during the first round of screening (Table 3.3). These strains were selected for further study because they reduced gall numbers by more than 30% compared to the controls. Only strain T22 caused a statistically significant reduction in gall number during the first round of screening. This strain also caused a significant increase in shoot mass. Certain strains of rhizobacteria failed to grow on NA after being stored in 80% glycerol and could not be screened for biocontrol of *M. incognita.*

The number of replicates was increased from six to eight in the second round of screening. Strains T19, T22 and N04 had statistically significant effects on gall number and reduced the number of galls per plant by 67 %, 32 % and 44 %, respectively (Table 3.4). None of the strains caused a significant increase in plant biomass compared to the controls in the second round of screening. Treatment with the PGPR appeared to cause a reduction in gall size.

## 3.6. Discussion

Crops are most susceptible to plant-parasitic nematode damage during the early stages of plant growth. Bionematicides that are active during the early stages of plant growth may therefore be useful to growers, if used as part of an integrated management system (Sikora *et al.* 2008; Timper, 2014). Biocontrol agents tend to be less effective than traditional chemical nematicides but may be useful if the nematode population is below an economic threshold, or

if the biocontrol agent is combined with a chemical product (Hughes, 1996; O'Callaghan, 2016). Numerous promising biocontrol agents have been identified using *in vitro* and greenhouse experiments, but only a relatively small number of these agents have been found effective under field conditions. A commercially successful biocontrol agent should be compatible with common agricultural practises. Rhizobacteria applied onto the seeds of legumes should therefore be compatible with root-nodulating bacteria, for example (O'Callaghan, 2016).

In the current study, three rhizobacterial strains with activity against *M. incognita* on soybean were identified: *L. sphaericus* strain T19, *P. alvei* strain T22 and *P. fluorescens* strain N04. These strains reduced the number of galls on soybean seedling roots by more than 30 %, while it also appeared to reduce the size of the galls and may be compatible with the root-nodulating bacterium *B. japonicum* (according to an *in vitro* test). These strains may not necessarily be effective, or as effective as it had been in the *in vitro* tests, against *M. incognita* on soybean under field conditions and should, therefore, be evaluated in under prevailing environment conditions. Nematode gall numbers (as assessed in the current study) may be a useful indication of the efficacy of a nematode control practise but cannot be used as a substitute for nematode reproduction data (Dong *et al.* 2007). Results are reported in the following chapter pertaining to the efficacy of the selected rhizobacterial strains in reducing the reproduction of *M. incognita* on soybean. Different formulation and application methods should be evaluated in future studies, as the formulation and application methods used can affect the efficacy of a biocontrol agent (O'Callaghan, 2016).

Most of the rhizobacteria that were screened during this current study for compatibility with *B. japonicum* were found to be compatible. Polonenko *et al.* (1987) tested 18 rhizobacterium isolates for compatibility with *B. japonicum* using a greenhouse pot trial and similarly found that most rhizobacteria did not inhibit nodule formation.

Rhizobacterial strains T19 and N04 did not promote plant growth significantly in the seedling bioassays in the current study, while strain T22 promoted shoot growth in one of two seedling bioassays. Previous studies found that strains T19 and T22 promoted growth of cereals in greenhouse trials (Pretorius, 2012; Rudolph, 2014), while strain T19 promoted the growth of maize in field trials (Breedt, 2015). The rhizobacteria may have failed to cause a significant increase in plant growth due to the short duration of the seedling bioassays. It should be noted that coarse sand mixed with vermiculite, without fertiliser, was used in this study. Strain T19 may be most effective as a biofertiliser at suboptimal fertiliser levels (Breedt, 2015). Experiments would be conducted in the following chapter to test the effect of the selected strains of PGPR on soybean growth in soil with suboptimal fertiliser levels. The activity of the

selected rhizobacteria may also differ between different species of crops (Costa *et al.* 2014; Lawongsa *et al.* 2008).

Different methods should be investigated for the screening PGPR for biocontrol of phytonematodes in future studies. The galls observed on the roots of the soybean seedlings were small and difficult to count, possibly due to the short duration of the trials. Plants were harvested 17 days after nematode inoculation in the seedling bioassays in the current study, while Kloepper *et al.* (1992) recorded gall formation after six weeks. Future studies with the aim of screening PGPR for biocontrol of phytonematodes using an *in planta* assay would therefore require more greenhouse space, and should allow for formation of larger galls. Seedling bioassays have the advantage of including the interactions between the rhizobacterium and the crop (Knudsen, 1997), but *in vitro* methods are less laborious and allow high numbers of strains to be screened within a short time period (Kerry, 2001). Additionally, a greenhouse trial that indicates that a microorganism has biocontrol activity does also not necessarily indicate that the biocontrol agent will be successful under field conditions (O'Callaghan, 2016). Improved *in vitro* assays should therefore be developed to allow the high-throughput screening of biocontrol agents.

The results of this chapter were presented at the 6th International Congress of Nematology (Cape Town, South Africa, May 2014) (Conrad *et al.* 2014).

### 3.7. Conclusion

*Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens* strain N04 are promising PGPR for biocontrol of *M. incognita* on soybean. These strains should be tested further to determine the efficacy of these strains for control of *M. incognita* on soybean and promotion of soybean growth. The modes of action of these strains should also be determined and different formulation and application methods should be investigated. Furthermore, more than one population of *M. incognita* can also be used in future studies since differences in the injuriousness of the species can then also be brought into consideration and the efficacy of PGPR as potential biocontrol agents further optimized.

## 3.8 Tables and Figures

**Table 3.1 – *In vitro* compatibility of rhizobacterium strains with *Bradyrhizobium japonicum* strain WB 74**

| Strain | Compatibility | Strain | Compatibility |
|---|---|---|---|
| A05b | + | S6 | + |
| A07 | + | S7 | - |
| A08 | + | T06 | + |
| A10 | + | T10 | - |
| A16 | + | T11 | + |
| A26Y | + | T13 | + |
| A29 | + | T16 | + |
| A32 | + | T19 | + |
| A40 | + | T20 | + |
| N04 | + | T21 | + |
| N10 | + | T22 | + |
| N19 | + | T23 | + |
| N20 | + | T24 | + |
| N21 | - | T27 | + |
| N25 | - | T29 | - |
| N30 | - | T30 | + |
| S5 | - | | |

Key: '+' indicates that the rhizobacterium strain is compatible with *B. japonicum*, '-' indicates that clear zones were observed during the dual culture test

**Table 3.2 – Effect of different growth media on biomass and gall numbers of uninoculated and *Meloidogyne incognita*-inoculated soybean seedlings (means and standard deviations (SD) are indicated)**

| Growth medium | Treatment | Fresh root mass (g) | Fresh shoot mass (g) | Number of galls per plant |
|---|---|---|---|---|
| Mixture | *M. incognita* | 1.6a (SD 0.2) | 1.1a (SD 0.1) | 234a (SD 41) |
| Mixture | Control | 1.4a (SD 0.2) | 1.1a (SD 0.1) | 0b (SD 0) |
| Sand | *M. incognita* | 0.6b (SD 0.3) | 0.8b (SD 0.1) | 168a (SD 87) |
| Sand | Control | 1.1ab (SD 0.4) | 1.1a (SD 0.1) | 0b (SD 0) |

Controls consisted of plants treated with sterilised water. In each column, the means followed by the same letter are not significantly different according to Tukey's test at α = 0.05. Number of replicates (N) = 3.

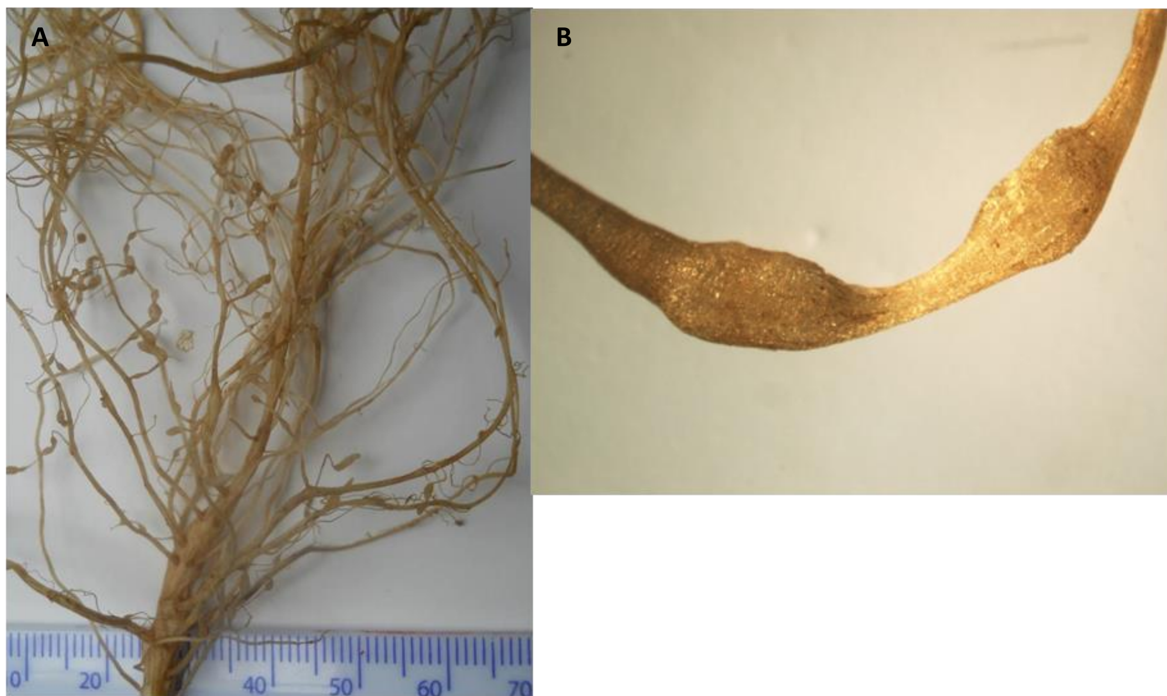


**Figure 3.1 – Root systems of soybean seedlings infected with *Meloidogyne incognita* showing galling at time of assessment during the seedling bioassay.**

A: Soybean root system with *M. incognita* galls (ruler marked in mm for scale).

B: Galls caused by *M. incognita* infected viewed under microscope (20 × magnification).

**Table 3.3 – Effects of rhizobacteria on biomass and gall numbersof uninoculated and *M. incognita*-inoculated soybean seedlings (means ± 1 standard error of the mean (SE) are displayed).**

| Treatment | N[1] | Fresh root mass (g) | Fresh shoot mass (g) | Number of galls per plant | % Decrease in gall number[2] |
|---|---|---|---|---|---|
| **Group 1** | | | | | |
| Control | 5 | 1.7 ± 0.1 | 1.6 ± 0.1 | 473 ± 46 | |
| A07 | 4 | 1.6 ± 0.2 | 1.5 ± 0.1 | 386 ± 45 | 18% |
| A10 | 5 | 1.6 ± 0.1 | 1.7 ± 0.1 | 385 ± 42 | 19% |
| N04 | 5 | 1.3 ± 0.3 | 1.5 ± 0.2 | 320 ± 91 | 32% |
| T19 | 4 | 1.6 ± 0.1 | 1.6 ± 0.2 | 327 ± 16 | 31% |
| T29 | 6 | 1.6 ± 0.1 | 1.5 ± 0.1 | 378 ± 28 | 20% |
| **Group 2** | | | | | |
| Control | 6 | 2.0 ± 0.3 | 1.8 ± 0.2 | 241 ± 37 | |
| A32 | 6 | 2.1 ± 0.1 | 2.0 ± 0.1 | 242 ± 13 | 0% |
| A40 | 6 | 1.9 ± 0.4 | 1.9 ± 0.2 | 210 ± 42 | 13% |
| T21 | 5 | 1.8 ± 0.2 | 1.7 ± 0.2 | 211 ± 27 | 12% |
| T27 | 6 | 1.9 ± 0.2 | 1.9 ± 0.2 | 229 ± 16 | 5% |
| **Group 3** | | | | | |
| Control | 6 | 2.1 ± 0.2 | 1.7 ± 0.1 | 414 ± 49 | |
| A05b | 7 | 2.1 ± 0.1 | 1.8 ± 0.1 | 451 ± 29 | -9% |
| A08 | 5 | 2.0 ± 0.3 | 1.7 ± 0.2 | 334 ± 71 | 19% |
| A26Y | 5 | 1.7 ± 0.4 | 1.6 ± 0.2 | 317 ± 60 | 23% |
| **Group 4** | | | | | |
| Control | 6 | 2.1 ± 0.3 | 1.6 ± 0.1 | 297 ± 25 | |
| A29 | 6 | 2.2 ± 0.3 | 1.8 ± 0.1 | 230 ± 27 | 23% |
| N19 | 5 | 2.3 ±0.1 | 2.0 ± 0.2 | 262 ± 15 | 12% |
| N20 | 5 | 2.4 ± 0.2 | 2.2 ± 0.2* | 329 ± 41 | -11% |
| T06 | 6 | 2.4 ± 0.1 | 2.1 ± 0.1 | 289 ± 32 | 3% |
| T22 | 6 | 2.3 ± 0.2 | 2.4 ± 0.1* | 184 ± 29* | 38% |
| **Group 5** | | | | | |
| Control | 3 | 2.0 ± 0.4 | 2.0 ± 0.4 | 260 ± 40 | |
| T11 | 2 | 1.7 ± 0.4 | 1.5 ± 0.5 | 360 ± 60 | -38% |
| T18 | 3 | 2.0 ± 0.3 | 1.7 ± 0.1 | 447 ± 32 | -72% |
| T30 | 3 | 1.4 ± 0.1 | 1.3 ± 0.2 | 327 ± 64 | -26% |
| **Group 6** | | | | | |
| Control | 5 | 2.1 ± 0.1 | 1.5 ± 0.1 | 337 ± 15 | |
| A01 | 6 | 2.2 ± 0.1 | 1.7 ± 0.1 | 362 ± 10 | -7% |
| S5 | 6 | 2.3 ± 0.1 | 1.6 ± 0 | 330 ± 8 | 2% |
| S7 | 5 | 1.7 ± 0.2 | 1.2 ± 0.1 | 289 ± 35 | 14% |

*Table continues onto following page.*

Controls consisted of plants treated with blank ¼-strength Ringer's solution. Greenhouse air temperature varied between 18 (min) and 37 °C (max) during groups 1 and 2, 18 (min) and 36 °C (max) during group 3, 19 (min) and 38 °C (max) during group 4 and 18 (min) and 39 °C (max) during groups 5 and 6.

[1]N = Number of replicates

[2] Percentage decrease = 100 × (control – treatment) / control

[*] Means of root and shoot masses followed by a star (*) are significantly higher than the control mean within a group, while means of gall numbers followed by a star are significantly lower than the control mean within a group. One-tailed Dunnett t-tests were used at α = 0.05.

**Table 3.4 – Effects of rhizobacteria on biomass and gall numbers of uninoculated and *Meloidogyne incognita*-inoculated soybean seedlings for a second screening (means ± SE are displayed).**

| Treatment | N[1] | Fresh root mass (g) | Fresh shoot mass (g) | Number of galls per plant[2] | % Decrease in gall number[3] |
|---|---|---|---|---|---|
| Infected control | 8 | 1.3 ± 0.1 | 1.4 ± 0.1 | 98 ± 7 (10 ± 0) | |
| N04 | 7 | 1.4 ± 0.1 | 1.4 ± 0.1 | 55 ± 10 (7 ± 1)* | 44% |
| T19 | 6 | 1.3 ± 0.1 | 1.2 ± 0 | 33 ± 7 (6 ± 1)* | 66% |
| T22 | 7 | 1.4 ± 0.1 | 1.4 ± 0.1 | 66 ± 5 (8 ± 0)* | 32% |
| Uninfected | 6 | 1.0 ± 0.2 | 1.1 ± 0.1 | 0 ± 0 (0 ± 0)* | 100% |

[1]N = Number of replicates.

[2]The number of galls per plant was subjected to square root transformation prior to analysis. Means and standard errors of raw data are displayed, followed by brackets containing means and standard errors of transformed data.

[3]Percentage decrease = 100 × (control – treatment) / control (a negative number therefore indicates an increase)

[*]Means of root and shoot masses followed by a star (*) are significantly higher than the control mean, while means of gall numbers followed by a star are significantly lower than the control mean. One-tailed Dunnett t-tests were used at $\alpha = 0.05$.

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## CHAPTER 4

## Biocontrol of *Meloidogyne incognita* on soybean, and promotion of soybean growth, by selected rhizobacteria in the greenhouse


### 4.1 Abstract

Plant growth-promoting rhizobacteria (PGPR) may be useful for amelioration of biotic and abiotic plant stress within integrated management systems. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens* strain N04 were tested in greenhouse trials for control of *M. incognita* on soybean. Strain T19 (applied as a Perlite powder seed treatment) produced the most consistent control of *M. incognita* on soybean. This strain reduced the number of egg masses on roots of soybean plants by 64 % and 86 %, respectively, in two experiments (compared to nematode-infected controls that were not treated with these PGPR). Separate greenhouse trials were also conducted to test the effects of the selected strains on soybean growth under nutrient-limited conditions. Strains T19 and T22, applied as Perlite powder seed treatments, increased dry shoot mass of plants by 84% and 124%, respectively, and leaf area by 84 % and 124 %, respectively, in experiments to test plant growth enhancement (the results of two experiments were pooled). The selected rhizobacteria did not promote soybean growth in quartzite sand with hydroponic fertiliser.


### 4.2 Introduction

Humans currently depend on both technology and ecosystem services for survival. Modern, high-input agriculture has allowed growth of the human population and has contributed to improved quality of life but has simultaneously contributed to degradation of different ecosystems. Sustainable agriculture should include rational application of agricultural remedies, and efficient use of water and chemical fertilisers (Tilman *et al.* 2002). Biological control may (in theory) facilitate reduced use of pesticides, while biofertilisers may facilitate reduced use of chemical fertilisers. Certain types of biological control have successfully been integrated into modern agriculture. Biological control has high potential for wider use within the near future, while biofertilisers have moderate potential for wider use (Wezel *et al.* 2014).

Certain strains of plant growth-promoting rhizobacteria (PGPR) ameliorate biotic and/or abiotic plant stresses and can be used as active microorganisms within various types of biological products. Biofertilisers improve nutrient acquisition by crops through nitrogen fixation and/or

nutrient solubilisation. Phytostimulators increase plant growth through phytohormone production (Reddy, 2014a). Rhizobacteria can suppress plant diseases, as well as nematode and arthropod population densities through different mechanisms, and may enhance the activity of other beneficial microorganisms (such as arbuscular mycorrhizal fungi). Genetic material from PGPR could be used to produce transgenic crops with increased resistance to plant stresses (Reddy, 2014a). The efficacy of different strains of PGPR is determined in part by the survival of the PGPR during seed treatment and the choice of inoculant formulation used (De Gregorio *et al.* 2017). The amount (dosage) of rhizobacteria applied and the amount of mineral nutrients in the soil may also influence the efficacy of different strains of PGPR (Breedt, 2015).

Soybean and other legumes are important sources of protein for humans and livestock. Consumption of soy protein instead of animal proteins would reduce the environmental impact of food production (Reijnders & Soret, 2003). *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 is the most important plant-feeding nematode that parasitize and damage soybean (and most other legumes and oil-rich seed crops) grown in South Africa. No nematicides are registered for use on soybeans (and certain other legumes) in South Africa (Van Zyl, 2016) and the cost of nematicide application may be prohibitive. The majority of widely grown soybean cultivars in South Africa lack resistance to *M. incognita* (Fourie *et al.* 2017). *Meloidogyne incognita* is also a significant cause of yield losses on crops grown in rotation with soybean in South Africa, such as maize (Mc Donald *et al.* 2017). Numerous studies have been published that report the application of PGPR for control of phytonematodes. Most of these studies have focused on root-knot nematodes (Genus *Meloidogyne*, Göldi, 1889) (Reddy, 2014b).

Different criteria have been used in experiments used to measure the success of nematode management practices. Different gall rating, gall index and egg mass index systems have been published (Dias *et al.* 2016; Hussey & Boerma, 1981; Kloepper *et al.* 1992). Genetic resistance to phytonematodes is usually measured using nematode reproduction data (Fourie *et al.* 2017). Certain studies have found that root-knot nematode reproduction is directly proportional to gall indices on different cultivars (Faske, 2013; Hussey & Boerma, 1981). Other studies have found cultivars with low gall indices exhibited high reproduction factors, indicating that different measures of nematode infection should be used in combination to assess nematode management practices (Fourie *et al.* 2015). Measures used to test the efficacy of PGPR against phytonematodes include nematode penetration, nematode reproduction, as well as gall and egg mass numbers. Plant growth-promoting rhizobacteria may also increase the tolerance of crops to nematode damage (Sikora *et al.* 2007).

## 4.3 Aims

The first aim of this study was to test rhizobacteria, selected according to results obtained in the previous part of this study (third chapter), for biocontrol of *M. incognita* on soybean in greenhouse trials. The second aim of this study was to test the selected rhizobacteria for promotion of soybean growth in greenhouse trials.

## 4.4 Materials and methods

### 4.4.1 Biological materials

Soybean seeds (cv. LS6248R) were obtained from Mrs Annelie De Beer (Agricultural Research Council – Grain Crops Institute, Potchefstroom: ARC-GCI). The seeds were stored at room temperature in paper bags and surface-sterilised when needed as described in Section 3.4.4 of this study. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens* strain N04 were stored in Microbank™ beads (Pro-Lab Diagnostics Inc., Richmond Hill, Canada) and revived when necessary by streaking the bacteria on nutrient agar (BIOLAB Inc., Budapest, Hungary) using the quadrant streak method. Broth cultures were produced as described in Section 3.4.1 of this dissertation. *Meloidogyne incognita* second-stage juveniles (J2) were also obtained and quantified as described in Section 3.4.1 of this study.

### 4.4.2 Pilot experiment: biocontrol of *M. incognita* on soybean with bacterial suspensions (in growth chamber)

A completely randomised design was used, with 8 replicates per treatment. The treatments were represented by strains *L. sphaericus* strain T19, *P. alvei* strain T22 and *P. fluorescens* strain N04, as well as *M. incognita*-infected controls and uninfected controls. Plastic pots (1.4 $dm^3$ capacity) and saucers were rinsed with hot water, allowed to dry and then sterilised with 1% sodium hypochlorite (NaOCl) overnight. Pots were then filled with playpen quartzite sand (F. H. Chamberlain (Pty) Ltd, Pretoria, South Africa) that had been autoclaved for 1 h at 121 °C. One soybean seed was planted in each pot at a depth of 1 cm. Seeds were treated with rhizobacteria suspended in quarter-strength Ringer's solution as described in Section 3.4.5 of this dissertation. The rhizobacteria were applied by pipetting 10 mℓ of bacterial suspension, containing $10^8$ cells $ml^{-1}$, onto each seed before the seed was covered with sand-

vermiculite mixture. Controls (those that would be inoculated with *M. incognita* J2, and those that would be treated with water without J2) were treated with blank quarter-strength Ringer's solution. Pots were maintained in a growth chamber (at Phytotron D at the experimental farm of the University of Pretoria). Temperatures in the growth chamber varied between 23 and 25 °C according to a thermograph (25 °C is an optimal temperature for *M. incognita* (Taylor & Sasser, 1978). Plants were exposed to 14 h of illumination and 10 h of darkness each day.

Plants were inoculated with 2000 *M. incognita* J2 2 weeks after planting using the method described in Section 3.4.4 of this dissertation. Control plants were inoculated with sterilised water. Pots were irrigated daily by adding 100 mℓ of sterilised tap water to the saucers. A fertiliser mixture (Table 4.1) was applied once per week (starting from one week after nematode inoculation) with 50 mℓ of Nutrifeed® (Starke Ayres (Pty) Ltd), Bredell, South Africa) at a rate of 1 g/ℓ sterilised tap water. Only 50 mℓ of irrigation water was applied on the days that fertiliser was applied.

Seven weeks after nematode inoculation, plants were harvested. Roots were excised, washed free of sand and then stained for 20 min in 0.015 % phloxine B (Merck KGaA, Darmstadt, Germany). Stained egg masses were counted using a commercial magnifying glass (magnification 2 ×). Eggs and J2 were then extracted using the method of Riekert (1995) and counted using a stereomicroscope (magnification 32 ×). Shoots and roots of plants were placed in a drying oven at 50 °C for 3 days and then weighed.

The nematode reproduction factor (Rf) was calculated using the formula Rf = (Pf + 1)/(Pi +1), where Pf is the number of eggs and J2 extracted at the end of the experiment and Pi is the initial amount of J2 used as inoculum (Wang *et al.* 2000). Egg laying females (ELF) index A was on a scale of 0 to 5, with 0 = 0 egg masses, 1 = 1 to2 egg masses, 2 = 3to 10 egg masses, 3 = 11 to30 egg masses,4 = 31 to 100 egg masses and 5 = more than 100 egg masses. Egg laying females index B was also on a scale of 0 to 5, but with 0 = 0 egg masses, 1 = 1 to 50 egg masses, 2 = 51 to 125 egg masses, 3 = 126 to 225 egg masses, 4 = 226 to 350 egg masses and 5 = more than 350 egg masses (Hussey & Boerma, 1981).

### 4.4.3 Pilot experiment: promotion of soybean growth (with bacterial suspensions)

A completely randomised design was used similar that in Section 4.4.2. Plastic pots and saucers were sterilised and then filled with playpen quartzite sand that had been autoclaved. One soybean seed was planted in each pot at a depth of 1 cm and then treated with 10 mℓ of bacterial suspension, containing $10^8$ cells $ml^{-1}$. Controls (without rhizobacteria) were treated

with blank quarter-strength Ringer's solution. Pots were maintained in the greenhouse. Irrigation water and fertiliser solution were applied as described in Section 4.4.2. Minimum and maximum temperatures in the greenhouse were recorded using a minimum maximum thermometer. The air-conditioning system in the greenhouse had been set to 25 °C, but temperatures deviated from this level. Shoots and roots of plants were excised, dried (as described in Section 4.4.2) and weighed. The experiment was repeated once.

### 4.4.4 Pilot experiment: biocontrol of *M. incognita* with different rhizobacterium formulations

A two-factor completely randomised block design was used. The two factors were i) formulation and ii) treatment, with 6 replicates per formulation-treatment combination. Three formulations were tested: A Perlite powder formulation (applied as a seed treatment), a hydroxyethyl cellulose gel formulation (applied as a seed treatment) and a drench treatment (with bacterial broth cultures prepared with nutrient broth). Controls with and without *M. incognita* were included.

The fluid gel inoculum was prepared using a method derived from Jawson *et al.* (1989). Hydroxyethyl cellulose 60000 (Unichem Services (Pty) Ltd, Durban, South Africa) was autoclaved and then stirred into aliquots of stationary phase bacterial cultures (at a rate of 1 g polymer / 100 mℓ bacterial broth culture). Bacterial broth cultures had been prepared using nutrient broth as described in Section 3.4.1 of this dissertation. The polymer-broth culture mixtures were incubated for 6 h at 37 °C in a rotary shaker (200 rpm) to allow stable gels to form. Soybean seeds were then coated with the gels (using 1 mℓ of gel per seed) before being transferred to pots using sterilised spatulas. Controls (with and without *M. incognita*) consisted of seeds treated with a hydroxyethyl cellulose gel prepared with sterilised nutrient broth.

The powder inoculum was prepared by injecting bags, each containing approximately 200 g of sterile perlite powder (Stimuplant CC, Pretoria, South Africa), with 21 mℓ of stationary phase bacterial broth culture. The bags were then sealed, kneaded until the powder was thoroughly mixed, and incubated for two weeks at room temperature. A packet of Stimulym® (Stimuplant CC, Pretoria, South Africa), containing 1.5 g of adhesive in powder form, was stirred into 150 mℓ of sterilised, demineralised water and incubated overnight in a shaking incubator at 37 °C (200 rpm). Soybean seeds were coated with the resulting Stimulym® adhesive (at a rate of 1 mL adhesive per seed, the same rate used for application of fluid gels on soybean seed by Jawson *et al.* (1989). Adhesive-coated seeds were then transferred to plastic bags and subsequently coated with the Perlite powder inoculum of the respective bacterial strains at a

rate of 20 g powder/kg seed. The powder-coated seeds were then left to dry in a laminar flow cabinet for 6 hours before being planted. Controls consisted of seed treated with perlite powder that had been injected with sterile nutrient broth (without rhizobacteria).

Plastic pots (1.4 $dm^3$ capacity) and saucers were sterilised. The pots were then filled with playpen quartzite sand (Massbuild, division of Massmart Holdings Ltd, Sandton, South Africa) that had been autoclaved for 1 h at 121 °C. Three soybean seeds were planted per pot for the seed treatment (with a powder formulation) and the seed priming treatment (with a gel formulation). One seed was planted per pot for the drench treatment, which entailed the application of 10 mℓ bacterial broth culture (prepared as described in Section 3.4.1 of this dissertation) per seed, by means of a pipette, before covering the seed with sand. All seeds were planted at a depth of 1 cm.

Plants were thinned to 1 plant per pot 2 weeks after planting. Plants were then inoculated with 1000 *M. incognita* J2 using the method described in Section 3.4.4 of this dissertation. Control plants were treated with sterilised water. Pots were irrigated with 100 mℓ of sterilised tap water daily and were fertilised four times per week with 20 mℓ of Nutrifeed® (Starke Ayres (Pty) Ltd), Bredell, South Africa) at a rate of 1 g/ℓ irrigation water and fertiliser solution was added to saucers as in previous experiments. Minimum and maximum temperatures in the greenhouse were recorded using a minimum-maximum thermometer. The air-conditioning system in the greenhouse had been set to 25 °C, but temperatures deviated from this level.

Plants were placed harvested 9 weeks after nematode inoculation. Enumeration of *M. incognita* females was attempted during this pilot experiment by using a modification of the bromothymol blue method (for staining endoparasitic nematodes) (Kirkpatrick & Mai, 1957). A sample of roots weighing 5 g was taken from each plant. The sample was bleached using 7 % NaOCL solution for 10 minutes. The root pieces were then washed with 50% ethanol. Each sample was then soaked in 20 mℓ of 50 % ethanol with 0.2 % bromothymol blue. Gram stain solution 5 was added to each sample as a counter-stain (at a rate of 1 uℓ / mℓ bromothymol blue solution). Roots were incubated in the stain solution for at least 2 weeks at room temperature. Stained root pieces were then transferred to 50 % ethanol with 0.2 % acetic acid. Nematodes were extracted from root samples by macerating the root piecesin 50 % ethanol with 0.2 % acetic acid, using a commercial blender. Mature females had been stained green and were counted within aliquots using a stereomicroscope. *Meloidogyne incognita* females remained intact after extraction from certain samples but were ruptured after extraction from other samples. Staining, extraction and counting of nematode females also proved to be more laborious than the staining and counting of egg masses on galled root surfaces. Gall and egg mass counts would therefore be used to measure the efficacy of rhizobacteria as biocontrol

agents within the following experiments being done during the current study. Nematode reproduction factors as well as root and shoot dry masses were determined as described in Section 4.4.2.

### 4.4.5 Greenhouse trial to determine the efficacy of selected PGPR for biocontrol of *M. incognita* on soybean

A two-factor completely randomised block design was used. The two factors were i) experiments and ii) treatments, with blocks nested within experiments and 6 replicates per treatment per experiment. Treatments included strains N04, T19 and T22, as well as infected controls (that would be inoculated with *M. incognita*) and uninfected controls.

Plastic seedling trays (20.5 cm × 14.5 cm × 5.5 cm), each with nine wells, were washed with detergent, rinsed with tap water, allowed to dry, sterilised with 90 % ethanol and again allowed to dry. Woven propylene cloth rectangles, secured over the bottoms with masking tape, was used to prevent the loss of planting medium from the seedling trays whilst allowing drainage. The seedling trays were filled with coarse vermiculite (Hygrotech SA (Pty) Ltd., Pyramid, South Africa) that had been autoclaved for 1 h at 121 °C. Soybean seeds were treated with Perlite powder inoculum of the respective rhizobacterial strains (as described in Section 4.4.4) and planted in the vermiculite-filled trays. The seeds were allowed to germinate in the greenhouse. Each well was irrigated with 5 mℓ of sterilised tap water daily.

Plastic pots (1.4 $dm^3$ capacity) and saucers were sterilised. The pots were then filled with playpen quartzite sand (0.3 mm, Silica Quartz (Pty) Ltd) that had been autoclaved for 1 h at 121 °C. Two weeks after the seeds had been planted in the seedling trays, the seedlings were transplanted to the pots. One seedling was planted per pot. Plants were then inoculated with 1000 *M. incognita* J2 using the method described in Section 3.4.4 of this study. Pots were irrigated daily by adding 50 mℓ sterilised tap water to each saucer. Three times per week, an additional 50 mℓ of water was sprinkled onto the surface of each pot to prevent surface crust formation. Pots were fertilised four times per week with 20 mℓ of Nutrifeed® (Starke Ayres (Pty) Ltd), Bredell, South Africa) at a rate of 1 g/ℓ sterilised tap water. Minimum and maximum temperatures in the greenhouse were recorded using a minimum-maximum thermometer. The air-conditioning system in the greenhouse had been set to 25 °C, but temperatures deviated from this level.

The chlorophyll content index (CCI) of each plant was measured seven weeks after nematode inoculation with a CCM-200 chlorophyll meter (Opti-Sciences, Inc.). Chlorophyll content index

measurements were obtained between 10h00 and 12h00, from the centres of the three uppermost mature leaflets of each plant, near the midribs of the leaflets. The mean CCI of each plant was then calculated. Plants were harvested 1 day after the chlorophyll content was measured. Roots were excised and washed free of sand. *Meloidogyne incognita* egg masses on root surfaces were stained with phloxine B and counted as described in Section 4.4.2, while galls were counted using a magnifying glass (2× magnification). Gall index A, ELF index A, gall index B and ELF index B was rated using the methods of Hussey and Boerma (1981). The dry masses of roots and shoots were also measured as described in Section 4.4.2.

### 4.4.6 Greenhouse trial to determine the efficacy of selected PGPR for promotion of soybean growth in soil with suboptimal (reduced) levels of fertiliser

A two-factor completely randomised block design was used as in Section 4.4.5. Rhizobacterial strains N04, T19 and T22 were tested Controls without rhizobacteria were included. Seeds were treated with Perlite powder inoculum and planted in vermiculite-filled seedling trays as described in Section 4.4.4. Plastic pots (1.4 $dm^3$ capacity) and saucers were sterilised. The pots were then filled with Huttons type soil (used within a PGPR trial with different fertiliser levels by Breedt (2015)) that had been pasteurised for 1 h at 85 °C. The soil consisted of 60.4 % sand, 26.9 % silt and 26.9 % clay. The chemical properties of the soil were as follows: pH 6, phosphorous content 11.7 mg/kg, calcium content 1493 mg/kg, potassium content 339 mg/kg, magnesium content 288 mg/kg. The soil had been amended with 12% single superphosphate (at a rate equivalent to 25 mg phosphate / kg soil) and ammonium nitrate (0.47 mℓ of 0.6 % stock solution / kg soil) after pasteurisation. The fertiliser amendments were equivalent to a phosphate application of 75 mg/ha and a nitrogen application of 140 mg/ha (Breedt, 2015). The superphosphate had been obtained from Omnia (Bryanston, South Africa). Two weeks after the seeds had been planted in the seedling trays, the seedlings were transplanted to the pots. One seedling was planted per pot. No *Bradyrhizobium japonicum* inoculant was added to the soil, so that the bioavailability of nitrogen was limiting to plant growth. Pots were irrigated daily by adding 50 mℓ sterilised tap water to each saucer. Three times per week, an additional 50 mℓ of water was sprinkled onto the surface of each pot to prevent surface crust formation. Minimum and maximum temperatures in the greenhouse were recorded using a minimum-maximum thermometer. The air-conditioning system in the greenhouse had been set to 25 °C, but temperatures deviated from this level.

The CCI of each plant was measured seven weeks after nematode inoculation as described in Section 4.4.4. The following day, the plants were harvested. A LI-3100 area meter (Li-Cor, Inc., Lincoln, Nebraska) was used to measure the total leaf area of each plant (in $cm^2$). The

roots were then excised, and the dry masses of roots and shoots were measured as described in Section 4.4.2.

#### 4.4.7 Statistical analysis of greenhouse trial results

Data were tested for normality using the Shapiro-Wilkinson test ($\alpha = 0.01$). Stem leaf plots, box plots and normal probability plots were also assessed. Extreme outliers were detected by computing the internally studentized residuals of the data and were excluded from analyses in this chapter if necessary. The UNIVARIATE procedure (SAS® University Edition, version university.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1) was used. Levene's test for homogeneity of variances ($\alpha$=0.01) was used to compare the variances between experiments before deciding whether to pool the data from different experiments. The data was subjected to an analysis of variance (ANOVA) and treatment groups were compared using Tukey's tests ($P \leq 0.05$). Gall and egg mass numbers as well as reproduction factors were subjected to square root transformation, if necessary, to ensure homogeneity of variances. The GLM procedure (SAS® University Edition, version university.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1) was used.

#### 4.4.8 Degradation of hydroxyethyl cellulose, used for gel seed treatment in experiment 4.4.4, by selected rhizobacteria

Selected rhizobacteria were tested for degradation of hydroxyethyl cellulose using a method similar to that described by Cazemier *et al.* (1997). Agar plates were prepared using the following recipe: $K_2HPO4$, 1.9 g/ℓ; $KH_2PO4$, 0.94 g/ℓ; KCl, 1.6 g/ℓ; NaCl, 1.43 g/ℓ; $NH_4Cl$, 0.15 g/ℓ; $MgSO_4·7H_2O$, 0.037 g/ℓ; $CaCl_2·2H_2O$, 0.017 g/ℓ; yeast extract (free of fermentable carbohydrates), 0.1 g/ℓ; hydroxyethyl cellulose 2 g/ℓ; Noble agar 15 g/ℓ; pH adjusted to 7.2. Each plate was poured using 17 mℓ of molten medium. Agar plates were inoculated with rhizobacteria using the quadrant streak method and incubated for 1 week at 25 °C. Plates were then stained using the method of Teather & Wood (1982). The surfaces of plates were flooded with 0.1% Congo red (Univar®, Saarchem (Pty) Ltd, Krugersdorp, South Africa) for 15 min before being flooded with 5.8 % NaCl. Plates were then checked for presence of clear zones around bacterial colonies, which is indicative of degradation of hydroxyethyl cellulose.

### 4.4.9 Analysis of seed germination during experiments 4.4.5 and 4.4.6.

Germination of seeds planted in vermiculite-filled trays (in preparation for experiments 4.4.5 and 4.4.6) was recorded. Assessment of seed germination had not been pre-planned, and experiments were therefore not designed using International Seed Testing Asssociation guidelines. The germination data was tested for normality and for homogeneity of variances as described in section 4.4.7. Treatments were then compared using Scheffe's test (α = 0.05) (The GLM procedure, SAS® University Edition, version university.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1). Scheffe's test is the most conservative test available for multiple comparisons and is suitable for unplanned data exploration (Ruxton & Beauchamp, 2008).

## 4.5 Results

### 4.5.1 Pilot experiment: biocontrol of *M. incognita* with bacterial suspensions (in growth chamber)

Soil drenches with *L. sphaericus* strain T19 and *P. fluorescens* strain N04 suspended in quarter-strength Ringer's solution significantly (P ≤ 0.05) reduced the reproduction of *M. incognita* by 67 % and 51 %, respectively, compared to nematode-infected controls. These reductions were statistically significant (Table 4.2). Application of strains T19 and N04 did, however, not reduce the ELF indices (A and B) or the number of egg masses significantly in this pilot experiment. Soil drenches with *P. alvei* strain T22 strain T22 did not reduce any *M. incognita*parameters used in this study.

Inoculation with *M. incognita* reduced the dry shoot and dry root masses of plants significantly (P ≤ 0.05) by 27 % and 56 %, respectively, compared to controls without nematodes. The rhizobacteria tested in this pilot trial did not increase shoot mass or root mass of soybean seedlings significantly, compared to *M. incognita*-infected controls without rhizobacteria.

### 4.5.2 Pilot experiments: promotion of soybean growth (with bacterial suspensions)

No significant interactions between the effects of experiments and treatments were observed (P ≤ 0.05). Levene's test for homogeneity of variances (α = 0.01) indicated that the variances of the two experiments were significantly different. Data from the two experiments was

therefore analysed separately. The selected strains of rhizobacteria did not significantly affect root dry mass or shoot dry mass in the two greenhouse experiments (Table 4.3). Sterilised quartzite sand had been used as growth medium. It should be noted that water-soluble fertiliser (containing nitrogen, phosphates, potassium and micronutrients) had been applied in these experiments. Variation in greenhouse conditions could possibly account for some of the differences in plant biomass between the two experiments.

### 4.5.3 Pilot experiment: biocontrol of *M. incognita* with different rhizobacterium formulations

Two formulations were assessed in this experiment, namely a hydroxyethyl cellulose gel seed treatment and a perlite powder seed treatment. The soil drench treatment could not be assessed due to poor seed germination. A significant ($P = 0.0074$; F-ratio = 4.22) interaction was observed between the effects of formulations and the effects of treatments on *M. incognita* reproduction ($P = 0.0074$). Only one formulation-treatment combination caused a significant ($P \leq 0.05$) decrease in nematode reproduction compared to its corresponding nematode-infected control group, namely the Perlite powder seed treatment with strain T19 (Table 4.4). Strain T19, applied as a powder seed treatment, reduced reproduction of *M. incognita* by 69 %. The Perlite powder treatment was therefore selected for further greenhouse experiments.

No formulation-treatment combination caused significant increases in shoot or root dry mass, compared to corresponding nematode-infected controls (Table 4.4). This finding mirrors that of Section 4.4.3. Nematode-infected controls had significantly ($P \leq 0.05$) lower mean shoot dry mass than non-infected controls. The shoot dry mass of nematode-infected controls (subjected to gel treatment) was 42 % lower than that of the corresponding uninfected control, while the shoot dry mass of nematode-infected controls (subjected to Perlite powder treatment) was 36 % lower. Root dry mass was not significantly affected by any treatments in this experiment.

### 4.5.4 Greenhouse trial to determine the efficacy of selected PGPR for biocontrol of *M. incognita* on soybean

The selected strains (N04, T19 and T22) were tested as perlite powder seed treatments for biocontrol of *M. incognita*. A significant interaction ($P = 0.0140$; F-ratio =3.57) was observed between the effects of the two experiments and the effects of treatments on the dry shoot mass of soybean. The two experiments were therefore analysed separately (Table 4.5). The

number of *M. incognita* eggs and J2 extracted from roots of soybean plants and counted was zero for all experimental units. Nematode reproduction may have failed to occur by the time of harvesting. This observation could be attributed to suboptimal environmental conditions in the greenhouse (which were unavoidable due to winter conditions). Minimum air temperature reached 10 °C. Galls and egg masses were however counted and have been used for comparison of treatments.

Application of strain T19 only reduced the number of galls per plant significantly ($P \leq 0.05$), by 40 % in the first experiment, compared to nematode-infected control (Table 4.5). For the second experiment, only Strain N04 significantly ($P \leq 0.05$) reduced gall numbers compared to the nematode-infected control. Strains N04, T19 and T22 reduced the number of egg masses per plant significantly ($P \leq 0.05$) (by 48 %, 64 % and 40 %, respectively) in the first experiment. Application of strain T19 reduced the number of egg masses per plant significantly ($P \leq 0.05$) (by 86 %) during the second experiment.

No rhizobacteria affected the *M. incognita* gall indices of plants significantly in either experiment, if gall index A was used for comparison of treatments (Table 4.5). However, all three selected rhizobacteria reduced gall index B significantly in the first experiment, while strains N04 and T22 reduced gall index B in the second experiment. Only strain T19 reduced egg-laying female index A significantly in the two experiments, while no rhizobacteria affected egg-laying female index B in either experiment.

The dry root mass of infected controls was significantly ($P \leq 0.05$) lower (38 %) than that of non-infected controls in the first experiment, while no significant differences were found between the dry root mass of different treatments in the second experiment (Table 4.5). No treatments affected dry shoot mass significantly ($P \leq 0.05$) in the first experiment, but strain N04 reduced dry shoot mass significantly ($P \leq 0.05$) in the second experiment. This finding indicates that strain N04 may have deleterious effects on the growth of soybean cv. LS 6248 R under certain conditions. No significant differences were found between the mean chlorophyll content indices of treatments in either experiment.

#### 4.5.5 Greenhouse trial to determine the efficacy of selected PGPR for promotion of soybean growth in soil with suboptimal (reduced) levels of fertiliser

The selected rhizobacterial strains were tested within a Perlite powder seed treatment for promotion of soybean growth under conditions of limited fertiliser levels. No significant interactions between the effects of experiments and treatments were observed ($P \leq 0.05$).

Levene's test for homogeneity of variances (α = 0.01) indicated that the variances of the two experiments were not significantly different. Data from two experiments were therefore pooled during the analysis (Table 4.6). Strains N04 and T22 increased root dry mass by 88 % and 127 %, respectively, compared to controls without rhizobacteria, while strain T19 did not increase dry root mass significantly. All three selected rhizobacteria increased dry shoot mass, as well as total leaf area, significantly ($P \leq 0.05$). Strains N04, T19 an T22 increased dry shoot mass significantly ($P \leq 0.05$) by 103 %, 84 % and 124 %, respectively, and total leaf area by 90 %, 97 % and 127 %, respectively.

### 4.5.6 Degradation of hydroxyethyl cellulose, used for gel seed treatment, by selected rhizobacteria

Clear zones were observed on agar plates which had been inoculated with strain N04. Strain N04 appears to be able to degrade hydroxyethyl cellulose under mesophilic conditions, whilst hydroxyethyl cellulose does not appear to be degraded by strains T19 and T22.

### 4.5.7. Analysis of seed germination during experiments 4.4.5 and 4.4.6

No significant interactions were observed between experiments and treatments ($P \leq 0.05$). Levene's test ($P \leq 0.01$) indicated that variances did not different significantly between experiments. Germination data from two biocontrol experiments and two growth promotion experiments were therefore pooled. T19 and T22 caused significant ($P \leq 0.05$) increases in the percentage of soybean seeds that germinated in vermiculite-filled seedling trays (Figure 4.1). Perlite powder seed treatment with strains T19 and T22 resulted in the normal germination of 43 % and 50 % of seeds, respectively. Treatment with perlite powder that had been injected with sterilised nutrient broth resulted in the germination of 25% of seeds. Strains T19 and T22 therefore increased seed germination by 68% and 97%, respectively.

## 4.6. Discussion

The primary aim of the current study was to test the efficacy of *L. sphaericus* strain T19, *P. alvei* strain T22 and *P. fluorescens* strain N04 for biocontrol of *M. incognita* on soybean in greenhouse trials. *Lysinibacillus sphaericus* strain T19 proved to be the most consistent-performing biocontrol agent among the selected strains. Strain T19 reduced the reproduction

of *M. incognita* significantly after being applied as a drench treatment and a Perlite powder seed treatment (in pilot experiments 4.4.2 and 4.4.4). The strain (applied as a Perlite powder seed treatment) reduced the number of *M. incognita* egg masses found on roots of plants seven weeks after nematode inoculation in two experiments (Section 4.4.5). The egg-laying female indices of plants (index A of Hussey and Boerma (1981)) were also reduced by strain T19 in both experiments (Section 4.4.5) in which quartzite sand had been used as growth medium.

The consistent performance of strain T19 mirrors the results of previous studies. Rudolph (2014) found that strain T19 was the only isolate, among a selection of PGPR studied at the University of Pretoria, that consistently controlled the plant pathogen *Rhizoctonia solani* on maize in seedling bioassays and pot trials. This author also found that T19 was the only strain that consistently promoted the growth of wheat plants in experiments with different levels of fertiliser. Rudolph (2014) reported that strain T19 was only effective for promotion of maize growth and biocontrol of *R. solani* when applied as a soil drench, and that T19 did not produce statistically significant results if applied as a perlite powder seed treatment. Based on these results, Rudolph (2014) concluded that the concentration of *L. sphaericus* T19 in seed coatings should be increased. Pretorius (2012) similarly concluded that further research on the formulation and application of rhizobacteria was needed. The amount of Perlite powder used to coat seeds was increased to 20 g powder /kg seed in the current study (from 4 g powder / kg seed in previous studies) as an interim solution. This increased rate is recommended by Stimuplant® (Pretoria, South Africa) for application of rhizobial inoculants on perennial soybean (*Neonotonia wightii* (Wight & Arn.) Lackey), as well as stylos (*Stylosanthes* spp.), clovers (*Trifolium* spp.), serradellas (*Ornithopus* spp.), *Lotus* spp., *Medicago* spp. and *Lespedeza* spp. (Anonymous, 2015).

In the greenhouse biocontrol trial (Section 4.4.5), *L. sphaericus* strain T19 applied as a Perlite powder seed treatment produced a statistically significant ($P \leq 0.05$) decrease in the number of egg masses per plant but did not increase plant mass significantly (compared to *M. incognita* infected controls). The bacterium reduced the reproduction of *M. incognita* in pilot experiments but did not increase plant mass significantly or reduce the RF to below 1. Plants treated with strain T19 therefore cannot be considered ‘highly resistant’ (Fourie *et al.* 2017) to the *M. incognita* population used in this study. The results obtained in the current study may be compared to the results of Baidoo *et al.* (2017). These authors found that the chemical nematicide furfuraldehyde and the biocontrol agent *Purpureocillium lilacinum* reduced the population density of *M. incognita* on an ornamental plant significantly but failed to significantly increase plant mass (compared to infected controls). Baidoo *et al.* (2017) concluded that furfuraldehyde or *P. lilacinum* should only be used for nematode control as part of an

integrated management system. The authors also suggested that the application of furfuraldehyde or *P. lilacinum* would be most effective at the beginning of the season and when the level of infestation by *M. incognita* is below an economic threshold. *Lysinibacillus sphaericus* strain T19 (applied as a Perlite seed treatment) could therefore be used as part of an integrated management system that involves the periodic monitoring of nematode pest population densities, and the implementation of different management tools as needed.

A hydroxyethyl cellulose fluid gel formulation was tested in Pilot Experiment 4.4.4. Strain T19 did not reduce *M. incognita* reproduction significantly when applied within the fluid gel formulation. The possible reasons for this result include an insufficient concentration of rhizobacteria within the gel formulation (Rudolph, 2014) and insufficient survival of bacterial cells after application. Hydroxyethyl cellulose is not degraded by strain T19, according to the results of the *in vitro* test. The Perlite seed coating appeared to have adhered to seed testae until after harvesting (in Experiments 4.4.4 and 4.4.5), possibly allowing the gradual release of bacteria around the germinating seed and the establishment of a rhizobacterium population. Different authors have found that solid-state formulations (such as Perlite powder) may allow improved survival of rhizobacteria (compared to liquid formulations). Peat was historically considered to be the best carrier for rhizobacteria, and Perlite may be a viable alternative to peat for formulation of inoculants (Albareda *et al.* 2008). Hydroxyethyl cellulose was tested in this study because this polymer differs from sodium carboxymethyl cellulose (which was used in certain previous studies at the University of Pretoria). Hydroxyethyl cellulose is non-ionic, and gels made with this polymer are therefore more stable than those made with carboxymethyl cellulose (in the presence of high concentrations of electrolytes). This polymer can be used to prepare gels with a wider range of viscosity than certain other cellulose derivatives (Anonymous, 2005; Guo *et al.* 1998). Further research might be needed before an effective gel seed treatment containing strain T19 is produced. Jawson *et al.* (1989) suggested that a seed treatment powder should be produced, consisting of a mixture of dried bacteria, a polymer carrier and nutrients. This powder would be hydrated immediately before use by the grower, and the nutrients within the powder would allow the bacteria to multiply in the fluid gel and the rhizosphere. Use of spray-dried bacteria could allow an increase in the concentration of bacteria applied to seeds. Certain nutrients may stimulate the germination of bacterial endospores and improve the consistency of the performance of biocontrol agents (Crane *et al.* 2014). Furthermore, certain micronutrients, such as molybdenum, may increase the nematode-suppressive activity of PGPR (Hamid *et al.* 2003).

The effectiveness of strain T19 (and other biocontrol agents) could be increased by combining the biocontrol agent(s) with selective chemical pesticides within a single seed treatment. Abamectin (Avicta®, Syngenta Crop Protection AG, Stein, Switzerland) has been used a

seed-applied nematicide in certain countries, such as the United States. This chemical has no significant antibacterial activity and could therefore be combined with different bionematicides (Becker & Morton, 2014). Fungicides that are compatible with the biocontrol agent may improve the survival of the biocontrol agent in the rhizosphere by reducing competition with, and predation of, the biocontrol agent (Taylor & Hartman, 2003). Strains N04 and T19 are compatible with mefenoxam (Apron® XL, Syngenta Crop Protection AG, Stein, Switzerland). The strains are also compatible with triazole fungicides, at lower concentrations (Makgolane, 2016).

The results obtained using gall- and egg mass index A and index B of Hussey & Boerma (1981) differed markedly (Table 4.5). Hussey & Barker (1981) found that index B was more sensitive for differences between soybean cultivars than index A. Authors citing Hussey & Boerma (1981) tend to only present index A. Dong *et al.* (2007) found that a gall rating index based on the percentage of galled roots allowed the identification of peanut cultivars with resistance to *Meloidogyne arenaria* Neal, 1889 (Chitwood, 1949) at an earlier harvest date after inoculation (and using lower amounts of nematode inoculum).The root-knot nematode gall rating system based on the percentage of galled roots should be investigated in a future study.

Strains T19 and T22 did not increase plant mass after being applied as drenches in quartzite sand in conjunction with water-soluble fertiliser applications (Section 4.5.2). These strains did, however, increase the dry shoot mass and the leaf area of soybean plants in *M. incognita*-free soil in conjunction with suboptimal fertiliser levels (Section 4.5.5) after being applied as perlite seed treatments. The selected strains also increased the percentage of seeds that germinated in trays after being applied as Perlite seed treatments (Section 4.5.7). These results support the conclusions of Breedt (2015), who found that the effectiveness of strain T19 was affected the dose (application rate) of rhizobacteria, the amount fertiliser in the soil and the soil type. Breedt (2015) found that strain T19 was most effective in soils with reduced amounts of fertiliser and in soils with lower clay content. The growth-promoting activity of strain T19 may be attributed, at least in part, to nitrogen fixation and indole-3-acetic acid production (Pretorius, 2012). The increase in plant growth due to strain T22 may be attributed in part to phosphate solubilisation (Pretorius, 2012). In future, the effects of strains T19 and T22 on germination of different legumes using guidelines provided by the International Seed Testing Association (Bassersdorf, Switserland) will be conducted. This future study could also include an investigation into combined applications of the nodule-forming bacterium *Bradyrhizobium japonicum* and the selected rhizobacteria. Measurement of the effects of the rhizobacteria on seed germination had not been one of the original aims of the current study.

*Pseudomonas fluorescens* strain N04 reduced plant mass significantly compared to *M. incognita*-infected controls in one experiment (Table 4.5). Secondary metabolites produced by rhizobacteria may inhibit plant growth at high doses (Zahir *et al.* 2004). Phytotoxic effects of rhizobacterial products may be pronounced in the quartzite sand medium (with a poor capacity to bind and gradually release chemicals) used in the current biocontrol experiments.

The ability of strains T19 and T22 to promote soybean growth under conditions of abiotic stress (nutrient-limited conditions) may increase the commercial viability of these strains. PGPR such as *P. alvei* strain T22 that increase the nutrient uptake of plants could reduce the required fertiliser application rates (Adesemoye *et al.* 2009) and could increase the tolerance of these plants to nematode damage (Sikora *et al.* 2007). Nematicides may have phytotoxic effects, even when applied in accordance with label instructions (Jones *et al.* 2017). Plant growth-promoting rhizobacteria could negate some of the yield loss caused by phytotoxic effects. Certain strains of PGPR may also improve the uptake of systemic pesticides by plants, allowing the use of reduced pesticide application rates (Myresiotis *et al.* 2014). A future study could also assess the use of strains T19 and T22 for improved phytostabilization of mine soils. Mine soils tend to have low amounts of nutrients and poor soil structure (in other words, conditions in which strain T19 might be most effective for promotion of plant growth). PGPR may increase the establishment, growth and stress tolerance of legumes, grasses, and other plants used for phytostabilization (Novo *et al.* 2018).

Future studies should investigate the nematode-suppressive effects of strains T19 and T22 in unsterilized as well as pasteurised Huttons-type soil with reduced fertiliser levels. Experiments should be conducted in greenhouses or controlled environment chambers with appropriate temperature control and lighting. Quartzite filter sand had been used as medium in the biocontrol experiments in the current study and hydroponic fertilizer had been applied in the biocontrol experiments. Sand was used as medium for the biocontrol experiments in the current section, because the reproduction of *M. incognita* is generally higher in soils with high sand content than in soils with low sand content (Jaraba *et al.* 2014). Cleaning of roots, enumeration of egg masses and extraction of nematode eggs was also found to be less laborious when sand was used as medium.

### 4.7 Conclusion

*Lysinibacillus sphaericus* strain T19, applied as a Perlite seed treatment, is promising for biocontrol of *M. incognita* on soybean. Strain T19 should be used within an integrated pest management system. *Lysinibacillus sphaericus* strain T19 and *P. alvei* strain T22 are

promising as active ingredients of biofertilisers for use on soybean. Future research could includethe development of seed treatments combining strain T19 with seed-applied nematicides. The effect of strain T19 on root-knot nematode reproduction in different soil types should also be investigated.

## 4.8 Tables and Figures

**Table 4.1 – Nutrient composition of Nutrifeed (Starke Ayres (Pty) Ltd, Bredell, South Africa)**

| **Nutrient** | **Content** |
|---|---|
| Nitrogen | 65 g/kg |
| Phosphorous | 13 g/kg |
| Calcium | 70 mg/kg |
| Copper | 20 mg/kg |
| Iron | 1500 mg/kg |
| Molybdenum | 10 mg/kg |
| Magnesium | 22 mg/kg |
| Manganese | 240 mg/kg |
| Sulphur | 75 mg/kg |
| Boron | 240 mg/kg |

**Table 4.2 – Effect of rhizobacteria on infection of soybean by *Meloidogyne incognita* in a growth chamber (pilot experiment)**

| Treatment | N[1] | RF[2] | | Dry shoot mass (g) | | Dry root mass (g) | | Egg masses | | ELF-IA[3] | | ELF-IB | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SE[4] | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| *M. incognita*-infected control | 8 | 88 a | 5 | 0.962 b | 0.024 | 0.564 b | 0.015 | 272 a | 18 | 5 a | 0 | 4 a | 0 |
| Uninfected control | 7 | 1 c | 0 | 1.326 a | 0.010 | 1.296 a | 0.069 | 0 b | 0 | 0 b | 0 | 0 b | 0 |
| Bacterial strain N04 | 5 | 43 b | 7 | 1.091 ab | 0.027 | 0.583 b | 0.015 | 148 ab | 20 | 5 a | 0 | 3 a | 0 |
| Bacterial strain T19 | 6 | 29 b | 2 | 1.077 b | 0.020 | 0.626 b | 0.011 | 215 a | 15 | 5 a | 0 | 3 a | 0 |
| Bacterial strain T22 | 7 | 53 ab | 2 | 1.084 b | 0.024 | 0.574 b | 0.012 | 249 a | 12 | 5 a | 0 | 4 a | 0 |

Controls consisted of plants that were treated with blank ¼-strength Ringer's solution and later inoculated with *M. incognita* juveniles or sterile water. Growth chamber temperature varied between 23 and 25 °C. Means followed by the same letter are not significantly different according to Tukey's test (α = 0.05). Certain extreme outliers have been excluded from the analysis.

[2]Rf denotes the reproduction factor, calculated with formula Rf = (Pf+ 1)/(Pi + 1), where Pf is the number of eggs and J2s extracted at the end of the experiment and Pi is the initial amount of J2 used as inoculum (Wang *et al.* 2000). The Rf values were subjected to square root transformation before analysis (means and standard errors of untransformed data is presented here).

[3]ELF-IA and ELF-IB denote egg laying female indices A and B (Hussey & Barker, 1981), respectively.

[4]SE denotes the standard error of the mean.

**Table 4.3 – Effect of rhizobacteria on growth of soybean in greenhouse experiments (pilot experiment)**

**Experiment 1**

| Treatment | N[1] | Dry root mass (g) | | Dry shoot mass (g) | |
|---|---|---|---|---|---|
| | | Mean | SE | Mean | SE |
| Non-inoculated control (without rhizobacteria) | 16 | 0.795 | 0.084 | 0.461 | 0.061 |
| Bacterial strain N04 | 6 | 0.755 | 0.153 | 0.403 | 0.093 |
| Bacterial strain T19 | 8 | 0.729 | 0.131 | 0.376 | 0.066 |
| Bacterial strain T22 | 8 | 0.905 | 0.054 | 0.480 | 0.039 |

**Experiment 2**

| Treatment | N[1] | Dry root mass (g) | | Dry shoot mass (g) | |
|---|---|---|---|---|---|
| | | Mean | SE | Mean | SE |
| Non-inoculated control (without rhizobacteria) | 16 | 1.415 | 0.208 | 1.318 | 0.217 |
| Bacterial strain N04 | 8 | 1.287 | 0.264 | 1.065 | 0.174 |
| Bacterial strain T19 | 8 | 2.043 | 0.105 | 1.608 | 0.160 |
| Bacterial strain T22 | 7 | 1.401 | 0.361 | 0.923 | 0.270 |

The variances of experiments were not homogeneous according to Levene's test ($\alpha = 0.01$). Experiments were therefore analysed separately. Air temperature in the greenhouse varied between 18 and 38 °C. There were no significant differences between treatments according to Tukey's test ($\alpha = 0.05$) within any columns in any experiments. Certain extreme outliers have been excluded from the analysis.

[1]N denotes the number of replicates.

[2]SE denotes the standard error of the mean.

**Table 4.4 - Effect of rhizobacteria (applied using different rhizobacterium formulations) on infection of soybean roots by *Meloidogyne incognita* (pilot experiment in greenhouse)**

| Rhizobacterium formulation | Treatment | N[1] | Rf[2] | | Dry root mass (g) | | Dry shoot mass (g) | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | SE[3] | Mean | SE | Mean | SE |
| Seed treatment with hydroxyethyl cellulose gel | *M. incognita*-infected control | 6 | 46 ab | 6 | 0.683 a | 0.089 | 1.252 b | 0.062 |
| | Bacterial strain N04 | 4 | 36 ab | 5 | 0.711 a | 0.086 | 1.255 b | 0.172 |
| | Bacterial strain T19 | 6 | 46 ab | 6 | 0.680 a | 0.059 | 1.371 b | 0.111 |
| | Bacterial strain T22 | 2 | 59 a | 26 | 0.630 a | 0.037 | 1.046 b | 0.030 |
| | Uninfected control | 3 | 1 c | 0 | 0.634 a | 0.065 | 1.799 a | 0.157 |
| Seed treatment with perlite powder | *M. incognita*-infected control | 6 | 54 a | 7 | 0.578 a | 0.107 | 1.154 b | 0.097 |
| | Bacterial strain N04 | 5 | 31 abc | 2 | 0.709 a | 0.129 | 1.360 b | 0.181 |
| | Bacterial strain T19 | 5 | 17 bc | 3 | 0.486 a | 0.063 | 1.214 b | 0.089 |
| | Bacterial strain T22 | 6 | 34 ab | 6 | 0.608 a | 0.085 | 1.253 b | 0.087 |
| | Uninfected control | 4 | 1 c | 0 | 0.805 a | 0.108 | 1.962 a | 0.087 |

Controls consisted of plants that were treated with hydroxyethyl cellulose gel mixed with sterilised nutrient broth (in the case of the gel formulation) or perlite powder injected with sterilised nutrient broth (in the case of the seed treatment with the powder formulation). Uninfected controls were inoculated with sterilised water instead of the suspension containing *M. incognita* J2 applied to other plants. The air temperature in the greenhouse varied between 18 and 38 °C. Means followed by the same letter are not significantly different according to Tukey's test (α = 0.05).

[1]N denotes the number of replicates.

[2]Rf denotes the reproduction factor, calculated with formula Rf = (Pf + 1)/(Pi + 1), where Pf is the number of eggs and J2 extracted at the end of the experiment and Pi is the initial amount of J2 used as inoculum (Wang *et al.* 2000).

[3]SE denotes the standard error of the mean.

**Table 4.5 – Efficacy of rhizobacteria for biocontrol of *Meloidogyne incognita* on soybean in greenhouse experiments**

**Experiment 1**

| Treatment | N[1] | Dry root mass (g) | | Dry shoot mass (g) | | Galls | | Egg masses | | CCI[2] | | G-IA[3] | | ELF-IA | | G-IB | | ELF-IB | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SE[4] | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Infected control[5] | 6 | 0.550 b | 0.019 | 1.795 a | 0.038 | 70 a | 3 | 25 a | 1 | 6.883 a | 0.131 | 4 a | 0 | 3.2 a | 0.2 | 1.8 a | 0.2 | 1 a | 0 |
| Non-infected control | 6 | 0.838 a | 0.035 | 1.879 a | 0.040 | 0 c | 0 | 0 c | 0 | 6.650 a | 0.064 | 0 b | 0 | 0 c | 0 | 0 b | 0 | 0 b | 0 |
| Bacterial strain N04 | 4 | 0.648 ab | 0.015 | 1.912 a | 0.084 | 55 ab | 3 | 13 b | 1 | 7.225 a | 0.203 | 4 a | 0 | 2.5 ab | 0.3 | 1.5 b | 0.3 | 1 a | 0 |
| Bacterial strain T19 | 5 | 0.690 ab | 0.020 | 1.751 a | 0.033 | 42 b | 3 | 9 b | 1 | 7.180 a | 0.167 | 4 a | 0 | 2.2 b | 0.2 | 1.2 b | 0.2 | 1 a | 0 |
| Bacterial strain T22 | 5 | 0.640 ab | 0.021 | 1.635 a | 0.055 | 52 ab | 3 | 15 b | 1 | 7.340 a | 0.087 | 4 a | 0 | 2.8 ab | 0.2 | 1.6 b | 0.2 | 1 a | 0 |

**Experiment 2**

| Treatment | N | Dry root mass (g) | | Dry shoot mass (g) | | Galls | | Egg masses[6] | | CCI | | G-IA | | ELF-IA | | G-IB | | ELF-IB | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Infected control | 6 | 0.510 ab | 0.020 | 1.820 a | 0.095 | 59 a | 17 | 22 a | 7 | 10.717 a | 1.198 | 3.8 a | 0.4 | 2.8 a | 0.6 | 1.5 a | 0.2 | 0.8 a | 0.4 |
| Non-infected control | 6 | 0.698 a | 0.087 | 1.870 a | 0.097 | 0 b | 0 | 0 b | 0 | 8.733 a | 0.769 | 0 b | 0 | 0 b | 0 | 0 c | 0 | 0 b | 0 |
| Bacterial strain N04 | 6 | 0.363 b | 0.091 | 1.189 b | 0.229 | 23 b | 5 | 6 ab | 3 | 9.333 a | 0.899 | 3.2 a | 0.3 | 1.3 ab | 0.5 | 1 b | 0 | 0.7 ab | 0.5 |
| Bacterial strain T19 | 6 | 0.598 ab | 0.084 | 1.869 a | 0.140 | 24 ab | 8 | 3 b | 2 | 12.000 a | 1.389 | 2.8 a | 0.4 | 1 b | 0.4 | 1.2 ab | 0.2 | 0.5 ab | 0.5 |
| Bacterial strain T22 | 6 | 0.580 ab | 0.055 | 1.667 ab | 0.091 | 25 ab | 8 | 7 ab | 3 | 12.750 a | 1.250 | 3 a | 0.5 | 1.7 ab | 0.6 | 1 b | 0 | 0.7 ab | 0.5 |

Air temperature in the greenhouse varied between 10 and 30 °C. Means followed by the same letter (within a column in an experiment) are not significantly different according to Tukey's test ($\alpha = 0.05$).Certain extreme outliers have been excluded from the analysis.[1]N denotes the number of replicates.[2]CCI denotes the chlorophyll content index, measured using a CCM-200 chlorophyll meter (Opti-Sciences, Inc.).[3]G-IA, G-IB, ELF-IA and ELF-IB denote gall index A, gall index B, egg-laying female index A and egg-laying female index B (Hussey & Barker, 1981) respectively.[4]SE denotes the standard error of the mean.[5] Infected controls were inoculated with *M. incognita* J2.[6]The numbers of egg masses in the second experiment were subjected to square root transformation (untransformed values shown).

**Table 4.6 – Efficacy of rhizobacteria for promotion of soybean (cv. LS 6248R) growth in greenhouse experiments, in soil with suboptimal (reduced) fertiliser levels**

| Treatment | N[2] | Dry root mass (g) | | Dry shoot mass (g) | | Leaf area ($cm^2$) | | CCI[1] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SE[3] | Mean | SE | Mean | SE | Mean | SE |
| Non-inoculated control (without rhizobacteria) | 13 | 0.459 b | 0.093 | 0.732 b | 0.129 | 110.59 b | 20.56 | 7.031 a | 0.480 |
| Bacterial strain N04 | 13 | 0.851 a | 0.095 | 1.486 a | 0.171 | 210.32 a | 19.83 | 6.946 a | 0.266 |
| Bacterial strain T19 | 13 | 0.741 ab | 0.096 | 1.347 a | 0.150 | 217.74 a | 24.68 | 7.577 a | 0.494 |
| Bacterial strain T22 | 13 | 1.044 a | 0.117 | 1.641 a | 0.128 | 251.19 a | 30.07 | 6.785 a | 0.439 |

Data from two experiments has been pooled in this analysis. Controls consisted of plants that were treated with Perlite powder injected with sterilised nutrient broth. The air temperature in the greenhouse varied between 10 and 30 °C. Means followed by the same letter are not significantly different according to Tukey's test (α = 0.05).

[1]CCIdenotes the chlorophyll content index, measured using a CCM-200 chlorophyll meter (Opti-Sciences, Inc.).

[2]N denotes the number of replicates.

[3]SE denotes the standard error of the mean.

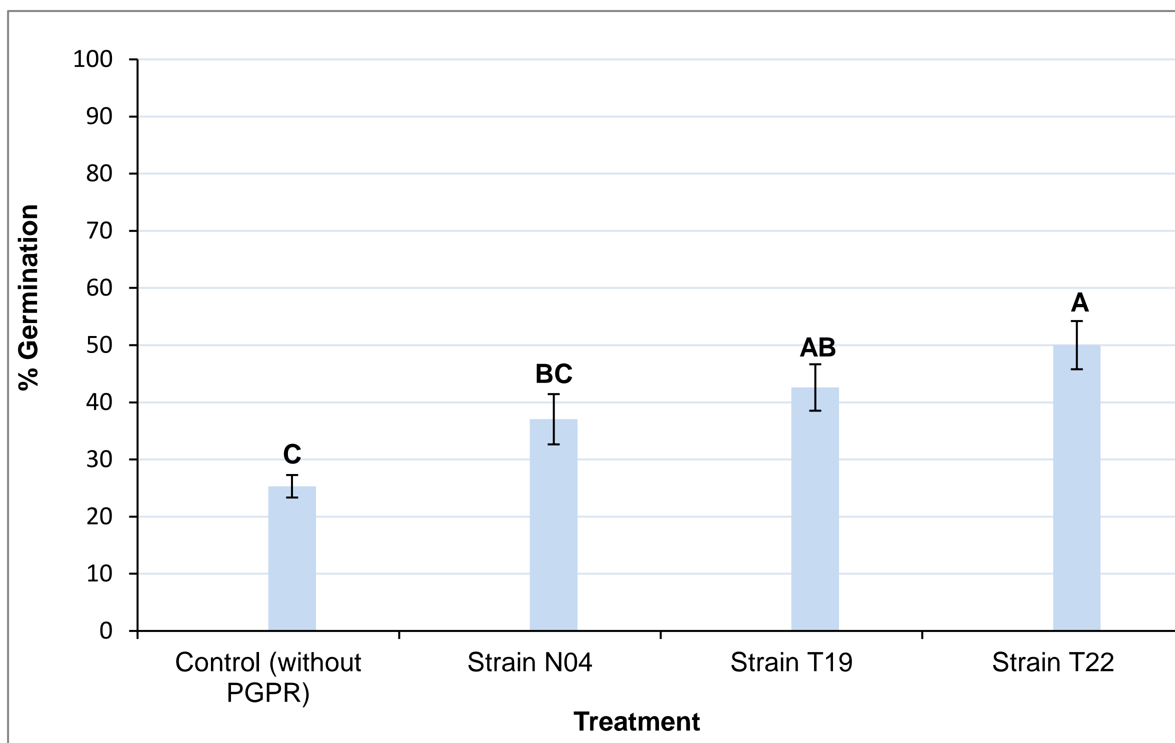


**Figure 4.1–Effect of selected strains of rhizobacteria on germination of soybean seeds (cv. LS 6248R) in vermiculite-filled seedling trays.**

Data from four experiments have been pooled in this analysis. Means of twelve replicates are presented for strains N04, T19 and T22, while the mean of eighteen replicates is presented for the non-inoculated control. Each replicate consisted of a seedling tray with nine cells. Error bars indicate standard error of the means. Means with the same letter are not significantly different according to Scheffe's test ($\alpha = 0.05$).

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# CHAPTER 5

## Modes of action of selected rhizobacteria for biocontrol of *Meloidogyne incognita* on soybean

### 5.1 Abstract

The ability of selected rhizobacteria to produce nematode-suppressive metabolites were assessed with *in vitro* assays, while the ability of the rhizobacteria to induce resistance against *M. incognita* was assessed using a split-root assay. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens strain* N04 appear to produce metabolites (in broth culture) that reduce motility as well as hatching of *M. incognita* second-stage juveniles. Culture filtrate produced by strain T19, at a concentration of 50 %, reduced J2 motility by 54 % and 77 %, respectively, compared to water controls, in two experiments. T19 culture filtrate, at a concentration of 50 %, reduced J2 hatching by 91 % and 94 %, respectively, in two experiments. At a concentration of 10 %, T19 culture filtrate reduced J2 hatching by 66 % and 82 %, respectively, in two experiments. Strain T22 induced resistance against *M. incognita* in soybean in one of two split-root experiments. The split-root assay should be repeated in a future study with a different plant growth medium. Future studies should attempt to assess the sub-lethal effects of secondary metabolites produced by strain T19 on phytonematodes. The amount of antibiotics produced by strain T19 in the rhizosphere should be investigated.

### 5.2 Introduction

In terms of economic impact, root-knot nematodes (Genus *Meloidogyne* Göldi, 1887) are the most important group of plant-feeding nematodes worldwide. Yield losses due to root-knot nematodes are likely to continue to increase in future due to climate change, spread of quarantine species and deregistration of chemical nematicides. Root-knot nematodes infect numerous crops, and cause reductions in yield as well as water and fertiliser use efficiency. The marketability of root and tuber crops may be affected by root-knot nematodes. Profit margins of growers are affected by the high costs of nematode control (including the costs of soil sampling and analysis). Some growers may fail to obtain phytosanitary certificates due to the presence of quarantine root-knot nematode species (Onkendi *et al.* 2014; Wesemael *et al.* 2011). Further research on the management of root-knot nematodes is therefore warranted.

Biopesticides could be an alternative to conventional chemical pesticides. Products that contain biocontrol agents have several advantages over other products, including low risk to users, short pre-harvest intervals, and multiple modes of action (theoretically allowing management of resistance to pesticides) (Kiewnick, 2007). Extensive research has been conducted on the application of plant growth-promoting rhizobacteria (PGPR) for control of phytonematodes. The modes of action of PGPR against *Meloidogyne* spp. were discussed in Section 2.7 of this dissertation, and include production of nematode-suppressive metabolites, priming of plant defences and increased tolerance of nematode damage (Mendoza *et al.* 2008; Sikora *et al.* 2007).

A tiered approach should be used when studying the bioactivities of metabolites produced by biocontrol agents, so that the cost of research, development and product registration is minimised. This tiered approach involves different experiments that precede any attempts to characterise the transcriptome and/or metabolome of the biocontrol agent (George *et al.* 2015; Scheepmaker *et al.* 2012). Studies on the modes of action of PGPR against phytonematodes typically include *in vitro* assays that assess the effects of crude mixtures of metabolites on the hatching and motility of infective juvenile stages. Split-root assays are most commonly used to test PGPR for induction of resistance against phytonematodes (Alcals, 2007; Schrimsher, 2013). Such studies could be followed by metabolomics studies aimed at isolation of proteins or metabolites with nematode-suppressive activity (Zeng *et al.* 2015). Different methods have been used for the*in vitro* assessment of the effects of microbial extracts and fermentation products on the biology and/or physiology of phytonematodes (Mendoza *et al.* 2008; Meyer *et al.* 2004; Pankaj *et al.* 2010; Xiang & Lawrence, 2016). Methods for the *in vitro* assessment of nematode- or trematode-suppressive chemicals include direct measurement of the activity or physiology of the nematode or staining the target nematode with vital or non-vital dyes to determine if it is alive or dead (Peak & Hoffmann, 2011). Relatively few studies have been published that include split-root experiments that study the interactions between soybean and root-knot nematodes. Different methods have been used to carry out soybean split-root experiments for various purposes (Heron & Pueppke, 1987; Schaarschmidt *et al.* 2013; Schrimsher, 2013).

Complete elucidation of the modes of action of a biocontrol agent may be a time-consuming process, as illustrated by the case of *Pseudozyma flocculosa* (Traquair, L. A. Shaw & Jarvis), a basidiomycete used to control powdery mildew fungi on different crops. Elucidation of the modes of action of the biocontrol agent has taken approximately three decades and has required biochemical as well as transcriptomic research. Early studies indicated that antibiosis was the primary mode of action of the biocontrol agent, and lead to the discovery of a novel fungicidal compound, flocculosin (Bélanger *et al.* 2012). Later studies indicated that antibiosis

is of secondary importance for this biocontrol agent. The organism now appears to controlpowdery mildew through a novel process, hyperbiotrophism, which is only expressed in tri-trophic interactions (Laur *et al.* 2018). Results of *in vitro* tests on the modes of actionof biopesticides should therefore be interpreted with caution.

According to Bélanger *et al.* (2012), some scientists have been misinformed that biocontrol agents that function through antibiosis would be impossible to register. Certain research groups have therefore undertaken projects aimed at proving that the biocontrol agents they investigated and/or developed do not function through antibiosis. However, several biocontrol agents that suppress phytonematode reproduction or their population densities through secondary metabolite production (such as *Bacillus firmus* and *Bacillus pumilus*) have been commercialised successfully. Few research groups have attempted to isolate and characterise the nematode-suppressive metabolites, due to the cost and difficulty of such projects (Engelbrecht *et al.* 2018).

Application of chemicals (or transgenes), discovered through the study of biocontrol agents, may solve the problem of variable biopesticide efficacy. An example of a promising chemical discovered through research on PGPR is the volatile compound 2,3-butanediol. This compound can be used to induce resistance against different diseases and pests while promoting plant growth (in contrast to certain resistance-inducing chemicals that impair plant growth) (Kong *et al.* 2018). The volatile organic compound dimethyl disulfide is produced by different soil bacteria and has been commercialised as a broad-spectrum fumigant (Paladin®, Arkema Inc., Philadelphia) (Tyc *et al.* 2017). Dimethyl disulfide is an effective alternative to methyl bromide and has an acceptable toxicological profile (Han *et al.* 2017).

## 5.3 Aims

The primary aim of this chapter was to identify the modes of action of PGPR that showed biocontrol activity, *viz*. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens strain* N04, against *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 on soybean (in Chapter 3 of this dissertation). This chapter has been limited to the evaluation of the effects of cell-free culture filtrates of the three selected PGPR on *M. incognita* eggs and second stage juveniles (J2), and the evaluation of the effects of the selected rhizobacteria in split-root systems.

## 5.4 Materials and methods

### 5.4.1 *In vitro* test to determine the effect of cell-free culture filtrates on *M. incognita* second-stage juvenile (J2) motility

Multiwell plate-based bioassays were conducted to determine the effect of bacterial culture filtrates of three PGPR that showed potential in suppressing *M. incognita* gall formation and in Chapter 3, namely *L. sphaericus* strain T19, *P. alvei* strain T22 and *P. fluorescens strain* N04, on the motility of *M. incognita* J2. The assay was planned as a two-factor completely randomised design. The two factors were i) experiments and ii) treatments. The experiment was conducted twice, with 6 replicates per treatment per experiment.

Culture filtrates were prepared by growing the rhizobacteria in nutrient broth as described in Section 3.4.1, centrifuging the cultures (at 6000 g for 10 min at 4 °C) and filter-sterilising the supernatants using 0.45- and 0.22-µm pore size syringe filters (Millipore Corporation, now affiliated with Merck KGaA, Darmstadt, Germany). Antibiotic solution was prepared by dissolving 20 mg of nystatin (Duchefa Biochemie, Haarlem, the Netherlands), 26.4 mg of gentamycin sulphate (Melford Biolaboratories Ltd, Suffolk, England) and 26.4 mg of streptomycin sulphate (Calbiochem®, Merck KGaA, Darmstadt, Germany) in 16 mL of ReNu® (Bausch & Lomb Inc., Rochester, New York State) solution. The product ReNu® contains 0.001% m/v DYMED™ (polyaminopropyl biguanide) and 0.03 % m/v HYDRANATE™ (hydroxyalkylphosphonate). ReNu® can be used to surface sterilise nematodes without significant loss of nematode viability (Chen & Caswell-Chen, 2004). This product was used in this experiment primarily because it has surfactant and potentiating activities (Richard & Heiler, 1997). Nystatin is poorly soluble in water, but surfactants may be used to dissolve nystatin and increase its bioactivity (Tallury *et al.* 2007). The final volume of the antibiotic solution was adjusted to 100 mℓ using sterilised, demineralised water.

Six-well assay plates with 10 mℓ wells were cleaned and sterilised with 90% ethanol and allowed to dry. The antibiotic solution was kept in a state of agitation using a magnetic stirrer, while 500 µℓ was transferred to each well. *Meloidogyne incognita* J2 were obtained as described in Section 3.4.1 of this dissertation. An aliquot containing approx. fifty J2 was added to each well. Culture filtrates of the three selected PGPR (see first paragraph of this section) were tested at two concentrations: 10% and 50%. Controls comprised water, 10% sterile nutrient broth and 50% sterile nutrient broth, respectively. Water was added to each well to produce final volumes of 2 mℓ per well. Final concentrations of antimicrobial compounds were as follows: 0.067 mg/mℓ gentamycin sulphate and 0.067 mg/mℓ streptomycin sulphate (used by Mendoza *et al.* 2008), as well as 0.05 mg/mℓ nystatin (used by Ingham *et al.* 2015) and

0.00004 mg/mℓ DYMED™. The assay plates were sealed with aluminium foil and incubated at 25 °C for 1 day before being assessed.

The percentage of motile J2 was determined for each well. A pipette was used to blow air into a well before the mixture in the well was transferred to a counting dish. The counting dish was then viewed with a stereomicroscope (32 × magnification). Second-stage juveniles with a straight to arcuate habitus, that did not move when touched with a fine needle, were considered immotile. Nematodes with a sinusoidal or curling habitus were considered motile.

### 5.4.2 *In vitro* tests to determine the effect of cell-free culture filtrates on hatching of *M. incognita* second-stage juveniles (J2)

The same experimental design was used as described in Section 5.4.1, and the same mixture of antibiotics was added to each well. Culture filtrates of the same three PGPR selected and used in the J2 motility assays (see Paragraph 5.4.1) were also tested at 10% and 50% concentrations, and water controls as well as broth controls were included.

*Meloidogyne incognita* eggs were obtained for this experiment by staining tomato cv. Moneymaker roots (that had previously been inoculated with *M. incognita*) with 0.015% phloxine B (Merck KGaA, Darmstadt, Germany) for 20 minutes. Individual egg masses were then picked from roots using a sharp needle and placed in tap water. Eggs were extracted from the egg masses using the sodium hypochlorite method described in Section 3.4.1 of this dissertation. Eggs were added to the six-well assay plates at a rate of 200 eggs per well. The final volume of fluid in each well was adjusted to 2 mℓ using sterilised tap water.

Each six-well assay plate was sealed with aluminium foil and incubated at 25 °C for 7 days. The contents of each well were then transferred to a counting dish and viewed with a stereomicroscope (32 × magnification). The number of hatched J2 was counted.

### 5.4.3 Split-root assay

A two-factor completely randomised design was used for this experiment. The two factors were experiments and bacterial strains, with 6 replicates per strain per experiment. Strains of the PGPR N04, T19 and T22 were tested, as well as controls with and without nematodes.

The method used to prepare split-root plants was derived from Schaarschmidt *et al.* (2013). Soybean cv. LS 6248 R, which is susceptible to *M. incognita* (Marais *et al.* 2017). Seeds were

germinated using a modified top-of-paper method (Anonymous, 2006). Whatman® qualitative filter paper discs (GE Healthcare Life Sciences, Chicago, Illnois) were autoclaved for 1 hour at 121 °C. Two filter paper discs were placed in each of five 90 mm diameter sterile Petri dishes. A Sporekill® (ICA International Chemicals (PTY) Ltd, Stellenbosch, South Africa) solution was prepared by adding 20 µℓ of Sporekill® to 1 ℓ of demineralised water. The Sporekill® solution was filter-sterilised with a 0.22 µm pore size syringe filter. The filter paper discs in each dish were moistened with 3 mℓ of the Sporekill® solution (sufficient to moisten the paper discs without causing water flow when the papers were pressed with a finger). Sporekill® contains didecyl dimethyl ammonium chloride (120 g/ℓ), and has contact bactericidal and fungicidal activity (Anonymous, 2018). The disinfectant was used because *Bacillus* seed decay had been problematic while attempting to germinate soybeans on paper in a pilot trial (results not shown). Ten soybean seeds were placed in each Petri dish. The seeds were incubated at 25 °C in darkness for two days. The tips of the seedling roots were then excised with a sterilised scalpel, and the germinating seeds were incubated at 25 °C overnight to allow the cut surfaces to harden. The following day, the seedlings were transferred to 450 mℓ pots filled with sterilised playpen quartzite sand. Each swollen seed was planted at a depth of 1 cm. These pots were maintained in the greenhouse for ten days and were watered daily. Quartzite sand from F. H. Chamberlain (Pty) Ltd (Pretoria, South Africa) was used during the first experiment. During the second experiment, playpen sand from Massbuild (Massmart Holdings Ltd, Sandton, South Africa) was used due to the lack of availability of sand from the previous supplier. Minimum and maximum temperatures in the greenhouse were recorded using a minimum-maximum thermometer. The air-conditioning system in the greenhouse had been set to 25 °C, but temperatures deviated from this level.

After the 10-day period, during which the plants were allowed to form lateral roots, each plant was transplanted to two 750 mℓ pots filled with quartzite sand as illustrated in Figure 5.3. Approximately half of each root system was transferred to each of the two pots. The stem of each plant was tied to a bamboo skewer using rubber bands, while the bamboo skewer and the two pots were secured together using masking tape. One half of each root system was inoculated with 10 mℓ of rhizobacteria suspended in quarter-strength Ringer's solution (prepared as described in Section 3.4.3 of this dissertation). Ten days later, the other half of each root system was inoculated with an aliquot containing approximately 1000 *M. incognita* J2 (prepared as described in Section 3.4.1 of this dissertation. Controls without J2 were inoculated with sterilised water only. Pots were fertilised three times per week with 15 mℓ of half-strength Nutrifeed® (Starke Ayres (Pty) Ltd), Bredell, South Africa).

Plants were harvested 23 days after inoculation. Roots were excised, washed free of sand and placed in plastic containers with ± 200 mℓ tap water. Nematode galls were counted using

a commercial magnifying glass (magnification 2×). Gall indices A and B were calculated as described in Section 4.4.2 of this dissertation, using the methods of Hussey & Boerma, 1981. Shoots and roots of plants were washed, blotted dry and weighed.

### 5.4.4 Statistical analysis of results

Data were tested for normality using the Shapiro-Wilkinson test (α = 0.01). Stem leaf plots, box plots and normal probability plots were also assessed. Extreme outliers were detected by computing the internally studentised residuals of the data, and were excluded from analysis if necessary (The UNIVARIATE procedure, SAS® University Edition, version university.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1). Levene's test (α = 0.01) was used to compare the variances between experiments before deciding whether to pool the data from different experiments. The data was subjected to an analysis of variance (ANOVA) and treatment groups were compared using Tukey's tests (P ≤ 0.05). The percentages of J2 that hatched were subjected to angular transformation if necessary (The GLM procedure, SAS® University Edition, versionuniversity.cny.sas.com@sas:university-3p.2/3p.2.f23fd5825fb4-1-1).

## 5.5 Results

### 5.5.1 *In vitro* test to determine the effect of cell-free culture filtrates on *M. incognita* second-stage juvenile (J2) motility

A significant interaction (F-ratio = 14.18; P < 0.0001) was observed between the effects of experiments (initial and repeat) and treatments. Experiments were therefore analysed separately. During the first experiment, culture filtrates of strains N04, T19 and T22 at both concentrations (10 % and 50 %) reduced the percentage motile *M. incognita* J2 significantly, compared to all controls (α = 0.05) (Figure 5.1). The culture filtrates at a concentration of 10% caused less inhibition of *M. incognita* J2 motility than at a concentration of 50%. Culture filtrates of strains N04, T19 and T22 reduced J2 motility by 11%, 15% and 13%, respectively, at a concentration of 10 %, and by 51 %, 54 % and 77 %, respectively, at a concentration of 50%, compared to water controls. During the second experiment, only the culture filtrates at a concentration of 50% caused significant reductions in J2 motility. Culture filtrates of strains N04, T19 and T22 reduced J2 motility by 26 %, 77 % and 47 %, respectively, at a concentration

of 50%. The percentage of motile J2 did not differ significantly between the water controls, the 10% broth control and the 50% broth control in either experiment.

### 5.5.2 *In vitro* test to determine the effect of cell-free culture filtrates on the hatching of *M. incognita* second-stage juveniles (J2)

A significant interaction (F-ratio = 17.41; P < 0.0001) was observed between the effects of experiments (initial and repeat) and treatments. Experiments were therefore analysed separately. During the two experiments, the culture filtrates of strains N04, T19 and T22 at both concentrations (10% and 50%) reduced the percentage of *M. incognita* J2 that hatched significantly compared to the corresponding broth controls, as well the water controls (α = 0.05) (Figure 5.2). Within this experiment, the effect of the culture filtrates at a concentration of 10% was also significantly lower than that of the culture filtrates at a concentration of 50% (as observed in Section 5.4.1). In the first experiment, culture filtrates of strains N04, T19 and T22 reduced J2 hatching by 68 %, 82 % and 76 %, respectively, at a concentration of 10 %, and by 97%, 94% and 94%, respectively, at a concentration of 50%, compared to water controls. In the second experiment, culture filtrates of strains N04, T19 and T22 reduced J2 hatching by 25 %, 66 % and 69 %, respectively, at a concentration of 10%, and by 91 %, 91 % and 94 %, respectively, at a concentration of 50%, compared to water controls.

In the first experiment, the percentage of J2 that hatched was significantly lower in the 50 % broth control than in the other controls, while the water control and 10 % broth control did not differ significantly. Within the second experiment, a different result was observed: the 10% broth control appeared to have a stimulating effect on J2 hatching compared to the water control, while the 50% broth control caused a reduction in J2 hatching compared to both controls.

### 5.5.3 Split-root assay

Significant interactions were observed between the effects of experiments (initial and repeat) and treatments on root mass (F-ratio = 3.86; P = 0.0052), shoot mass (F-ratio = 2.74; P=0.0406), galls (F-ratio = 3.71; P=0.0111), gall index A (F-ratio = 4.91, P = 0.0031) and gall index B (F-ratio = 3.55, P = 0.0159). Experiments were therefore analysed separately. The selected rhizobacteria did not affect root mass, shoot mass significantly in any of the two experiments (Table 5.1). Strain T22 reduced the number of galls per plant as well as gall index A, significantly (P ≤ 0.05) during the first experiment, but not during the second experiment.

This result indicates that strain T22 induced resistance during the first experiment, but not during the second experiment. Numerous experimental units had to be discarded during the second experiment due to the development of phytotoxic symptoms on leaves. The cause of the phytotoxic symptoms may have been caused by a chemical contaminant present within the batch of quartzite sand used.

### 5.6 Discussion

The results of the current study suggest that *L. sphaericus* strain T19, *P. alvei* strain T22 and *Pseudomonas* sp. strain N04 may produce metabolites (in broth culture) that inhibit the motility and hatching of *M. incognita* J2. The concentrations at which the culture filtrates reduced *M. incognita* J2 motility and hatching are comparable to those concentrations at which Mendoza *et al.* (2008) detected inhibition of *M. incognita* J2 motility and hatching. The secondary metabolites responsible for these activities are yet to be identified, but strain N04 is known to produce hydrogen cyanide during fermentation (personal communication, Stacy Lovell, 2016). Hydrogen cyanide may play a role in the nematode-suppressive activity of several strains of *Pseudomonas* spp. (Kang & Kim, 2018). Yang *et al.* (2012) found that a strain of *Lysinibacillus mangiferahumi* sp. nov. that produces volatiles that suppress motility of *M. incognita* J2. The metabolites produced by *Lysinibacillus sphaericus* strain T19 are being investigated by other students (in other studies) at the University of Pretoria.

The results of the *in vitro* assays may indicate that the amounts of metabolites produced by the rhizobacteria during fermentation, or the susceptibility of the *M. incognita* J2 to these metabolites, varied between experiments (Table 5.1). The production of secondary metabolites by microorganisms tends to be variable and is affected by different environmental factors (Laur *et al.* 2018). The effects of the nutrient broth (used in this study) on J2 hatching also varied between experiments (Table 5.2). Previous studies have also found that liquid culture media affect the J2 hatching of phytonematodes (Meyer *et al.* 2004).

Strain T19 was the most promising biocontrol agent against *M. incognita* (among the selected strains) according to the results reported in Chapter 4 of this dissertation. Culture filtrate produced with strain T19, at a concentration of 50 %, reduced J2 motility by 54 % to 77 % (compared to water controls), whilst reducing J2 hatching by 91 % to 94 %. This culture filtrate, at a concentration of 10%, reduced J2 hatching by 66 % to 82 % (compared to water controls). These results are comparable to that obtained by Mendoza *et al.* (2008) with *B. firmus*.

*Paenibacillus alvei* strain T22 controlled *M. incognita* less consistently than strain T19 as reported in Chapter 4 of this dissertation. Strain T22 appeared to induce systemic resistance against *M. incognita* in one of two experiments reported in the current chapter. Furthermore, this strain was effective for promotion of soybean growth in soil with suboptimal fertiliser levels, as suggested in Chapter 4 of this dissertation. Kloepper *et al.* (2004) suggests that members of the Bacillaceae that control diseases through induced resistance may be most effective if applied at several time-points in a season. The results of the split root assay in the current study should be viewed as preliminary results. In the future, the split-root assay should be repeated with soil with suboptimal fertiliser levels instead of playpen sand.

Within several pilot *in vitro* experiments (of which the results have not been reported here), chemical dyes were used to stain *M. incognita* J2 to assess the effect of rhizobacterium culture filtrates on *M. incognita* viability. Methylene blue (uniLAB®, Merck KGaA, Darmstadt, Germany) was used as a non-vital dye according to the method of Gold (1997). Culture filtrates at concentrations of 10% or 50% did not cause significant increases in J2 mortality after 1 day of incubation (according to the results with the methylene blue method. Methylene blue is broken down in living trematodes by an enzyme-catalysed reaction to form a colourless compound, while dead trematodes remain dark blue after a period of incubation (Peak & Hoffmann, 2011). Nematodes that were killed heat remained dark blue after being incubated with methylene blue, while motile nematodes were not stained (results not shown). Killing nematodes with heat would lead to denaturation of the enzymes inside the nematodes, but a chemical that paralyses nematodes might not cause short-term changes in the activity of certain enzymes.

Neutral red was tested as an alternative to methylene blue using the method of Zetsche & Meysman (2012), but no differences could be seen between the colour of motile and heat-killed *M. incognita* J2 after being stained with neutral red, using the stereomicroscope being used for the research. The stain was used successfully, however, with (larger) bacterivorous nematodes (results not shown).

Results of *in vitro* tests should be interpreted with caution, as mentioned earlier in the Introduction. Certain microorganisms may produce metabolites that reduce nematode motility within conventional *in vitro* tests but have sub-lethal effects on nematodes at lower concentrations (Nandi *et al.* 2015). In this chapter, an experiment was attempted with the aim of assessing the effects of selected rhizobacteria on the attractiveness of root exudates to nematode J2. The ability of the metabolites from the selected rhizobacteria to repel *M. incognita* J2 was not successfully assessed. A future study should assess the sub-lethal

effects of metabolites from strain T19 on root-knot nematodes, and the amounts of antibiotics produced in the rhizosphere.

### 5.7 Conclusion

In the current study, culture filtrates of *L. sphaericus* strain T19, *P. alvei* strain T22 and *Pseudomonas sp.* strain N04 reduced the motility and hatching of *M. incognita* J2. *Paenibacillus alvei* strain T22 may induce resistance to *M. incognita* in soybean, but the efficacy of this strain as a bionematicide is variable. Further research should attempt to assess the sub-lethal effects of strain T19 on root-knot nematodes, and to identify metabolites of interest produced by strain T19. A future study should include an attempt to assess the vital dye neutral red for use in multi-well plate assays with *M. incognita*, using an inverted microscope.

## 5.8 Tables and figures

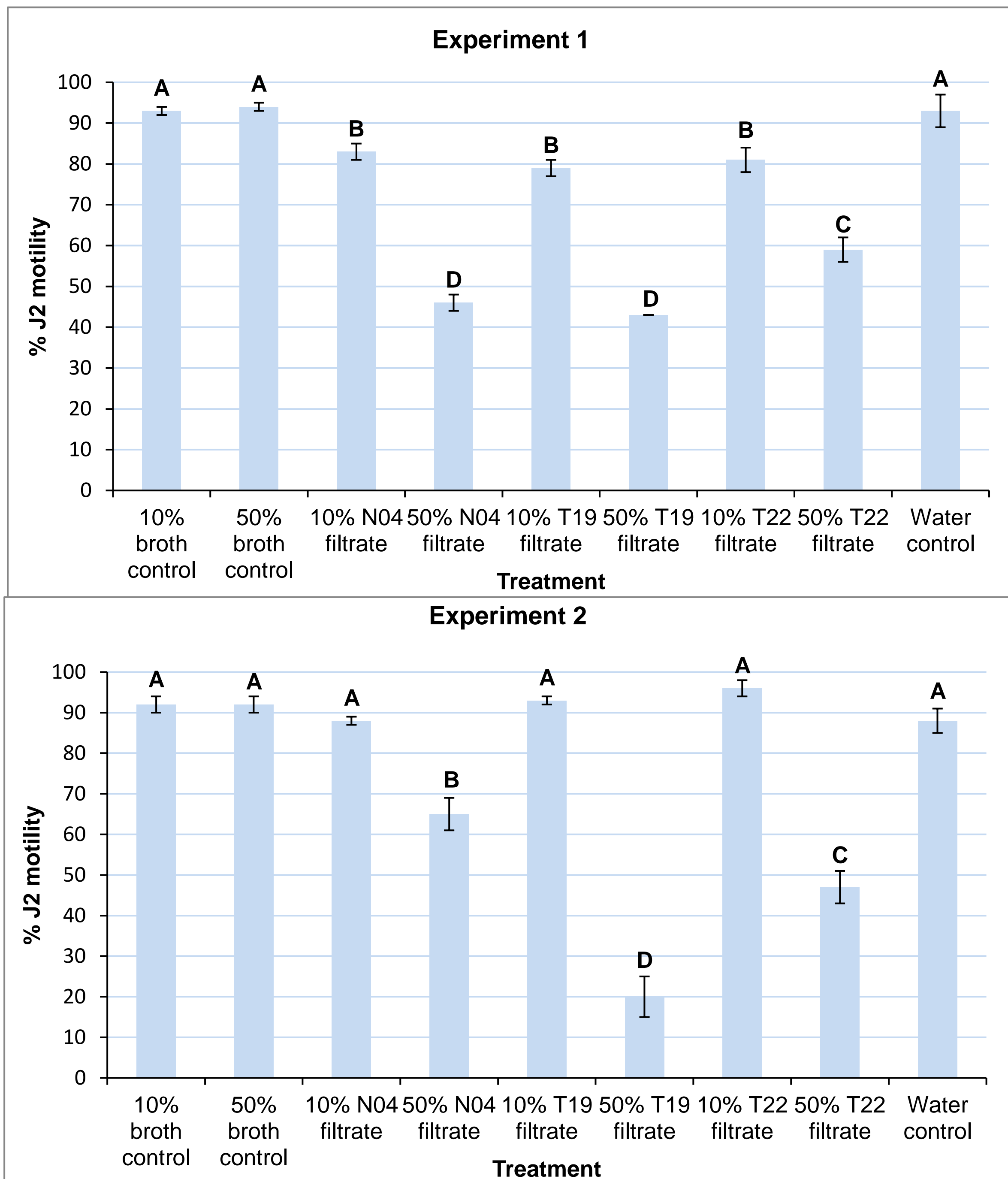


**Figure 5.1 - Effect of culture filtrates of selected rhizobacteria on *Meloidogyne incognita* second-stage juvenile (J2) motility in a multi-well plate assay.**

Juvenile motility was measured after 1 day of incubation at 25 °C. Means of six replicates are shown for treatment group. Errors bars indicate the standard error of the mean. Means with the same letter are not significantly different according to Tukey's test (α = 0.05).

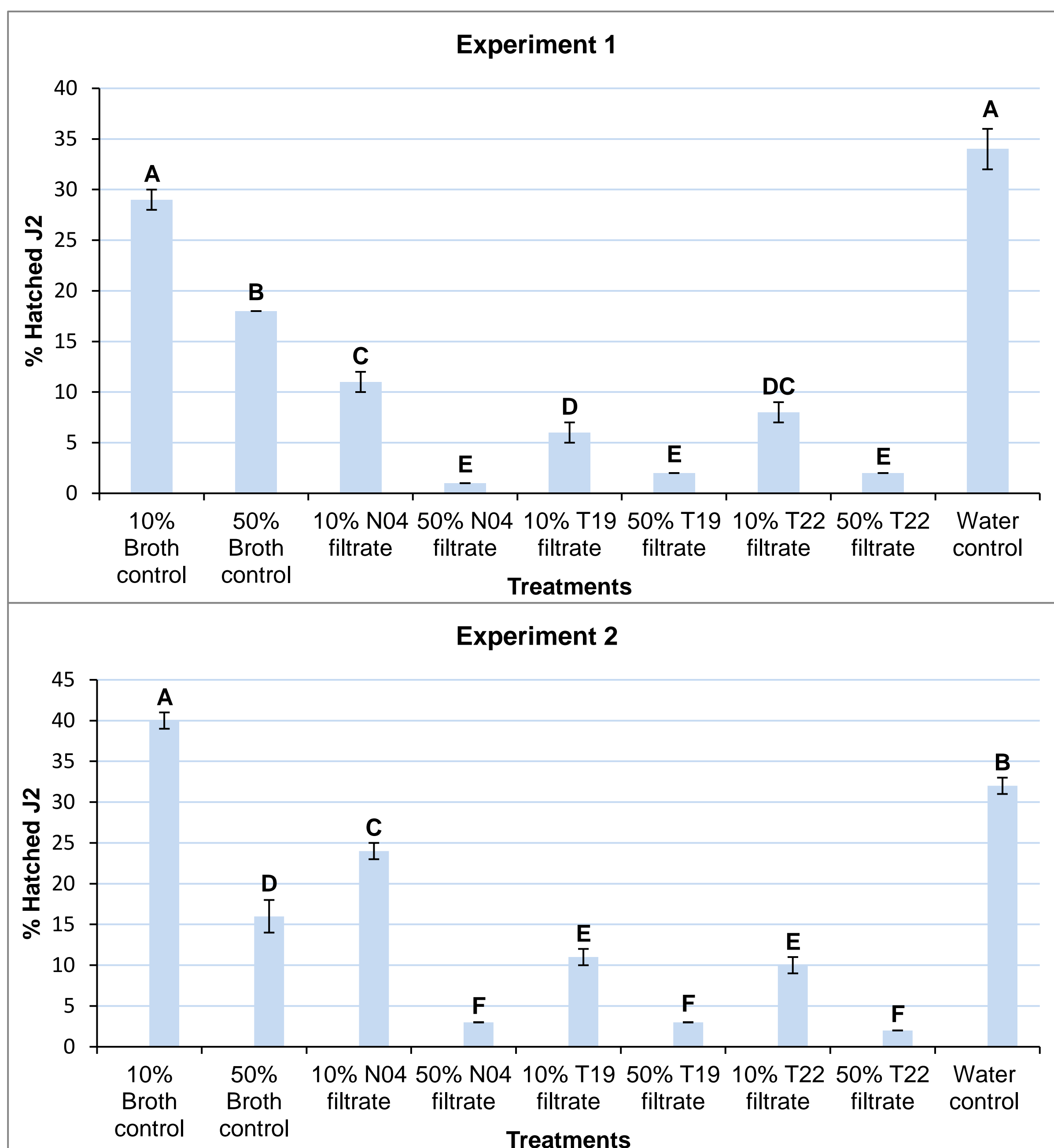


**Figure 5.2 - Effect of culture filtrates of selected rhizobacteria on *Meloidogyne incognita* second-stage juvenile (J2) hatching in a multi-well plate assay.**

Juvenile motility was measured after 7 days of incubation at 25 °C. Means of six replicates are shown for treatment group (except for the water control in the first experiment, from which one extreme outlier was excluded from analysis). Errors bars indicate the standard error of the mean. Means with the same letter are not significantly different according to Tukey's test ($\alpha = 0.05$). Data in the first experiment was subjected to angular transformation before analysis (summary data for untransformed values are presented here).

**Table 5.1 – Results of split-root assays to evaluate whether selected rhizobacteria could induce resistance in soybean against *Meloidogyne incognita* second-stage juveniles (J2)**

| Treatment | N[1] | Fresh root mass (g) | | Fresh shoot mass (g) | | Number of galls per root system | | Gall index A | | Gall index B | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Means | SE[2] | Means | SE | Means | SE | Means | SE | Means | SE |
| **Experiment 1** | | | | | | | | | | | |
| Control without J2 | 7 | 3.789 a | 0.529 | 3.444 a | 0.543 | 0 c | 0 | 0.0 c | - | 0.0 b | - |
| Control with J2 | 8 | 3.629 a | 0.352 | 3.448 a | 0.341 | 25 a | 11 | 3.1 a | 0.1 | 1.0 a | - |
| N04 | 8 | 4.056 a | 0.391 | 3.693 a | 0.708 | 19 ab | 8 | 2.9 ab | 0.1 | 1.0 a | - |
| T19 | 8 | 3.381 a | 0.299 | 3.459 a | 0.576 | 20 ab | 4 | 3.0 ab | 0.0 | 1.0 a | - |
| T22 | 8 | 3.832 a | 0.380 | 3.369 a | 0.733 | 13 b | 7 | 2.6 c | 0.2 | 1.0 a | - |
| **Experiment 2** | | | | | | | | | | | |
| Control without J2 | 2 | 6.938 a | 1.408 | 4.129 a | 0.088 | 0 c | 0 | 0.0 a | 0.0 | 0.0 a | - |
| Control with J2 | 3 | 4.460 a | 0.284 | 3.204 a | 0.116 | 9 a | 2 | 2.3 a | 0.3 | 1.0 a | 0.0 |
| N04 | 3 | 7.120 a | 0.553 | 4.386 a | 0.919 | 3 ab | 1 | 1.7 a | 0.3 | 1.0 a | 0.0 |
| T19 | 3 | 7.480 a | 1.329 | 5.547 a | 0.873 | 4 ab | 2 | 1.3 a | 0.7 | 0.7 a | 0.5 |
| T22 | 3 | 4.299 a | 0.665 | 4.552 a | 0.529 | 3 ab | 2 | 1.3 a | 0.7 | 0.7 a | 0.5 |

Temperatures in the greenhouse varied between 18 and 38 °C. Means followed by the same letter are not significantly different according to Tukey's test ($\alpha = 0.05$).

[1]N denotes the number replicates.

[2]SE denotes the standard error of the mean (the SE of certain gall index means could not be calculated, because the standard deviation was 0).

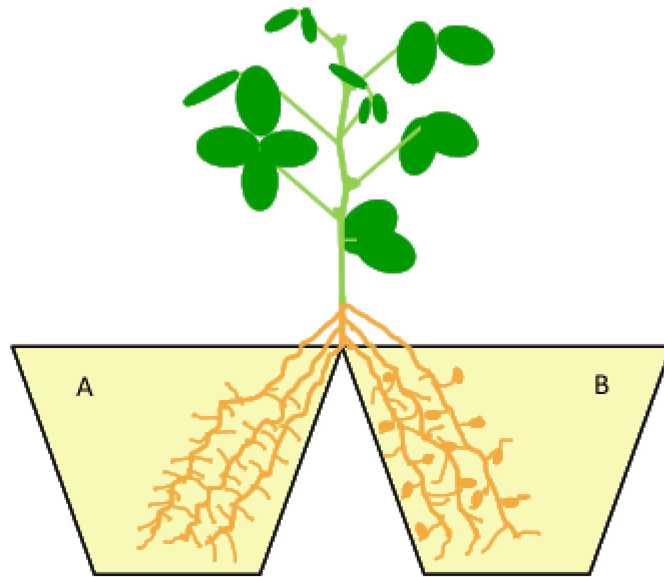


**Figure 5.3–** Illustration of the setup of split-root experiments in which selected rhizobacteria were applied (inducer side A) to determine whether they could induce resistance in soybean infected with *Meloidogyne incognita* second-stage juveniles (J2) (responder side B).

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# CHAPTER 6

## General conclusion

Biological control has high potential for wider use within the near future (Wezel *et al.* 2014). Biocontrol agents tend to be less effective than traditional chemical nematicides but may be useful if the nematode population is below an economic threshold, or if the biocontrol agent is used as part of an integrated management system (Barker & Koenning, 1998; Hughes, 1996). The modes of action through which plant growth-promoting rhizobacteria (PGPR) suppress phytonematodes are not fully understood, and may include priming of plant defences, production of hydrolytic enzymes, and production of mixtures of nematode-suppressive toxins (Kloepper *et al.* 2004; Lee *et al.* 2013; Meyer *et al.* 2009). Study of biocontrol agents may lead to the discovery of compounds or genes with commercial value (Kong *et al.* 2018).

In Chapter 3 of this dissertation, a seedling bioassay was used to screen 22 strains of PGPR for biocontrol of *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 on soybean (*Glycine max* L. Merrill) seedlings. The bacteria were applied as drench treatments, and the seedling bioassay was repeated with strains that reduced the number of *M. incognita* galls per seedling by 30% or more. *Lysinibacillus sphaericus* strain T19, *Paenibacillus alvei* strain T22 and *Pseudomonas fluorescens* strain N04 reduced the number of *M. incognita* galls per plant by 31 %, 38 % and 32 %, respectively, during the first round of screening, and by 67 %, 32 % and 44 %, respectively, during the second round. This study underscored the need for high-throughput methods to screen PGPR against phytonematodes.

In Chapter 4, *L. sphaericus* strain T19, *P. alvei* strain T22 and *P. fluorescens* strain N04 were tested in greenhouse trials. Strain T19 proved to be the most consistent-performing biocontrol agent among the selected strains. The strain (applied as a Perlite powder seed treatment) reduced the number of *M. incognita* egg masses on roots of soybean plants by 64% and 86%, respectively, in two experiments (compared to nematode-infected controls that were not treated with PGPR). The bacterium did not, however, increase plant mass significantly (compared to *M. incognita* infected controls) within the biocontrol experiments. The efficacy of strain T19 could be increased by increasing the concentration of bacteria in the Perlite powder formulation (Rudolph, 2014). A new formulation could be developed, which could include amendments that increase the biocontrol activity of strain T19 (Hamid *et al.* 2003). Strain T19 could also be combined with a seed-applied nematicide such as abamectin (Avicta® 500FS; Syngenta, Basel, Switzerland) (Becker & Morton, 2014), or a resistance-inducing chemical such as chitosan (Meyer, 2003). Strains T19 and T22, applied as Perlite powder seed

treatments, increased dry shoot mass of plants by 84 % and 124 %, respectively, and leaf area by 84 % and 124 %, respectively, in an experiment to test plant growth enhancement. Soil with suboptimal (reduced) fertiliser levels had been used. This experiment indicates that strains T19 and T22 have potential for use as active microorganisms within biofertilisers on soybean. In future, interactions between these strains and the nitrogen-fixing bacterium *Bradyrhizobium japonicum* could be tested in pot trials with reduced fertiliser levels. Strains T19 and T22 can potentially also be investigated for use in phytostabilization of mined soils.

In Chapter 5, the ability of selected rhizobacteria to produce nematode-suppressive metabolites was assessed during *in vitro* assays. *Lysinibacillus sphaericus* strain T19, *P. alvei* strain T22 and *P. fluorescens strain* N04 appear to produce metabolites (in broth culture) that reduce motility as well as hatching of *M. incognita* second-stage juveniles. The *in vitro* activity of strain N04 may be attributed, at least in part, to production of hydrogen cyanide. In this chapter, a split-root assay was attempted. Strain T22 induced resistance against *M. incognita* in soybean in one of two split-root experiments. The split-root assay should be repeated in a future study in different plant growth substrates. The secondary metabolites produced by strain T19 and their sub-lethal effects on phytonematodes, should be further investigated in a future study.

In general, this study demonstrates that *L. sphaericus* strain T19 has potential for use in the integrated management of *M. incognita* on soybean. This biocontrol agent was only assessed against one population of *M. incognita* and on one soybean cultivar in the current study. Field trials would be needed to assess the performance of strain T19 under natural conditions. Further research is also warranted to improve the formulation used before field trials are undertaken. A mixture of fermentation products produced by strain T19, similar in concept to DiTera® (Valent BioSciences LLC, Libertyville, Illnois), could be commercialised as an alternative nematode-suppressing product (DiTera® is a mixture of fermentation products produced by an isolate of the fungus *Myrothecium verrucaria* (Copping & Duke, 2007)).

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