# Metabolite profiling of *Bacillus* species with nematicidal activity

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# ABSTRACT

Root-knot nematodes continue to be a global problem in agriculture causing major economic losses. Various *Bacillus* spp. have the potential to inhibit the *Meloidogyne* root-knot nematode populations. Although many studies conclude that the secondary metabolites of relevant *Bacillus* spp. are responsible for nematicidal activity, the specific metabolites are not characterised. Subsequently, the efficacy and reproducibility of biocontrol products are questionable. The aim of this study was to compare the metabolic profiles of *Bacillus* spp. with known nematicidal activity to bacteria without nematicidal activity. For this purpose, four *Bacillus* spp. known to exhibit inhibitory effects towards second-stage juveniles (J2) of *Meloidogyne incognita,* namely *B. cereus*, *B. firmus*, *B. subtilis* and *B. pumilus* were compared to two bacteria without known nematicidal activity – *Escherichia coli* and *B. soli*. Bacterial strains were cultivated in two types of media, namely Luria-Bertani (LB) broth as a complex medium and minimal broth (MB) as a simpler medium. This was done to evaluate the effect of the medium composition on bacterial metabolism and also on metabolomics analyses performed using liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). The first step was to do nematicidal bioassays to confirm the nematicidal activity of cell-free filtrates obtained from the bacteria. During the bioassays the motile and paralysed J2 nematodes were quantified after exposure to different concentrations of cell-free filtrates presumably containing secondary metabolites of the bacteria. From the results obtained it was evident that all of the selected *Bacillus* spp., as well as *E. coli*, had nematicidal effects on the *M. incognita* J2. When cultivated in LB broth with optimised incubation times, *B. firmus* and *B. pumilus* showed the highest nematode paralysis in the bioassays. However, when cultivated in MB, *B. firmus* and *B. cereus* showed the highest nematode paralysis. Due to the nematicidal activity observed for *E. coli* and *B. soli*, the bioassays were repeated using *E. coli* OP50 as control. This strain caused the lowest levels of paralysis in all assays and was therefore a more appropriate control that should be used in further studies. Untargeted metabolomics distinguished between metabolite profiles from the different *Bacillus* spp. Moreover, there was a clear difference between profiles when bacteria were cultivated in the different media. Bacterial cultures produce extracellular metabolites in response to their surrounding environment and it is critical that specific bacterial species are matched with the optimal cultivation media to ensure reproducible production of compounds of interest before identification of metabolites is attempted.

**Keywords:** *Bacillus*; biocontrol; *Meloidogyne incognita*; metabolomics analysis; root-knot nematodes

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| | |
|---|---|
| amu | atomic mass unit |
| ANOVA | analysis of variance |
| BSTFA | N,O-bis(trimethylsilyl)trifluoroacetamide |
| cm | centimetre |
| °C | degrees Celsius |
| $CO_2$ | carbon dioxide |
| $CaCl_2$ | calcium chloride |
| DNA | deoxyribonucleic acid |
| EI | electron impact ionisation |
| ESI | electron spray ionisation |
| eV | electron volt |
| FAME | fatty acid methyl ester |
| $FeSO_4.7H_2O$ | ferrous sulphate heptahydrate |
| g | gram(s) |
| g’s | gravitational force |
| GC-MS | gas chromatography mass spectrometry |
| h | hour(s) |
| HILIC | hydrophobic interaction chromatography |
| iPCA | interactive Principal Component Analysis |
| IS | internal standard |
| J2 | second stage juveniles |
| KCl | potassium chloride |
| $KH_2PO_4$ | monopotassium phosphate |
| LC-MS | liquid chromatography mass spectrometry |
| L/min | liter per minute |
| MeBr | methyl bromide |
| MeOX | methoxyamination solution |
| mg/ml | milligram per millilitre |
| min | minute(s) |
| mL/min | millilitre per minute |
| ml | millilitre |
| mm | millimetre(s) |
| mRNA | messenger ribonucleic acid |
| m/z | mass to charge ratio |
| $MgSO_4.7H_2O$ | magnesium sulphate heptahydrate |
| $MnSO_4.4H_2O$ | manganese(II) sulphate tetrahydrate |
| NaCl | sodium chloride |
| $Na_2HPO_4$ | disodium phosphate |
| NaOCl | sodium hypochlorite |

| | |
|---|---|
| $(NH_4)_2SO_4$ | ammonium sulphate |
| nm | nanometre |
| NMR | nuclear magnetic resonance |
| OECD | Organisation for Economic Co-operation and Development |
| OD | optical density |
| PCA | Principal Component Analysis |
| (Pty) Ltd | property limited |
| psi | pounds per square inch |
| PPN | plant parasitic nematode |
| RKN | root-knot nematode |
| RNA | ribonucleic acid |
| ROS | reactive oxygen spp. |
| rpm | revolutions per minute |
| sec | second(s) |
| TMCS | trimethylchlorosilane |
| TOF-MS | time-of-flight mass spectrometry |
| Tris-HCl | tris hydrochloride |
| UK | United Kingdom |
| USA | United States of America |
| V | volts |
| v/v | volume to volume |
| w/v | weight per volume |
| µg/ml | microgram per millilitre |
| µl | microliter(s) |
| µm | micrometre(s) |

# CHAPTER 1 INTRODUCTION

## 1.1 *Meloidogyne incognita* and possible biocontrol agents

Plant parasitic nematodes (PPNs), specifically root-knot nematodes (RKNs), are of major global economic importance due to their devastating effect on a variety of agricultural crops including tomato and cucumber. One of the most important RKNs is *Meloidogyne incognita* due to its worldwide geographical distribution, large host range and its ability to form microbial symbiotic relationships with fungi and bacteria that reduce plant pathogen resistance (Terefe *et al.*, 2009; Xiong *et al.*, 2015). *Meloidogyne incognita* infections lead to altered levels of various amino and organic acids as well as reduced levels of chlorophyll within the infected plant. Thus *M. incognita* infections cause reductions in the bioactive compounds produced by the roots and leaves of crops (Huang *et al.*, 2016). The infection process of *M. incognita* starts with the J2 of the RKNs penetrating crop roots to establish a permanent feeding site and cause knots in root tissues that prevent the plant from nutrient and water uptake (Eloh *et al.*, 2015). The J2 of RKNs are hatched in eggs. These eggs are encapsulated in egg masses laid by females on the infected roots. Thereafter, hatched J2 migrate into the soil to find new roots (Hashem & Abo-Elyousr, 2011).

Root-knot nematodes inhabit soil and are subject to infection by indigenous fungi and bacteria. Some of these microorganisms have been identified as having nematicidal effects, making them potential eco-friendly biocontrol agents (Xiong *et al.*, 2015). Research into biological control has been ongoing for many years and continues to be important as more chemical pesticides are banned due to their detrimental effects on environmental health. Different microorganisms affect nematodes by various modes of action, including parasitising, producing nematicidal toxins, competing for nutrients, interfering with nematode-plant recognition, inducing systemic resistance of plants and nematode trapping (Demain, 1998; Terefe *et al.*, 2009; Xiong *et al.*, 2015).

Specifically, there are several *Bacillus* spp. that have shown nematicidal activity against *Meloidogyne* populations. These include *B. pumilus, B. cereus, B. firmus, B. megaterium, B. nematocida, B. subtilis* and *B. thuringiensis* (Lee & Kim, 2016). Some of the modes of action of these microorganisms, such as that of *B. thuringiensis* have been well documented but many still require clarification and literature often refers simply to “toxins” or “secondary metabolites” as possible modes of action against RKNs. Other studies have tested the effect of *Bacillus* isolates and concluded that they “hold immense potential” as biocontrol agents, but without contributing to what the mode of action is (Giannakou *et al.*, 2004; Mendoza *et al.*, 2008; Terefe *et al.*, 2009; Xiong *et al.*, 2015; Zhang *et al.*, 2016).

## 1.2 Importance of metabolomics

For a long time, the discovery of new natural products and bioactive compounds were limited by a lack of high throughput analytical techniques capable of identifying novel structures. Until now, the selection of bacterial strains with biocontrol potential has been based predominantly on phenotypic qualities instead of laboratory production of a wide range of secondary metabolites (Hou *et al.*, 2012; Lee & Kim, 2016). Now, metabolomics is providing new opportunities to address these limitations and to fill the knowledge gap. The advances in analytical instruments, development of specific software and on-line databases have contributed towards the standardisation of metabolomics approaches (Aliferis & Chrysayi-Tokousbalides, 2010).

Metabolomics has been defined as the comprehensive qualitative and quantitative profiling of the metabolites of a biological system (Aliferis & Chrysayi-Tokousbalides, 2010). Metabolic products can serve as indicators of the interactions between a cell's genome and its environment, making it possible for metabolomics to provide an unbiased valuation of a cellular state in a certain environment (Tang, 2011). Microbial metabolomics aim to analyse the metabolome of bacterial cells which consist of an estimated 200 to 2 000 metabolites (Liebeke *et al.*, 2012). Metabolites produced from bacterial metabolism can be classified into primary and secondary metabolites of which the former are involved in growth, development and reproduction of the organism, while the latter are not. These secondary metabolites rather contribute to the survival of the bacterial cells (Willey *et al.*, 2011).

Untargeted metabolomics was defined by Liebeke and Lalk (2014) as the "relative quantification of a complete set of both known and unknown metabolites in a biological sample, in an unbiased way and without the need for prior information of metabolite identities". In this investigation untargeted metabolomics was used to determine the presence of secondary metabolites that may be responsible for the nematicidal activity. There are many bacterial species from different genera that show potential as bionematicides and many of these can be studied in more detail than before with the addition of metabolomics data to existing knowledge. The discovery of bioactive compounds with modes of action different than commercially-used pesticides is of practical importance for predicting effects on non-target organisms, but also for combating pest resistance (Aliferis & Chrysayi-Tokousbalides, 2010). Firstly, the study aimed to assess nematicidal activity of selected strains and cultivation media by means of bioassays. This was followed by profiling of the metabolite features of the *Bacillus* spp. that exhibited nematicidal activity. The study was restricted to *Bacillus* spp. to limit variation between cultures due to diverse types of cultivation media and differences in metabolomes between genera. The present investigation will also serve as the basis for future investigations of a similar nature to extend the number of bacteria that will be characterised in this manner for potential biocontrol applications.

## 1.3 Problem statement

Global agricultural losses due to the destructive activities of RKNs amount to billions of US dollars annually. In the past, chemical pesticides were used to control *Meloidogyne* spp. (Nicol *et al.*, 2011). However, the use of chemical pesticides has led to pest resistance, pollution and concerns for human health. Subsequently, biopesticides became the preferred choice for pest control. However, not all biopesticides have been fully developed and there is a continuous search for effective, environmentally friendly products (Lee & Kim, 2016).

Contrary to chemical pesticides, biopesticides are host-specific and do not have the same detrimental effects on non-target species (Mnif & Ghribi, 2015). There are many organisms, specifically bacteria in the case of RKNs, that are potential biopesticides but not all have been studied to the same extent, and the mode of action of many bacteria that exhibit nematicidal activity is still unclear. This is problematic since a number of biological control products currently available claim to contain strains of microorganisms with nematicidal activity. Yet, the active ingredients are not quantified, and the efficacy are therefore doubtful and hard to replicate.

To gain insight into the properties of nematicidal compounds produced by *Bacillus* spp., it is necessary to study their metabolite production. The arrival of metabolomics as an essential component of the functional genomics approach has brought a new dimension to the study of biological systems. Within the context of agriculture and crop protection, metabolomics is a valuable tool for high throughput screening of naturally produced bioactive substances to discover those with unique modes of action, high selectivity and acceptable ecotoxicological/toxicological profiles (Aliferis & Chrysayi-Tokousbalides, 2010; Macintyre *et al.*, 2014).

## 1.4 Research aims and objectives

The aim of this investigation was to compare the metabolic profiles of *Bacillus* spp. with known nematicidal activity, to bacteria without nematicidal activity. Specific objectives included:

1. Assessing and selecting appropriate culture media and conditions for nematode bioassays.
2. Testing *B. cereus, B. firmus, B. pumilus, B. subtilis, B. soli,* and *E. coli* for nematicidal activity against *M. incognita* J2.
3. Evaluating the application of LC-MS and GC-MS for analysing complex (Luria-Bertani broth) and simpler (minimal broth) cultivation media and the bacterial metabolites produced in each.
4. Profiling the bacterial metabolites from filtrates tested against *M. incognita* J2.

### 1.5 Chapter layout

**This dissertation represents a combination of published and unpublished work, presented as follows:**

**Chapter 1** is the current chapter and provides an introduction to the study which describes the use of microbial species as biocontrol agents against nematodes, specifically *M. incognita*. The role of metabolomics in the characterisation of bacterial metabolites is also introduced. In addition, this chapter provides the problem statement, aim and specific objectives of the study.

**Chapter 2** reviews the literature available on the impact that *M. incognita* has on agriculture and different modes of action that can be used by *Bacillus* spp., especially metabolites that will aid in the battle against *M. incognita*. This chapter also discusses the role of metabolomics methods that can be used to profile and identify nematicidal metabolites. Parts of this chapter have been published in *Biocontrol Science and Technology* under the title “Bacillus-based bionematicides: development, modes of action and commercialisation” (full reference is provided in the chapter).

**Chapter 3** describes the methods used in this study. This includes cultivation of bacterial species, rearing of nematode populations, bacterial filtrate separation, nematode bioassays, metabolomics analyses and statistical analyses.

**Chapter 4** describes the results obtained from the evaluation of different cultivation media to use for further analysis, as well as bioassays of bacterial filtrates for their efficacy in paralyzing J2 of *M. incognita*. This chapter also includes growth curves of the bacteria to validate the incubation period required to obtain the stationary growth phase of bacterial species.

**Chapter 5** describes the untargeted metabolomics results obtained from the bacterial filtrates of the species under investigation when cultivated in LB broth and MB, respectively.

**Chapter 6** provides a general discussion of all the stated objectives and a conclusion with recommendations for future studies.

**References** are provided at the end of the dissertation.

**Appendix A** includes a table with replicate results and statistical significance between the nematode body shapes after being exposed to filtrates of bacterial species cultivated in LB broth.

**Appendix B** includes a table with replicate results and statistical significance between the nematode body shapes after being exposed to filtrates of bacterial species cultivated in MB.

**Appendix C** contains the title page of the published article included in Chapter 2.

# CHAPTER 2 LITERATURE REVIEW

**Part of this chapter was published as:**

Engelbrecht, G., Horak, I., Jansen van Rensburg, P.J. & Claassens, S. 2018. Bacillus-based bionematicides: development, modes of action and commercialisation. *Biocontrol Science and Technology*, 28(7):629-653.

## 2.1 Plant parasitic nematodes

According to Kenney and Eleftherianos (2016), the nematode phylum is vast, consisting of a large amount of parasitic and opportunistic species. Figure 1 shows the classification of selected plant parasitic nematodes (PPNs). These species are capable of infecting plant hosts. Haegeman *et al.* (2012) define PPNs as microscopic organisms that largely parasitise on the plant roots and other plant organs. When these PPNs feed on plant roots, they do not enter the plant tissue, while those feeding on plant organs penetrate plant tissues. Plant parasitic nematodes that stay outside the hosts are known as ectoparasites, while those that penetrate plant tissues are known as endoparasites (Haegeman *et al.*, 2012). Various ectoparasitic nematodes such as *Paratrichodorus minor* and *Xiphinema elongatum* can be found across Western and Southern Africa causing extensive damage to crops such as sugarcane (Meyer, 2017; Rimé *et al.*, 2003), while endoparasitic species include the sedentary endoparasite *Meloidogyne*, with a global distribution and wide host range, and *Pratylenchus* (migaratory endoparasite) (Jones *et al*., 2013; Williamson & Hussey, 1996).

Plant parasitic nematodes possess the ability to damage and/or degrade the cell wall of the host leading to manipulation of the host signalling pathways. This is possible as PPNs use a needle-like structure, called a stylet, to damage the rigid structure of the plant cell wall as it penetrates the cell during feeding. Other nematode genera, such as *Meloidogyne*, can be more elusive as they utilise their stylet to separate adjacent cell walls as the nematode moves into the apoplastic spaces that exist between these cells. This is the initial step in the infection process of host plants by the PPNs (Haegeman *et al.*, 2012).

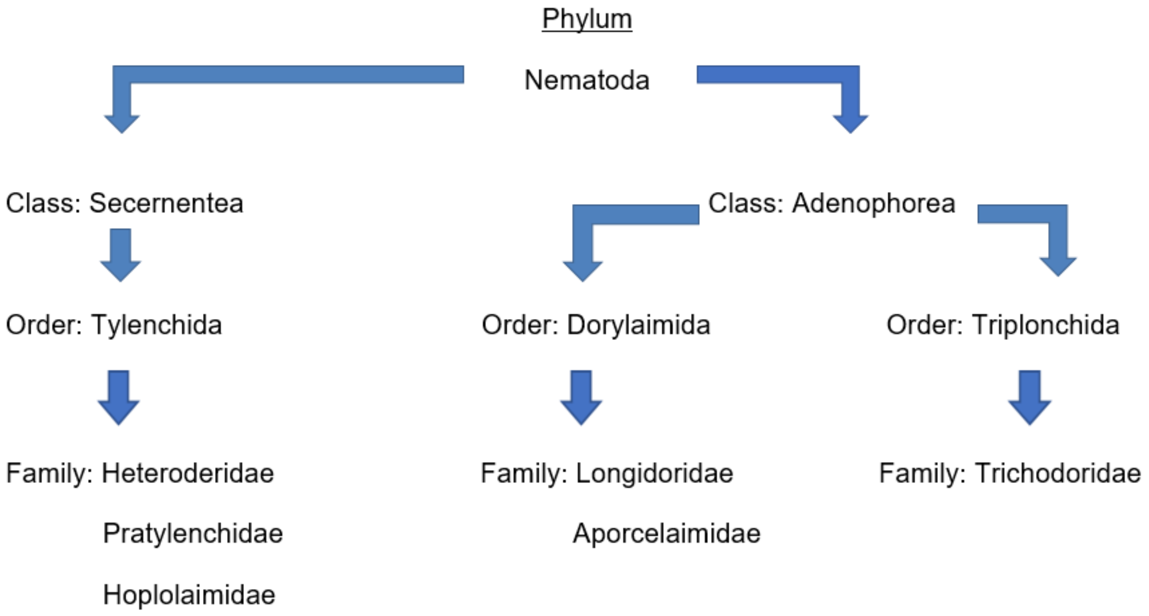

*Figure 1*: *The classification of selected PPN families (adapted from Van den Berg et al., 2017).*

## 2.2 The impact of *Meloidogyne* spp. on agricultural crops

The loss of agricultural crops may be caused by various biotic and abiotic environmental factors, including droughts, elevated temperatures and plant parasites. These factors will cause a reduction in crop yields, leading to a lower actual yield than the site-specific achievable yield/production for specific crops. Furthermore, the productivity, efficiency and quality of crops grown for consumption are at risk due to the increased occurrence of plant parasitic pests (Oerke, 2006). Plant parasitic nematodes alone cause global crop losses of an estimated $78 billion (Lima *et al.*, 2017). Of these PPNs, the nematode species that are commonly known as RKNs (*Meloidogyne incognita, Meloidogyne javanica*, *Meloidogyne arenaria* etc.) can be the most harmful. Due to their global distribution and wide range of host plants, they can cause substantial amounts of damage to various economically important crops, such as potatoes, grain, oilseed, industrial and fruit crops (Jones *et al.*, 2017). Of all documented *Meloidogyne* spp, 22 are reported to occur in Africa. These species include *M. incognita* that threatens economically important crop yields (Onkendi *et al.*, 2014) of certain African countries, such as Kenya. In a study done by Karuri *et al.* (2017) in Kenya, *M. incognita* was found to be present in all of the sampled sweet potato fields. Since sweet potato is an important staple food in Kenya and serves as an income source for many families (Claessens *et al.*, 2008), increased levels of *M. incognita* in these crops can contribute to national food security issues. Crop yields in other countries, such as South Africa

(Onkendi *et al.*, 2014) and Spain (Giné *et al.*, 2014), are also severely impacted by *Meloidogyne* infestations. However, *Meloidogyne* spp. are not a recent threat to agriculture. As early as 2001 the threat of *M. incognita* was already being studied. A study conducted in South Africa by Fourie *et al.* (2001) found *Meloidogyne* spp., especially *M. incognita*, to be present in 16 of the 17 studied soybean sites. These results correspond with other reports that list *M. incognita* as the most infective RKN of soybean crops globally (Fourie *et al.*, 2001).

Soybeans are not the only crops affected by *Meloidogyne* infestations. In Spain, cucumber is an important crop with yields of up to 664 975 tons annually. Even under protected cultivation, *Meloidogyne* spp. can cause up to 88% yield loss of cucumber crops (Giné *et al.*, 2014). The impact that *Meloidogyne* spp. can have on crop yields is completely miscalculated according to Onkendi *et al.* (2014). When compared to the rest of the world, these miscalculations occur more in Africa, making it plausible for annual crop losses to be much higher (Coyne *et al.*, 2006). Several factors contribute to the lack of available information on the economic impact of *Meloidogyne* spp. on crop production across Africa (Onkendi *et al.*, 2014). Firstly, the effects of these pathogens tend to go unnoticed. Furthermore, the lack of resources devoted to the development of new nematicides and large projects to assess the *Meloidogyne* spp. situation in Africa is also of concern (De Waele & Elsen, 2007; Onkendi *et al*., 2014).

### 2.3 The *Meloidogyne incognita* life cycle

Root-knot nematodes are mostly found within plant roots when they are actively feeding on the host cells (Williamson & Hussey, 1996). For infection of a host plant to occur, PPNs such as the RKN genus *Meloidogyne,* must first engage in a process known as host seeking. *Meloidogyne* spp. are known to be endoparasitic and therefore should be present inside plant roots before they can start feeding (Figure 2). In plant roots the ethylene signalling pathway attracts PPNs; however, it has not yet been determined if the ethylene signalling directly regulates the production of substances that attract specific PPNs (Fudali *et al.*, 2012). Other factors such as low pH created by growing roots and $CO_2$ levels might also attract *Meloidogyne* spp. (Robinson, 1995; Wang *et al.*, 2009).

During the infection of host cells, populations of this RKN can increase quickly. This increase is due to the competition between as many as several generations during a single growing season; in combination with high levels of female fertility (Calderón-Urrea *et al.*, 2016). Such competition drives faster development. The number of eggs that can be produced by a single female can range from 500 to 2000 eggs (Calderón-Urrea *et al.*, 2016). Inside the eggs first stage juveniles

(J1) develop into J2 which then are hatched from the eggs. The infective J2 are attracted to the elongation areas of the roots/other below ground plant parts where they migrate into the plant host cells, where only the J2 and females actively feed (Trudgill & Blok, 2001; Wang *et al.*, 2009). This migration into the host cells (Figure 2) is made possible by continuous stylet movements that destroy the epidermal cells (Wyss *et al.*, 1992). However, the duration of life cycles of RKNs are dependent on the type of species, soil temperature and various other factors (Heyns, 1971).

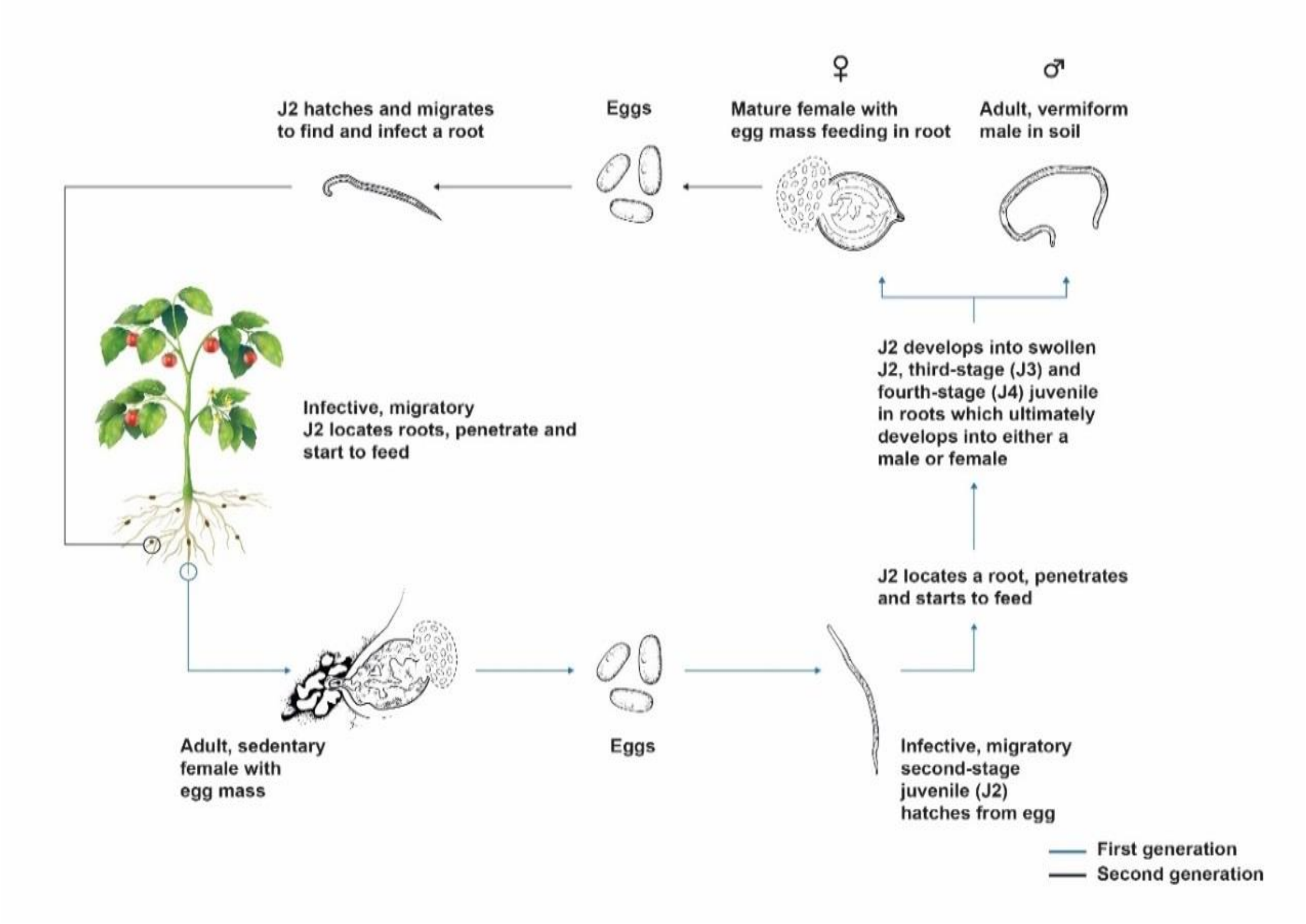

*Figure 2: Life cycle of Meloidogyne spp. (Mashela et al., 2017).*

## 2.4 Protection of plant parasitic nematodes against host defences

It is natural for plants to defend themselves against infections by pathogens using various genetically inherited defence strategies. These strategies include the production of different anti-pathogen compounds (Haegeman *et al.*, 2012). Studies such as the one conducted by Campbell *et al.* (2001) found that animal parasitic nematodes contain glutathione-S-transferases genes that aids in the detoxification of various anti-pathogen compounds produced by hosts. Likewise, the economically important RKN, *M. incognita* contains several glutathione-S-transferase genes and at least one will be expressed and secreted into the host cells. These secreted glutathione-S-transferase genes play important roles in the detoxification of nematicidal compounds secreted by the host cells (Dubreuil *et al.*, 2007; Haegeman *et al.*, 2012).

Other defences against nematode infection include the production of reactive oxygen species (ROS) (Waetzig *et al.*, 1999). However, *M. incognita* produces an antioxidant, superoxide dismutase, which can metabolise the ROS produced by the host (Bellafiore *et al.*, 2008). Although hosts have their own defences against PPN infection, *M. incognita* remains a large threat to agricultural activities worldwide (Haegeman *et al.*, 2012; Naz *et al.*, 2015). Therefore, it is important to obtain new RKN management methods and biocontrol options that can be less harmful towards the environment and humans (Castillo *et al.*, 2013).

## 2.5 Biocontrol agents

Due to the high number of PPNs that are soil-borne root pathogens, the management thereof remains difficult (Xiong *et al.*, 2015). Many chemical nematicides have elevated levels of toxicity contributing to environmental and human safety concerns and various chemical nematicides are now being removed from international markets (Naz *et al.*, 2015). However, chemicals remain the most common method for RKN management (Schneider *et al.*, 2003). This calls for the urgent development of more environmentally friendly PPN control methods. Since the PPNs inhabit soil, they can easily be subjected to infection by indigenous soil microbiota which are possible biocontrol agents (Terefe *et al.*, 2009).

Biocontrol agents are not only environmentally friendly, they also have different modes of action when compared to chemical pesticides (Ongena & Jacques, 2008), making it possible for them to be applied when other control options are not feasible. Biocontrol agents/biopesticides are divided into three groups according to their active substance: 1) microorganisms, 2) biochemicals and 3) semiochemicals (Chandler *et al.*, 2011). Examples of different biocontrol agents/biopesticides are listed in Table 1.

Various microorganisms have been progressively studied to determine their usefulness as biocontrol agents against PPNs (Xiong *et al.*, 2015). These include bacterial species such as *B. thuringiensis, Pasteuria penetrans* and various other microorganisms such as the fungal species *Purpureocillium lilacinum* (Timper, 2014; Timper *et al.*, 2016; Zhang *et al.*, 2016). For these biocontrol agents to be effective in the control of nematode pests, Anastasiadis *et al.* (2008)

indicated that they should be applied to the soil before crops are planted. This is done to ensure that microorganisms can establish themselves in the rhizosphere and produce the necessary nematicidal substances (Anastasiadis *et al.*, 2008).

*Table 1: Examples of biocontrol agents/biopesticides used against nematodes*.

| Biocontrol agent/biopesticide | Example(s) | Reference |
|---|---|---|
| • **Opportunistic fungi** | • *Purpureocillium lilacinum* and *Pochonia* | • Timper (2014) |
| • **Predacious fungi** | • *Arthrobotrys* spp. | • Degenkolb and Vilcinskas (2016) |
| • **Endoparasitic fungi** | • *Myzocytiopsis*, *Nematoctonus* and *Catenaria* | • Yang and Zhang (2014) |
| • **Parasitic bacteria** | • *Pasteuria penetrans* | • Flor-Peregrín *et al.* (2014) |
| • **Non-parasitic rhizobacteria** | • *Bacillus* spp. and *Streptomyces* spp. | • Pertot *et al.* (2016) |
| • **Products with pheromones or other semiochemicals as the active ingredient** | • Straight-chained lepidopteran pheromone | • Czaja *et al.* (2015) |
| • **Plant-extract- and vegetable-oil-based products** | • Citronella oil, orange oil and garlic extract | • Czaja *et al.* (2015) |

## 2.6 Advantages and limitations of biocontrol agents vs. chemical pesticides

De Waele and Elsen (2007) emphasised the need for food security for a world population that increased by 3.5 billion people between 1970 and 2006. Estimations made by the United Nations place the current world population at 7.6 billion (UNDESA, 2017), thus making the need for global food security greater than ever before. The necessity of reducing crop yield losses is therefore of major importance. Chemical nematicides have been used against PPNs since the 1950s and produced satisfactory results (Giannakou *et al.*, 2004; Mnif & Ghribi, 2015; Nicol *et al.*, 2011). Carbofuran, furfural, oxamyl, organophosphates, carbamates and halogenated compounds have been used as the primary nematicides to combat RKN infections (Fourie *et al.*, 2017; Singh *et al.*, 2017). Methyl bromide (MeBr) has also been one of the most commonly used chemical pesticides to control soil-borne pests and pathogens over the past four decades (Strauss & Kluepfel, 2015). However, since the 1990s various authors including Ristaino and Thomas (1997) indicated that MeBr could be implicated in the depletion of the ozone layer. This trait of MeBr also caused it to be included in the Montreal Protocol amongst substances with high ozone-depleting potential (Giannakou *et al.*, 2004). Other chemical pesticides such as the MeBr alternatives 1,3-dichloropropene and chloropicrin also face increased regulatory restraints (Strauss & Kluepfel, 2015). The use of MeBr and its alternatives are beneficial in some ways. Not only are these pesticides easily acquired and effective against a wide range of pests, but they are also more cost effective in comparison to other pesticide alternatives (Pertot *et al.*, 2016; Ware & Whitacre, 2004).

According to Arthurs and Dara (2018), a total of 356 active biopesticide ingredients for use against mites, insects and nematodes are registered in the United States. Of these 356 ingredients, 57 originate from microbial species and/or strains. This highlights the significance of microorganisms in biological control. Studies also found that successful biocontrol can be achieved by directly adding the living antagonistic strain as an inoculum to the soil. Alternatively, a combination of microbial enzymes and metabolites of biocontrol strains can be formulated, produced and optimised. This approach will overcome certain limitations in the use, maintenance and storage of living organisms (Berini *et al*., 2018).

The use of biopesticides is accompanied by various advantages including decreased pesticide residues in food, which reduces the risk to the consumer. Other advantages include their increased levels of decomposition and high levels of specificity (Czaja *et al.*, 2015). However, early reports suggested that although biopesticides have gained attention, they have not yet lived up to the promise of becoming major players in the pesticide market despite successful results (Copping & Menn, 2000). To increase the effectiveness of biopesticides such as microorganisms and microbial products, Lacey *et al.* (2001) suggested several aspects to be addressed. Firstly, there should be higher levels of efficiency in their production and improvements should be made in their application and performance in changing environmental conditions. Biopesticides that use microorganisms and/or their metabolites as pesticidal substances (OECD, 2003) must also adhere to the following basic requirements set by the Organisation for Economic Co-operation and Development (OECD):

- Microorganisms or metabolites used should not pose any pathogenic or toxic threats to non-target organisms that might be exposed to the substance.

- The microorganism used should not produce any genotoxins.
- Any substances added to the manufactured product should have low levels of toxicity, causing very little to no risk to human or environmental health.

If these improvements are made and all requirements met, the use and acceptance of biopesticides will increase. Over the past few years there has been an increase in the demand for biopesticides, resulting in a market that will have an estimated value of $4.5 million by 2019 (Pertot *et al.*, 2016). This growth in market value can be attributed to a number of factors, including increased concerns around the impact of various synthetic chemical pesticides, increased demand for control of pests and pathogens due to a rising global food demand and especially the increased levels of chemical pesticide resistance. Biopesticides are fast becoming the preferred choice for pest control (Lee & Kim, 2016; Pertot *et al.*, 2016).

## 2.7 ***Bacillus*** **spp. as biocontrol agents**

The *Bacillus* genus forms part of the family *Bacillaceae* of the order Bacillales. They are Gram-positive, rod-shaped bacteria that can usually be isolated from soil environments. The *Bacillus* genus has several important physiological characteristics, such as their ability to produce highly resistant endospores in aerobic conditions. This characteristic makes it possible for *Bacillus* spp. to tolerate extreme environmental conditions. The metabolism and metabolic properties of this aerobic endospore-forming genus differs greatly between its species, making it possible for them to act as opportunistic or obligate pathogens (De Vos *et al.*, 2009). Along with these characteristics, *Bacillus* not only represents one of the dominant genera in soil microbial communities but might act as the first line of defence against RKNs (Tian *et al.*, 2007; Tiwari *et al.*, 2017).

The use of bacterial strains is an alternative method for the prevention of RKN infections (Ashoub & Amara, 2010) and can reduce the damage done to economically important crops. Specifically, there are several *Bacillus* spp. that have been shown to exhibit nematicidal activity against *Meloidogyne* populations including *B. pumilus*, *B. cereus*, *B. firmus*, *B. subtilis*, *B. thuringiensis, B. coagulans*, *B. megaterium*, *B. nematocida* and *B. amyloliquefaciens* (Abbasi *et al.*, 2014; Jamal *et al.*, 2017; Lee & Kim, 2016; Metwally *et al.*, 2015; Niu *et al.*, 2006b). These qualities made it possible to use *Bacillus* spp. as the active substance in various bionematicidal products (Table 2).

*Table 2: Bionematicidal products containing Bacillus spp. as the active substance (Engelbrecht et al., 2018).*

| ***Bacillus* spp.** | **Product** | **Reference** |
|---|---|---|
| - ***B. firmus*** | - Bio-Nemax; BioNem-WP; BioSafe; VOTiVO; Nortica | - Castillo *et al.* (2013); Li *et al.* (2015) |
| - ***B. amyloliquefaciens*** | - BioYield<sup>TM</sup>; RhizoVital®42 li | - Cawoy *et al.* (2011); Tian *et al.* (2007) |
| - ***B. subtilis*** | - Rhizo Plus; *B. subtilis* IIHR BS-2 enriched vermicompost; SERENADE® | - Cawoy *et al.* (2011); Margolis *et al.* (2013); Rao *et al.* (2017) |
| - ***B. subtilis* + *B. velezensis*** | - BioYield | - Burkett-Cadena *et al.* (2008) |
| - ***B. subtilus* + *B. licheniforms* + *B. megaterium* + *B. coagulans*** | - Pathway Consortia | - Askary (2015) |
| - ***Bacillus* spp.** | - Nemix; Biostart | - Hallmann *et al.* (2009); Li *et al.* (2015) |

*Bacillus* spp. can serve as biopesticides due to the various modes of action affecting RKNs (Table 3). These mechanisms of action include parasitising of nematodes, production of nematicidal compounds, competing for nutrients, encouraging plant resistance, nematode trapping and interfering with nematode-plant recognition (Cawoy *et al.*, 2011; Mendoza *et al.*, 2008; Pertot *et al.*, 2016; Siddiqui & Mahmood, 1999; Terefe *et al.*, 2009). According to Xiong *et al.* (2015), *B. firmus* shows potential as a nematicidal bacterium due to its high level of lethality against *Meloidogyne* spp. Of all the *Bacillus* spp. that have been studied as potential bionematicides, the mechanisms of action used by *B. thuringiensis*, *B. nematocida* and *B. megaterium* are some of the most thoroughly studied and best understood (Huang *et al.*, 2010; Li *et al.*, 2015).

*Bacillus thuringiensis* and the toxins produced by it are the most commonly used method of biocontrol against RKN infections in agriculture (Mohammed *et al.*, 2008). Although Table 3 lists some of the *Bacillus* spp. and their possible nematicidal effects, there is still little known about the active compounds they produce. The lack of information regarding these active compounds and their nematicidal effects could be problematic for the development of biocontrol products. The acceptance and effectiveness of biocontrol agents also depend on their ability to survive in various environments and to successfully colonise infected plant roots (Castaneda-Alvarez *et al.*, 2016; Nemec *et al.*, 1996).

*Table 3: Certain Bacillus spp. and proposed effect on PPN (Engelbrecht et al., 2018).*

| ***Bacillus* spp.** | **Mechanism of action** | **References** |
|---|---|---|
| • ***B. pumilus*** | • Produces possible nematicidal toxins including protease and chitinase as secondary metabolites, while stimulating plant growth. | • Lee and Kim (2016); Nagesh *et al.* (2005); Ramezani Moghaddam *et al.* (2014) |
| • ***B. cereus*** | • Produces sphingosine, a nematicidal toxin, as secondary metabolite. The production of kanosamine, zwittermycin A, C16 sphingosine and phytosphingosine inhibit the growth of phytopathogens, such as nematodes. | • Emmert and Handelsman (1999); Gao *et al.* (2016); Nagesh *et al.* (2005) |
| • ***B. firmus*** | • Causes paralysis and inhibition of egg hatching of PPNs. This is likely caused by the production of secondary metabolites, such as serine protease Sep1, with known nematicidal potential. Certain strains can also promote plant growth. | • Geng *et al.* (2016); Giannakou *et al.* (2004); Mendoza *et al.* (2008); Xiong *et al.* (2015) |
| • ***B. megaterium*** | • Reduces J2 hatching and infection rate of *M. incognita* by producing nematicidal compounds such as: Benzeneacetaldehyde, 2-nonanone, Decanal, 2-undecanone and dimethyl disulphide | • Huang *et al.* (2010) |
| • ***B. subtilis*** | • Acts as a plant growth promoter while it produces various antibiotics with a broad spectrum of activity including suppression of plant pathogens. These antibiotics are largely lipopeptides that can be divided into surfactin, iturin or | • Nagórska *et al.* (2007); Ongena and Jacques (2008); Yanfei *et al.* (2011); |

| | | |
|---|---|---|
| | fengycin families. Nematicidal activity is also linked to the *purL* gene, the possible presence of the Trojan horse mechanism and the production of enzymes such as protease, chitinase and gelatinase. | Zaghloul *et al.* (2015); Zheng *et al.* (2016) |
| • ***B. thuringiensis*** | • Produces crystal proteins (Cry proteins) that act as toxins causing mortality of nematode J2 and hatching inhibition thereof. | • Li *et al.* (2015); Wei *et al.* (2003) |
| • ***B. nematocida*** | • Produces two extracellular alkaline serine proteases, Bace16 and a neutral protease Bae16, which causes degradation of the nematode cuticle. This mechanism is also known as the Trojan horse, as degradation occurs after the nematodes ingest the bacteria. | • Li *et al.* (2015); Niu *et al.* (2007) |
| • ***B. amyloliquefaciens*** | • Production of the nematicidal toxins plantazolicin and the dipeptide cyclo(D-Pro-L-Leu). | • Chowdhury *et al.* (2015); Jamal *et al.* (2017); Liu *et al.* (2013) |
| • ***Bacillus spp.*** | • Production of various catabolic enzymes such as proteases, chitinases, glucanases and peptide antibiotics might cause nematicidal activity. | • Lian *et al.* (2007); Niu *et al.* (2006a) |

## 2.8 Other possible biocontrol agents and their mechanisms of action

Biopesticides can be divided into three types according to their active substances. The first type is microorganisms (Chandler *et al.*, 2011). Biocontrol microorganisms are not restricted to the use of *Bacillus* spp., but include a wide range of microorganisms and products (Table 1). Numerous studies have attempted to use antagonistic bacteria and fungi to control the damage done by *M. incognita* (Hashem & Abo-Elyousr, 2011). It is also possible that these antagonistic microorganisms can produce compounds that have nematicidal activity and inhibit hatching and/or kill the J2 (Ashraf & Khan, 2010).

One of these nematicidal fungi has been studied by Khan *et al.* (2003) who found that *Purpureocillium lilacium* is able to penetrate the eggs of nematodes through the production of the lytic enzymes, serine protease and chitinase. Pertot *et al.* (2016) found that *Gliocladium catenulatum*, a naturally-occurring saprophytic fungus, can also be used as a possible biopesticide. This antagonistic fungus can control damping-off, seed- root- and stem-rots, and wilt diseases caused by *Rhizoctonia, Pythium, Phytophthora* and many other pests and pathogens (Pertot *et al.*, 2016). Some fungi, including *P. lilacinum* do not produce any mycotoxins and paecilotoxins like other strains, but rather parasitise on the nematodes (Pertot *et al.*, 2016; Timper, 2014). Antagonistic bacteria have repeatedly shown to be promising microorganisms to use as potential biocontrol agents against PPNs (Giannakou *et al.*, 2004). One important genus of these antagonistic bacteria is the aerobic, Gram-negative bacteria *Pseudomonas*. Various *Pseudomonas* spp. have been tested as possible biopesticides against *Meloidogyne* spp. According to Siddiqui and Mahmood (1999), *Pseudomonas* can promote plant growth in a way that leads to systemic resistance against PPNs. In a study conducted by Hashem and Abo-Elyousr (2011) they also found that *P. flourescens* and *P. putida* can reduce *M. incognita* infections in glasshouse conditions.

The second type of biopesticides is known as biochemicals. Biochemical pesticides use substances that occur naturally to control pests instead of synthetic chemicals (Neale, 2000). Some of the metabolites produced by plants can also be used as biopesticides. This type of biopesticide includes the fast-acting insecticidal compounds, known as pyrethrins, which are produced by *Chrysanthemum cinerariaefolium.* This leads to the production of synthetic pyrethrins known as pyrethroids (Chandler *et al.*, 2011; Silvério *et al.*, 2009). This type of biopesticide also includes plant-extract- and vegetable-oil-based products (Table 1), such as neem oil that can be extracted from *Azadirachta indica*.

The last type of biopesticides is semiochemicals. They can be defined as a type of chemical signal an organism is able to produce that can cause behavioural changes in organisms from different species (Chandler *et al.*, 2011). Semiochemicals that are mostly used for crop protection are sex pheromones such as sordidin, a male pheromone produced by *Cosmopolites sordidus* that is used in mass trapping of various pests (Reddy *et al.*, 2009).

## 2.9 Bacterial metabolism

Since van Leeuwenhoek first observed “animalcules” and Pasteur studied their isolation in pure culture, bacterial metabolism studies have been of great importance (Muñoz-Elías & McKinney, 2006). Soil contains various genera of bacteria and the metabolic activities of these soil bacteria caused them to be of major importance in agriculture. Bacterial metabolism of beneficial soil microorganisms can provide crops with various nutrients and stimulates growth. One of these beneficial soil bacteria genera is *Bacillus,* as some of its species can fixate nitrogen and enhance phosphorus levels of crops, therefore increasing the productivity of the crops (Hayat *et al.*, 2010).

Due to the large diversity of soil microorganisms, they can be classified into five metabolic groups, namely photolithotrophs, photoorganotrophs, photoautotrophs, chemolithotrophs and chemoorganotrophs (McGill, 2007). Each of these groups utilise specific electron sources and acceptors. The phototrophs utilise light as a metabolic energy source; in contrast, chemotrophs can obtain their metabolic energy from chemical compounds (McGill, 2007). These energy sources are important as they activate reactions and metabolite production needed for microbial growth and reproduction (primary metabolites) and provide bacteria with a competitive edge in their environments (secondary metabolites) (Sansinenea & Ortiz, 2011; Willey *et al.*, 2011).

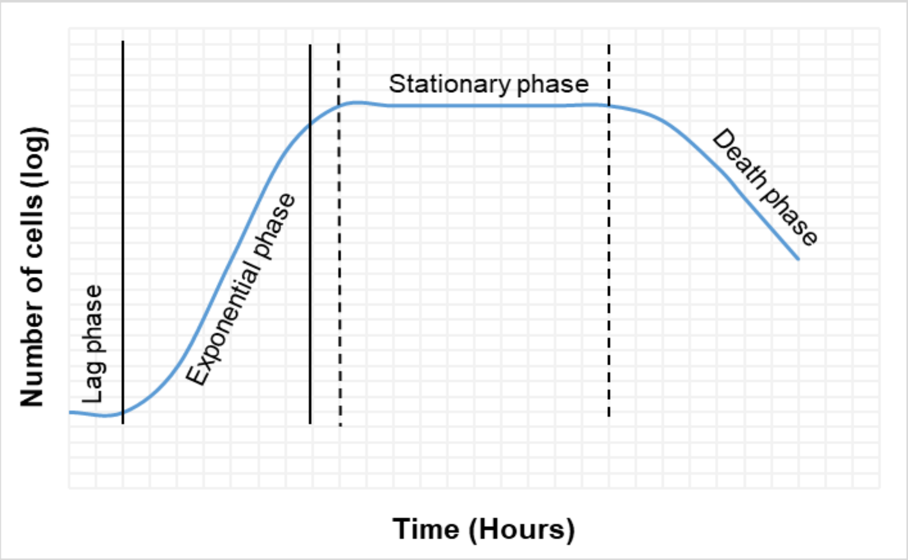

*Figure 3*: *An illustration of a theoretical bacterial growth curve under ideal conditions (Willey et al., 2011).*

### 2.9.1 Primary metabolites

During the exponential growth phase of microbes (Figure 3), products known as primary metabolites are produced and serve as fundamental products in the normal growth phase. Primary metabolites include various intermediates and end-products of anabolic metabolism. These metabolites are used in the formation of indispensable macromolecules (e.g. amino acids) or are converted to other vital molecules such as various coenzymes (Sanchez & Demain, 2008). However, not all primary metabolites result from anabolic metabolism. During catabolic metabolism, other primary metabolites including acetic acid and citric acid are formed. They are not used to form cellular constituents, but their production is essential for growth as they provide energy and utilise various substrates (Sanchez & Demain, 2008; Willey *et al.*, 2011).

### 2.9.2 Secondary metabolites

Microbial secondary metabolites are low molecular weight compounds that microorganisms produce during the stationary phase of microbial growth (Figure 3). These metabolites include various antibiotics, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, pesticides and growth promoters amongst others (Demain, 1998). However, the expression of secondary metabolites is influenced by environmental conditions such as nutrient depletion. This will lead to a decrease in the growth rate of a bacterial community resulting in the production of secondary metabolites (Ruiz *et al.*, 2010). These metabolites do not play important roles in bacterial growth, but rather contribute to the survival of microorganisms in their environments (Sansinenea & Ortiz, 2011).

The structures of secondary metabolites are often unusual, and their formation is regulated by nutrient availability and enzyme inactivation and induction (Demain, 1998). Secondary metabolites can be separated into volatile organic compounds and soluble compounds. Volatile organic compounds evaporate and diffuse easily and include terpenes, various nitrogen compounds, pyrazines, indole and sulphur-containing volatiles. Soluble compounds have higher polarities making them more soluble in water and include bacteriocins, non-ribosomal peptides, siderophores, lipopeptides, polyketides and PKS–NRPS hybrid compounds (Tyc *et al.*, 2017). It is well-documented that the secondary metabolites produced by microorganisms include very important anti-infective drugs such as antibiotics (Ruiz *et al.*, 2010). Since the early 21st century secondary metabolites produced by microorganisms have not only been increasingly researched as possible biopesticides (Copping & Menn, 2000). There has also been increased research done by the pharmaceutical industry with regards to secondary metabolites and their possible applications (Sansinenea & Ortiz, 2011).

## 2.10 The use of ‘omics’-based methods

‘Omics’-based methods consist of a wide range of tools and approaches, each with their own specific protocols (Beale *et al.*, 2016) and can be used for structural and functional analysis of mixed microbial cultures (Figure 4). These ‘omics’-based methods have gained significant interest since the Human Genome Project in the 1990s and ‘multi-omics’ (a combination of ‘omics’-based methods) has been applied increasingly from 2008 (Beale *et al.*, 2016). Some of the major ‘omics’-based methods include: metagenomics, proteomics, transcriptomics (Beale *et al.*, 2016; Fondi & Liò, 2015) and metabolomics. Handelsman (2004) described metagenomics as the isolation and analysis of DNA from a mixed population of microorganisms. Thus, enhancing our understanding of genetic composition and potential expressions (of genes, proteins and metabolites) from both cultivatable and uncultivatable microorganisms. In comparison to metagenomics, metatranscriptomics can be used to analyse short-term microbial changes due to environmental factors. Therefore, metatranscriptomics includes the analysis of RNA molecules such as mRNA, which serves as an indication of these short-term changes in microbial populations (Pascault *et al.*, 2015).

Studies that aim to determine the activity of mixed microbial populations can use metaproteomics and metabolomics. Metaproteomics is the characterisation of proteins expressed by a microbial community in an environment (Douterelo *et al.*, 2014). The use of metaproteomics can determine microbial community activities in various ecosystems such marine environments (Morris *et al.*, 2010). Metabolomics is the study of small molecules known as metabolites, which microbial cells produce under certain conditions (Tang, 2011). Metabolomics is an important part of systems biology and plays a key role in the Human Microbiome Project and various environmental studies. These include determining which metabolites are produced by specific microorganisms and the application thereof for improving human, animal and plant health (Barkal *et al.*, 2016).

Each of the meta-omics approaches (Figure 4) provides information regarding a specific characteristic of a microbial community. The DNA, and to a certain extent RNA, provides information about genes present in a community, which can be associated with potential microbial function and activity. By using metatranscriptomics, metaproteomics and metabolomics, active metabolic pathways can be identified (Abram, 2015). This can be associated with microbial community functions. In addition, metaproteomics and metabolomics can be used to determine the real-time activity present in a microbial community (Abram, 2015; Douterelo *et al.*, 2014).

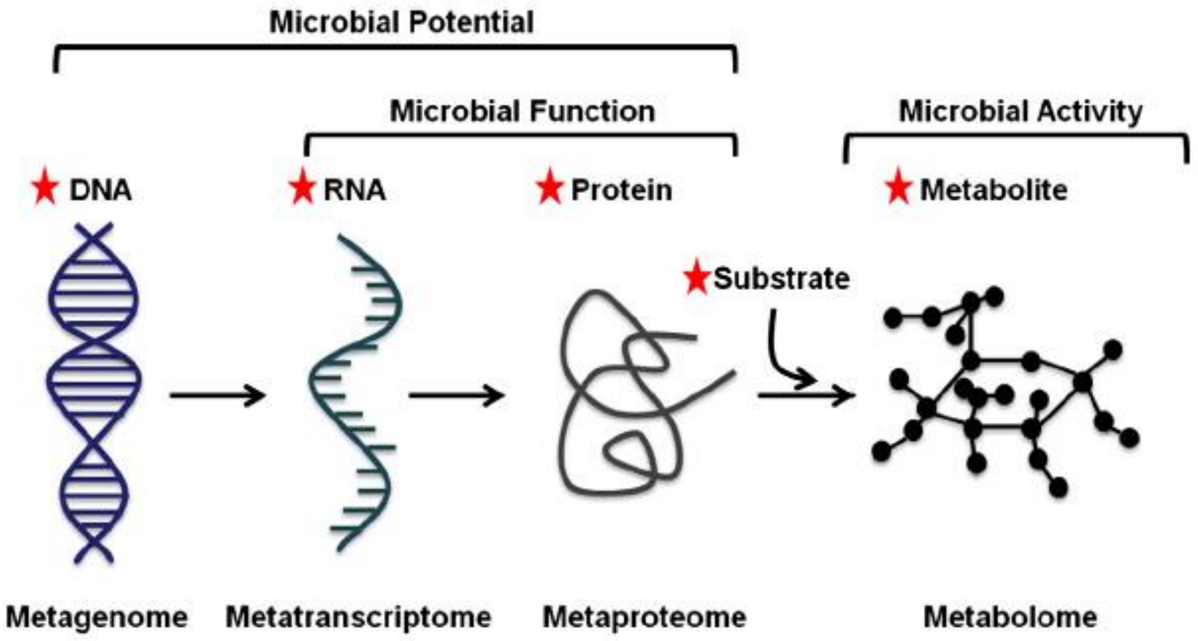

*Figure 4*: *Overview of a meta-omics approach (Abram, 2015).*

### 2.10.1 Metabolomics as an integrated study of microbial metabolism

Metabolomics is defined as the study of molecules that have low molecular weights (<1000 Da) (Carnicer *et al.*, 2016; Macintyre *et al.*, 2014). Thus, when studying the metabolome of organisms, it refers to the complete analysis of metabolites (primary and secondary) produced by an organism. This will reflect the numerous enzymatic and metabolic pathways that are encoded within the genome of that organism (Tang, 2011). In addition, the metabolites indicate the interaction between various developmental processes and environmental changes over the lifetime of the organism. Metabolites play key roles as energy carriers in cellular metabolism (Werf *et al.*, 2005) and can provide an accurate indication of an organism's physiological state (Garcia *et al.*, 2008; Tang, 2011). The information obtained from the metabolome varies from that of the genome because the genome represents "potential function" of a cell while the metabolome represents the expressed function of a cell (Figure 4) and can provide the most relevant information regarding biological functioning of organisms (Werf *et al.*, 2005).

The analytical platform is central to metabolomics analyses and the choice of platform depends on several factors, including physical and chemical properties of samples, the research question, and available instrumentation. Common bioanalytical techniques for metabolome analysis include liquid- and gas chromatographic methods coupled to mass spectrometry (LC-MS and GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. The main benefit of NMR is the ability to quantify identified metabolites by integration of proton NMR signals. Major drawbacks include lower sensitivity and separation than LC-MS or GC-MS (Liebeke *et al.*, 2012).

Unfortunately, none of these established techniques are sufficient to cover the complete diversity of metabolites of an organism when used alone (Aliferis & Chrysayi-Tokousbalides, 2010; Liebeke *et al.*, 2012). Each holds advantages and disadvantages and should be viewed as complementary (Halouska *et al.*, 2013), and therefore a combination of these approaches is proposed. The analytical platform is not the only critical consideration in metabolomics analysis. Methods for sample generation and the first steps of sample treatment can greatly influence the quality of results (Liebeke *et al.*, 2012; Liebeke & Lalk, 2014). Furthermore, previous studies have recommended that for appropriate metabolome analysis of microbial samples, an evaluated sampling protocol for each organism is needed (Liebeke *et al.*, 2012; Meyer *et al.*, 2013).

### 2.10.2 Untargeted vs. targeted metabolomics

Before a metabolomics study can be done, the first consideration should be whether to implement "untargeted" or "targeted" analyses. When referring to untargeted metabolomics, samples are analysed without using any authentic standards. This will allow the measurement of all features, as no prior decision was made to exclude any measurable features. However, the absence of standards might cause discrepancies in the quantitative accuracy and confidence of feature identification (Baig *et al.*, 2016). A recent study used untargeted metabolomics analysis to aid in the classification of variation in tomato plant low-molecular-weight polar metabolites after two months of being infected with *M. incognita* (Eloh *et al.*, 2016). When targeted metabolomics analyses/metabolic profiling are preformed, certain features are pre-selected for analysis. The quantification and identification of these features are done by including the use of standards (Baig *et al.*, 2016; Zhu *et al.*, 2013). When conducting the metabolic profiling of *Bacillus pumilus* 15.1, Garcia-Ramon *et al.* (2016) found that the bacterium produces parasporal crystals similar to that of *B. thuringiensis*, resulting in the potential application of *B. pumilus* 15.1 as an insecticide.

### 2.10.3 Common bioanalytical techniques for metabolome analysis

The use of metabolomics provides an understanding of various bacterial functions due to the link between the bacterial metabolome and phenotype (Werf *et al.*, 2005). Many analytical methods have been established that are limited towards the identification and quantification of multiple targets. However, with advances in chromatographic methods, it became easier to use retention times for identification of various peaks in complex matrices (Fiehn, 2002). These advanced separation techniques were joined to sensitive detectors with highly dynamic quantification ranges in order to obtain metabolomics data (Covington *et al.*, 2017; Fiehn, 2002). By combining gas chromatography (GC) (Gao *et al.*, 2016) and liquid chromatography (LC) with mass spectrometry (MS), metabolome analyses can be performed. The results are viewed as “features” that are ions with a determined mass to charge ratio (m/z) (Covington *et al.*, 2017).

One of the favoured hyphenated MS platforms used for untargeted metabolomics studies is LC-MS (Halket *et al.*, 2005). Analyses can be divided into two main stages (Berg *et al.*, 2013). Firstly, chromatographic separation of natural products (in a liquid phase) by using either reversed phase LC or hydrophobic interaction chromatography (HILIC). Separation is typically performed based on hydropathy using either a water–acetonitrile, or water–methanol gradient (Covington *et al.*, 2017). Hydrophobic interaction chromatography has an advantage over reversed phase LC. When using HILIC, the polar metabolites will be retained, while lipophilic metabolites will elute moderately faster. When using reversed phase LC, polar metabolites will elute first and apolar metabolites will be retained for longer (Kamleh *et al.*, 2008). The second part of the platform is MS. When LC-MS is used for untargeted metabolomics, time-of-flight mass spectrometry (TOF-MS) with an electrospray-ionisation technique is sufficient. This technique will result in minimal fragmentation and accurate masses of molecular ions can be obtained (Liebeke *et al.*, 2012).

Obtaining LC-MS data can vary from a couple of minutes to hours per chromatographic separation. Other external factors such as environmental changes (column conditioning and instrumental sensitivity, etc.) might alter the quality of the data set (Covington *et al.*, 2017). Another drawback of LC-MS is its semi-quantitative analysis (Berg *et al.*, 2013). Systematic variability between LC-MS measurements such as variable ionisation and differences in retention times also remain problematic (Zelena *et al.*, 2009). The lack of linearity between LC-MS signals and metabolite concentrations (Jankevics *et al.*, 2012) can be explained by three factors (Berg *et al.*, 2013) namely, the type of column used, metabolite concentration and loading capacity of the column. The biggest advantage of the LC-MS platform, when compared to GC-MS platforms, is the absence of derivatisation during sample preparation. By derivatising a sample, various chemical changes can be induced causing authors such as Halket *et al.* (2005) and Sadkowska *et al.* (2017) to conclude that sample derivatisation should be avoided where possible.

Another commonly-used hyphenated MS platform in microbial metabolomics is GC-MS. The separation of volatile or medium-polar compounds present in a gaseous phase, forms the basis of GC. Detection of the separated compounds is done by MS with electron impact ionisation of 70 eV forming characteristic fragment patterns (Liebeke *et al.*, 2012; Wahl *et al.*, 1999). These results are combined with information from the chromatographic separation and compounds can be identified by comparison with a database of known compounds (Gao & Xu, 2015; Liebeke *et al.*, 2012). One possible drawback with this technique is that various metabolites can contain polar functional groups, resulting in them being non-volatile at temperatures required for separation. To overcome this, it is necessary to derivatise samples before analysis (Gao & Xu, 2015) by means of oximation and silylation (Werf *et al.*, 2005). Benefits of using GC-MS for metabolomics include diverse application range, consistency, and low cost of purchase, operation and maintenance (Fiehn *et al.*, 2000).

## 2.11 Conclusion

The application of metabolomics to characterise microbial biocontrol agents effective against RKNs entails the analysis, profiling and identification of metabolites that inhibit J2 hatching and survival. Metabolomics enables the detection of metabolite features from bacterial cultures followed by statistical analysis and visualisation of variation between metabolomes of different bacteria. In the end, characterising the metabolite profiles of potential nematicidal species will facilitate the formulation of bionematicides which are more specific towards and antagonistic against RKNs, without harming the environment.

# CHAPTER 3 MATERIALS AND METHODS

## 3.1 Chemicals and glassware

High purity solvents: chloroform, hexane and methanol (Honeywell Burdick & Jackson®) were obtained from Anatech Instruments (Pty) Ltd. (Olivedale, South Africa). N,O-bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + TMCS, 99:1), henceforth referred to as BSTFA-TMCS, isooctane (2,2,4-trimethylpentane), methoxyamine hydrochloride and pyridine were obtained from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). The LC-MS internal standard mix (IS mix) consisted of L-norleucine, 3-phenylbutyric acid, 2-acetamidophenol and nonadecanoic acid was also obtained from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). The internal standard mix was dissolved in methanol with a final concentration of 5 µg/ml. The internal standards for GC-MS consisted of a mixture of fatty acid methyl esters (FAMEs) from C8:0 to C24:0 as per the protocol proposed by Fiehn (2017).

Glassware as required was purchased from Lasec South Africa (Pty) Ltd. (Midrand, South Africa). Glassware and caps used for all procedures were washed in tap water with a phosphate-free detergent (Liqui-Nox®, Alconox Inc., Separations, Johannesburg, South Africa), thereafter rinsed thoroughly with tap, deionised and nano-pure water. The glassware was air-dried and rinsed with the abovementioned high purity solvents and caps were sonicated in acetone for 2 min (Willers *et al.*, 2016).

## 3.2 Experimental design

The experimental workflow is illustrated in Figure 5. Second stage juveniles of *M. incognita* were exposed to five pre-selected broths, at four different concentrations (Lee & Kim, 2016; Mendoza *et al.*, 2008) during the bioassay. Effects of these broths were determined by counting the number of motile, curved (those who are not completely paralysed) and paralysed J2. Broth(s) with the lowest levels of paralysed J2 and highest levels of motile J2 were used for further experiments. Bacterial strains were cultivated until they had reached their stationary growth phase (determined by growth curves); thereafter a second nematicidal assay was performed using different bacterial filtrate concentrations. Nematodes were exposed to the various filtrates (containing the secondary metabolites), of the six bacterial strains as well as a fresh (not inoculated or incubated) broth and an incubated (not inoculated) broth. The effect of the bacterial strains was again determined by counting motile and paralysed nematodes. Thereafter, untargeted metabolomics analyses were performed to determine the metabolite profiles of these strains.

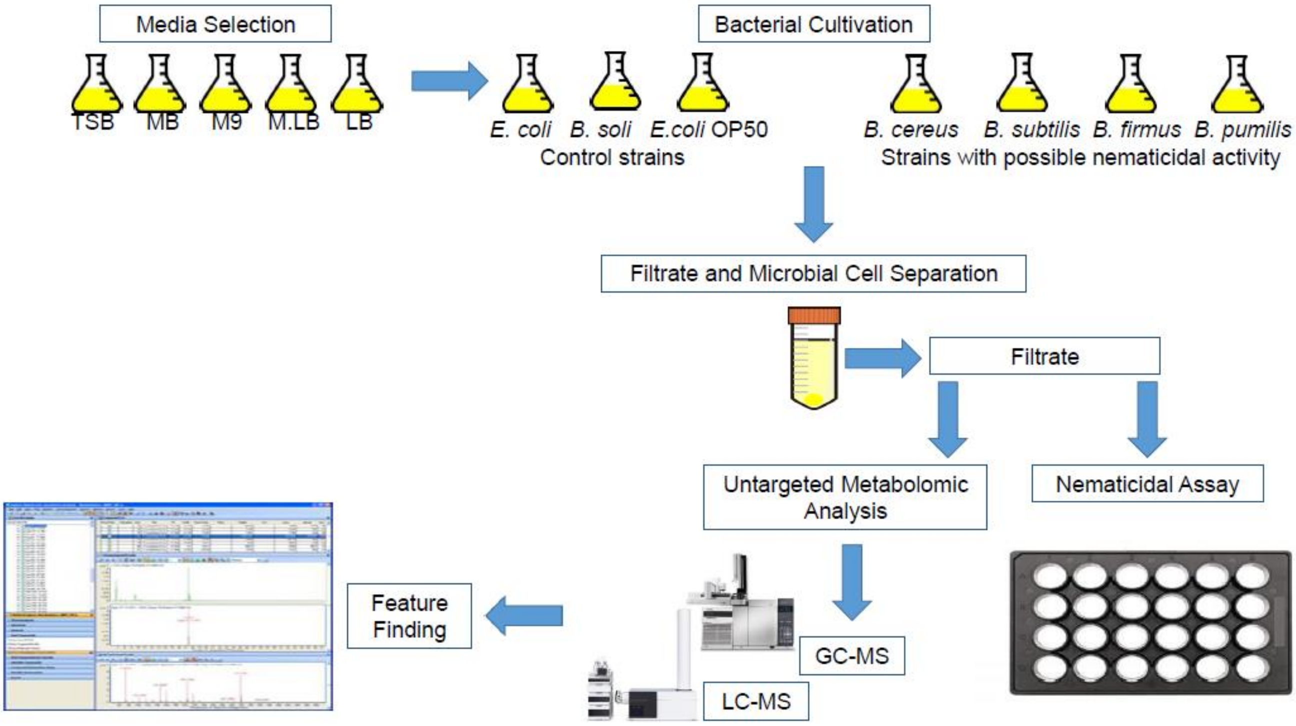

*Figure 5: Experimental workflow of in vitro biocontrol research.*

## 3.3 Media selection and bacterial cultivation

### 3.3.1 Media selection

Pure cultures were initially grown in nutrient broth as specified by Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) (DSMZ, 2007). Thereafter cultures were prepared by inoculating the different cultivation media and incubated for 48 h at 30°C on a rotary shaker (Labcon, California, USA) at 150 rpm. This was done to determine if the cultivation media would be suitable for *Bacillus* growth. The following cultivation media were tested because of their use in similar studies (Handtke *et al.*, 2014; ISO, 2010; Mohammed *et al.*, 2008; Xiong *et al.*, 2015):

- Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) obtained from Merck Millipore, Modderfontein, South Africa.

- Modified Luria-Bertani (M.LB) broth containing less salt than the LB broth (10 g/L tryptone, 5 g/L yeast extract and 5g/L NaCl).
- Minimal broth (MB) (1.98 g/L $(NH_4)_2SO_4$; 2.09 g/L $MgSO_4.7H_2O$; 2.01 g/L KCl; 2.06 g/L Sodium citrate tribasic dihydrate; 0.06 g/L Tris-HCl; 0.24 g/L $K_2HPO_4$; 0.22 g/L $CaCl_2$; 0.00032 g/L $FeSO_4.7H_2O$; 0.003 g/L $MnSO_4.4H_2O$; 0.66 g/L glutamate; 2g/L glucose; 4 µl biotin).
- M9-broth (6 g/L $Na_2HPO_4$; 3 g/L $KH_2PO_4$; 5 g/L NaCl; 0.25 g/L $MgSO_4{\cdot}7H_2O$).
- Tryptic soy broth (TSB) (17 g/L pancreatic digest of casein; 2.5 g/L $K_2HPO_4$; 2.5 g/L glucose; 5 g/L NaCl; 3 g/L papaic digest of soybean) obtained from Merck Millipore, Modderfontein, South Africa.

### 3.3.2 Bacterial cultivation

Type strains with possible nematicidal activity that were used for analyses were obtained from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and included: *Bacillus cereus* (DSM-31), *Bacillus firmus* (DSM-12), *Bacillus pumilus* (DSM-27), *Bacillus subtilis* (DSM-10) and *Bacillus soli* (DSM-15604). *Escherichia coli* (ATCC-10536) was obtained from the American Type Culture Collection (Virginia, USA) and served as a control strain along with *B. soli*. After it was found that both *B. soli* and *E. coli* exhibited nematicidal activity, *E. coli* OP50 was added as a control (obtained from the Caenorhabditis Genetics Center, Minnesota, USA). To ensure that bacterial cultivation is reproducible and provides a platform for unbiased metabolome analysis, the same growth medium must be used for cultivation and metabolomic fingerprinting (Halouska *et al.*, 2013). In agreement with the results obtained from the media selection procedures, two different cultivation media were selected for further use. Every strain was grown in triplicate in each medium and optical density was determined after 48 h (Abada *et al*., 2018) at 600 nm ($OD_{600}$) using a spectrophotometer (Jenway 7300, Lasec South Africa (Pty) Ltd., Midrand, South Africa).

## 3.4 Filtrate separation

For filtrate separation, 30 ml of each culture (in triplicate) was centrifuged (HERMLE Z32HK centrifuge, Lasec South Africa (Pty) Ltd., Midrand, South Africa) at 4800 g for 10 min at -8°C. This was done to remove bacterial cells from suspension by forming a cell pellet. Thereafter, the supernatant was filtered through a sterile 0.2 µm filter (Lasec, South Africa) to remove any remaining cells (Goodacre *et al*., 2017). For metabolomics analysis 150 µl aliquots of each filtrate was transferred to a 1.5 ml GC-vial (Chemetrix, South Africa) and 1.5 ml Eppendorf tube (Merck, South Africa) for liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analysis, respectively. All aliquots and the remaining bulk filtrates were flash frozen in liquid nitrogen and stored at -80°C until it was prepared for analysis (aliquots) or used for the nematicidal assays (bulk filtrates).

### 3.5 Nematode population

To obtain a single-species population of *M. incognita* eggs and J2 from the roots of infected tomato plants (cultivar Floradade) in a greenhouse at the Eco-Rehab facilities of the North-West University, Potchefstroom, Riekert's (1995) modified NaOCl-method was used. The identity of the population was verified with the use of both morphological and molecular methods (Visagie *et al*., 2018). The eggs and J2 were extracted from the roots of 30 to 40-day old tomato plants and suspended in aerated sterile distilled water on a 25 µm-mesh sieve at 26°C (Moura *et al.*, 1993; Riekert, 1995). The extracted eggs were placed on a 25 µm mesh sieve and submerged in a container filled with approximately 5 cm of tap water. The container with eggs was then incubated in an incubator at 26°C for 48 h. Hatchlings during the first 24 h were discarded and only the J2 that hatched during the last 24 h were used (Marais *et al.*, 2017). This was done to ensure optimal viability of J2.

### 3.6 Nematicidal bioassay

The nematicidal bioassays were performed based on the methods described by Eloh *et al.* (2015) and Lee and Kim (2016), with slight modifications as per Mendoza *et al*. (2008). Initial bioassays were performed to select an appropriate broth(s) for bacterial cultivation and further nematicidal bioassays. It was crucial to select a broth(s) that had the minimum impact on nematode motility. The broths selected for the screening assay were LB broth, M.LB broth, MB, M9-broth and TSB. Distilled water was used as a control.

To determine what effects the broths had on the motility of *M. incognita* J2, 20 to 30 J2 were exposed to four concentrations (25%, 50%, 75% and 100%) (Lee & Kim, 2016; Mendoza *et al*. 2008) of each broth. Exposures were done in sterile 24-well, flat bottom, culture treated plates (Greiner Bio-one), each well having a final volume of 1 ml. The four concentrations were made by diluting the broth with sterile distilled water. For example, a concentration of 50% contains 50% filtrate and 50% sterile distilled water, whereas a concentration of 100% only contains the bacterial filtrate. To avoid bacterial contamination, 100 µg/ml streptomycin and 100 µg/ml chloramphenicol were added to each well (Gao *et al.*, 2016). The plates were closed and incubated for 24 h at 25°C. Thereafter, the J2 in each well were counted and noted as motile (moving around), paralysed (laying completely straight) or curved (not motile or completely straight, body has a single curve). The broth(s) that showed the lowest level of paralysed J2 was used for bacterial cultivation and nematicidal bioassays of these bacteria. Using the extracted filtrates of each bacterial species, the second nematicidal bioassay was conducted following the same method used for the screening assay. Bioassays for all concentrations of each broth and filtrates of individual bacterial species cultivated in the media were replicated six times.

## 3.7 Sample preparation and workup for LC-MS and GC-MS

The preparation of samples for LC-MS was done by adding 300 µl acetonitrile to the Eppendorf tubes containing 150 µl frozen extracted filtrates and left to thaw. This was followed by vortexing and centrifugation at 11 000 rpm for 15 min at 4°C. 100 µl of the centrifuged samples were transferred to a GC-vial containing 200 µl LC-MS IS mix. Samples were vortexed and submitted to the laboratory (Analytical Platform, Human Metabolomics, North-West University, Potchefstroom) for LC-MS analysis.

To prepare samples for GC-MS analysis, the vials containing the frozen extracted bacterial filtrates were lyophilised before derivatisation. To the dried extract, 50 µl methoxyamination solution (MeOX) prepared from methoxyamine hydrochloride dissolved in anhydrous pyridine (20 mg/ml) was added to each vial, capped and incubated for 1 h at 60°C. Vials were removed from the oven, allowed to reach room temperature before the next step. This was followed by the addition of 50 µl BSTFA-TMCS, capped, vortexed and incubated for 1 h at 60°C. After incubation, vials were allowed to cool to room temperature and 50 µl isooctane containing the FAME mix, as described by Fiehn (2017), was added to each vial, mixed and transferred to a GC vial fitted with a 200 µl insert. Vials were capped and submitted to the laboratory (Analytical Platform, Human Metabolomics, North-West University, Potchefstroom) for GC-MS analysis (Fiehn, 2017; Venter *et al*., 2016).

## 3.8 LC-MS untargeted analysis

An Agilent 1200 LC system comprising a vacuum degasser, a column compartment, a thermostated autosampler and binary pump was used. The column compartment containing a

Cogent diamond hydride 2.1 mm x 10 mm, 4 µm particle size (MicroSolve Technologies, USA) was held at 50°C. The mobile phases consisted of (A) water containing 0.1% acetic acid (v/v) (pH 3.4) and 0.1% ammonium acetate (w/v) and (B) 90% acetonitrile (v/v) with 0.1% ammonium acetate (w/v) and 0.1% acetic acid (v/v). For optimum chromatographic separation (Callahan *et al.*, 2009) the following gradient was used: Starting at 100% B, then linearly decreasing to 40% B over 10 min, followed by a 1 min hold at 40%. The gradient then returned to 100% B and the column was re-equilibrated at 100% B for 6 min. Mass spectrometry was performed using an Agilent 6510 QTOF (Agilent, Santa Clara, USA) with a dual sprayer electron spray ionisation (ESI) source in positive ESI. A drying gas temperature of 300°C with a gas flow of 10 L/min and nebulizer pressure of 45 psi, capillary voltage of 4000V, fragmentor voltage of 150V and a skimmer voltage of 65V were used. The instrument was operated in the extended dynamic range mode and data collection in the m/z range of 70-1700 amu. For accurate mass reference a reference solution containing masses 121.050873 $[M+H]^+$ and 922.009798 $[M+H]^+$ was constantly infused (Callahan *et al.*, 2009).

### 3.9 GC-MS untargeted analysis

For analysis an Agilent GC-MSD instrument (Agilent Technologies, Santa Clara, USA) consisting of a 7890A gas chromatograph with a split/splitless injector (250°C), equipped with a 7683B auto sampler coupled to a 5975B inert XL mass selective detector was used. The gas chromatograph was equipped with a 30 m Agilent DB-5MS column, 0.25 mm internal diameter, and 0.25 µm film. For the carrier gas helium was used at a flow rate of 1.0 mL/min. A sample volume of 1 µL was injected with a 10:1 split ratio. The initial oven temperature was 60°C for 1 min, ramping to 325°C at 10 °C/min followed by a hold time of 10 min, resulting in a total runtime of 37.5 min per sample. The transfer line temperature was set to 290°C while the source temperature was set at 230°C. Acquisition was delayed for the first 5.9 min serving as a solvent delay. Data was captured with an acquisition rate of 20 spectra (50-600 m/z) per second, with electron ionisation energy of 70eV (Fiehn, 2017).

### 3.10 Statistical analysis

#### 3.10.1 Bioassay data processing

To determine what effects the broths and bacterial filtrates had on the motility of *M. incognita* J2, 20 to 30 J2 (Eloh *et al.*, 2015) were exposed to four concentrations (25%, 50%, 75% and 100%)

(Lee & Kim, 2016; Mendoza *et al*. 2008) of each broth and bacterial filtrate. To determine the amount of motile, curved and paralysed J2 for each concentration, the following calculations were done:

Initially, before exposure to the broths and bacterial filtrates, the number of J2 used per exposure concentration was determined by counting each replicate well and calculating the total average. After bioassays had been performed, the total number of motile, curved and paralysed J2 were determined for each of the exposure concentrations. This was done counting each J2 body shape type (motile, curved and paralysed) for every replicate of an exposure concentration. These motile, curved and paralysed J2 values were then expressed as percentages (%) of the original total J2 used per exposure concentration. Tukeys post-hoc test was used to determine whether the paralysis of *M. incognita* was statistically significant ($p<0.05$) between different concentrations of a single bacterial species when cultivated in each media.

### 3.10.2 Metabolomics data processing

Metabolomics can generate large volumes of data which is difficult to process and manually analyse (Lankadurai *et al.*, 2013). Various statistical tools can be used to initially manipulate raw data sets into a workable format (Issaq *et al.*, 2009). The following steps for the data transformation were used at the North-West University's Human Metabolomics laboratory to transform the data generated in this study into meaningful results: 1) Data extraction; 2) Data pre-processing; 3) Data normalisation; 4) Data pre-treatment and 5) Statistical analysis.

### 3.10.3 Data extraction

#### 3.10.3.1 LC-QTOF data

Data extraction was performed using Agilent's MassHunter Qualitative software (Version B.06, Chemetrix, Midrand, SA). The find by molecular feature extraction (FbMF) algorithm was used according to Agilent's specifications to aid with data extraction. Agilent's Mass Profiler Professional (MPP) (Version B.02.02) was used to align the data.

#### 3.10.3.2 GC-MSD data

GC-MSD spectra were identified by spectral analysis, using the Automated Mass Spectral Deconvolution and Identification System (AMDIS, Version 2.7) and the NIST 2011 mass spectral library. A match factor of ≥ 80 % was set. Data files created in AMDIS were imported into Agilent's MassHunter Mass Profiler Professional (Version B.02.02) (MPP), where chromatographic retention time aligned were performed across multiple data (Venter *et al*., 2015; Willers *et al.*, 2016).

### 3.10.4 Data analysis

After data extraction, pre-processing, normalisation and pre-treatment the "cleaned" data was transformed into a format usable for statistical analysis. The resulting data matrix were subsequently analysed using the software package MetaboAnalyst 3.0. (www.metaboanalyst.ca). Analysis included univariate and multivariate analyses, the determination of quantitative Variable Important in Projection (VIP) values and partial least squares discriminant analysis (PLS-DA) modelling (Alonso *et al*., 2015; Venter *et al*., 2015; Xia and Wishart 2016). Univariate analyses included Student's *t*-test and one-way ANOVA, while multivariate analyses included Principal Component Analysis (PCA) and interactive Principal Component Analysis (iPCA). The PCA is an unsupervised multivariate analysis that aims to find the directions that best explain the variance in a dataset and to enable visualisation of similarities between samples (Alonso *et al*., 2015). The iPCA visualisation summarises all the data into the first three principal components (PCs).

# CHAPTER 4 RESULTS AND DISCUSSION: *IN VITRO* NEMATODE BIOASSAYS

## 4.1 Introduction

Over the past few decades there has been substantial interest in the use of various antagonistic microorganisms for root-knot nematode (RKN) management within the rhizosphere (Demain, 1998; Terefe *et al*., 2009; Xiong *et al*., 2015). There is a high abundance of potentially beneficial microbes in the rhizosphere that could serve as sources of novel bioactive compounds with nematicidal potential for agriculture application (Saikia *et al*., 2013). Of all nematicidal *Bacillus* spp., *B. thuringiensis* is the most thoroughly studied and it is known that the mode of action of this bacterium can be attributed to toxic proteins (Table 3) (Mnif & Ghribi, 2015). Certain toxins produced by *B. pumilus* have also been characterised (Ahmadian *et al.*, 2007; Akbulut *et al.*, 2013), although not to the same extent as for *B. thuringiensis*. For many other nematicidal *Bacillus* spp., the exact modes of action remain unclear. Numerous studies have evaluated commercially available bionematicides containing *B. firmus* as the active ingredient against *Meloidogyne incognita*, concluding that the nematicidal effect can be attributed to the production of secondary metabolites by the bacterium (Giannakou *et al.*, 2004; Mendoza *et al.*, 2008; Terefe *et al.*, 2009; Xiong *et al.*, 2015). The activities of suspected nematicidal compounds remain largely untested against RKNs and therefore the efficacy of specific metabolites produced by *B. firmus, B. pumilus, B. cereus* and *B. subtilis* in biocontrol have not been determined. In the present study filtrates containing the secondary metabolites of *B. firmus, B. pumilus, B. cereus, B. subtilis, B. soli, Escherichia coli* and *E. coli* OP50 were evaluated against J2 of *M. incognita.* The results obtained for the *in vitro* nematode bioassays of the before-mentioned bacterial spp. are presented in this chapter.

## 4.2 Nematode bioassays for broth selection

When assessing and selecting appropriate culture media and conditions for nematode bioassays, the following criteria must be met: The media i) must enable growth of all selected bacterial strains (Hahn-Hägerdal *et al*., 2005); ii) should have little to no impact on the motility of *M. incognita* J2; and iii) should provide a comprehensive view on the metabolic capability of the bacterial strain without impacting metabolomics results (Handtke *et al*., 2014). Of all the broths selected for the initial screening tests (LB broth, M.LB broth, MB, M9 and TSB), TSB had low levels of nematode motility at all concentrations. The motility of *M. incognita* decreased from 35% to 24% as the concentration of the TSB increased from 25% to 100%. Although M9 broth has been used for exposures with the bacterivorous nematode *Caenorhabditis elegans* (Marudhupandiyan *et al*., 2017), in this study results of the bioassays using *M. incognita* resulted in high levels of J2 paralysis for all four concentrations (25%, 50%, 75% and 100%). The numbers of non-motile nematodes are determined by combining the amount of curved and paralysed nematodes. In TSB and M9 the numbers of non-motile individuals were high when compared to the other culture media and these two media were subsequently excluded for further analysis. The M.LB broth showed reduced motility levels (77% to 56%), as the broth concentration increased from 25 to 100% (Table 4). Luria-Bertani broth was the only broth tested that did not negatively impact motility of *M. incognita* as the concentration of the broth increased. At a concentration of 100%, LB broth had 74% motile *M. incognita*, while paralysed nematodes were limited to 8%. To determine if there were any statistically significant ($p<0.05$) differences, a post-hoc Tukey test was performed. Significant differences between different cultivation media concentrations were only found in the nematode body shapes when exposed to TSB and M.LB.

The observed paralysis caused by TSB, M9 and MB may be attributed to their pH range. Studies have shown that RKN reproduction occurs optimally at pH values of 5.0 to 5.6; however, these broths have pH ranges of 7.1 to 7.6 and it has been shown that such elevated pH levels can cause reduced levels of nematode reproduction and motility, while also increasing paralysis (Davide, 1980; Ruan *et al*., 2013). However, LB broth has a similar pH range as the other cultivation media, yet it had higher motility levels when compared to the other broths. Therefore, other factors contributing to paralysis of *M. incognita* J2 must be considered. These include salt gradients of $NH_4^+$, $K^+$, $Cl^-$ and $NO_3^-$ that are formed during metabolic activities (Castro *et al*., 1991). Since the M9, MB and TSB culture media all contain different salts, the formation of ions such as these will result in noticeable levels of nematode paralysis. Both LB broth and M.LB include more complex components such as yeast extract and they contain less salt and, in this study, these two broths were associated with more motile nematodes (Table 4).

Based on the observations LB and MB was chosen as the cultivation media for further analysis. LB broth had no negative effect on motility of *M. incognita* J2 and is therefore a good choice for future experiments. The possible effect of the complex nature of the media on metabolomics studies is noted and should be kept in mind. The composition of liquid media and possible presence of interferences (matrix) can cause problems with the operation of analytical equipment (Pinu & Villas-Boas, 2017). The second cultivation medium (MB) was included since it is far less complex and could be advantageous for the metabolomics approach although a negative effect was observed on the motility of *M. incognita* J2.

*Table 4: M. incognita J2 bioassay results obtained from exposures to different culture media.*

| | | **Nematode body shape (%)** | | | | |
|---|---|---|---|---|---|---|
| ***[Broth]*** | | ***M9*** | ***MB*** | ***TSB*** | ***LB*** | ***M.LB*** |
| ***25%*** | ***M*** | 55.8±11.9$^{a}$ | 61±3.7$^{a}$ | 35.3±7.3$^{a}$ | 68.3±5.2$^{a}$ | 77.5±4$^{a}$ |
| | ***C*** | 28.9±10.6$^{a}$ | 14.4±2.9$^{a}$ | 24.6±4.4$^{a}$ | 18.8±2.9$^{b}$ | 16.5±2.3$^{a,b}$ |
| | ***P*** | 15.4±5.4$^{a}$ | 24.6±3.6$^{a}$ | 40.1±4.5$^{a}$ | 13.1±2$^{a}$ | 6±0.8$^{a}$ |
| ***50%*** | ***M*** | 63.2±12.8$^{a}$ | 63.9±4.8$^{a}$ | 19.9±4$^{a}$ | 73.4±7.2$^{a}$ | 75.3±3.5$^{a}$ |
| | ***C*** | 32.6±9.9$^{a}$ | 11.5±2.4$^{a}$ | 38±3.3$^{a,b}$ | 8.7±1.1$^{a}$ | 14.1±2.9$^{a}$ |
| | ***P*** | 16.2±2.9$^{a}$ | 24.6±4.2$^{a}$ | 42.1±1.8$^{a}$ | 17.9±3.7$^{a}$ | 10.6±2.6$^{a,b}$ |
| ***75%*** | ***M*** | 59.7±5.8$^{a}$ | 52±3.1$^{a}$ | 17.5±4.6$^{a}$ | 75.3±6.6$^{a}$ | 72.7±5.1$^{a,b}$ |
| | ***C*** | 32.7±10.6$^{a}$ | 14.9±1.2$^{a}$ | 40.4±3.7$^{b}$ | 12.1±1.6$^{a,b}$ | 21.1±2.8$^{a,b}$ |
| | ***P*** | 19.4±4.7$^{a}$ | 33.1±5.1$^{a}$ | 42.1±4.1$^{a}$ | 12.6±3.3$^{a}$ | 6.2±0.8$^{a}$ |
| ***100%*** | ***M*** | 55.2±8.4$^{a}$ | 44.5±9.5$^{a}$ | 24.4±3.4$^{a}$ | 74.1±7.2$^{a}$ | 56.5±6$^{b}$ |
| | ***C*** | 41.9±8.3$^{a}$ | 20.9±5.6$^{a}$ | 38.4±3.4$^{a,b}$ | 17.7±3.7$^{a,b}$ | 27.3±3.3$^{b}$ |
| | ***P*** | 25±3.2$^{a}$ | 34.6±6.4$^{a}$ | 37.2±4.1$^{a}$ | 8.2±1.2$^{a}$ | 16.15±1.8$^{b}$ |

*Means ± standard deviations represent results obtained from sample replicates (n = 6). Statistically significant differences are indicated by alphabetic letters (p<0.05 using Tukeys post-hoc test). The same letters indicate no significant differences. Key to abbreviations: M, C and P indicates the body shape of the nematodes [M: Motile, C: Curved and P: Paralysed (straight); M9: M9 broth, MB: Minimal Broth, TSB: Tryptic Soy Broth, LB: Luria-Bertani broth and M.LB: Modified Luria-Bertani broth.*

## 4.3 Nematode bioassays with bacterial filtrates

The nematicidal activity of tested bacterial filtrates on the J2 individuals of *M. incognita* is summarised in Figure 6 (LB broth) and Figure 7 (MB). All the bacterial filtrates cultivated in both LB broth and MB showed nematicidal activity at all tested concentrations. Results from the bioassays indicated that nematicidal activity of the bacterial filtrates increased as the concentration of the filtrates increased. The highest paralysis levels of *M. incognita* J2 in LB broth was observed at filtrate concentrations of 100% for *B. soli* (69.0%) and *B. cereus* (68.9%) (Figure 6). Paralysis caused by *B. soli* at 100% filtrate concentration was significantly (*p*<0.05) different when compared to that of *B. cereus*, the control strain *E. coli* OP50 and the broth and water controls. Tukeys post-hoc test was used to determine whether the paralysis of *M. incognita* was statistically significant (*p*<0.05) between different concentrations of a single bacterial species cultivated in LB broth. This was done for each bacterial species. Statistically significant (*p*<0.05) paralysis of *M. incognita* was obtained at the 50, 75 and 100% concentrations of the filtrates from the *Bacillus* spp. cultivated in LB broth (Appendix A). The lowest percentage of *M. incognita* J2 paralysis recorded in the LB broth exposures was that of *E. coli* (11.3%), at a concentration of 25%. However, *E. coli* proved to have higher levels of nematode paralysis (Figures 6 and 7) as compared to that of *E. coli* OP50 at increasing concentrations of the filtrate. For further bioassays, *E. coli* was therefore discarded as a control strain. The *E. coli* OP50 strain usually serves as a food source for *C. elegans* (Ju *et al*., 2016) and in this study it was a suitable control strain for bioassays with *M. incognita.*

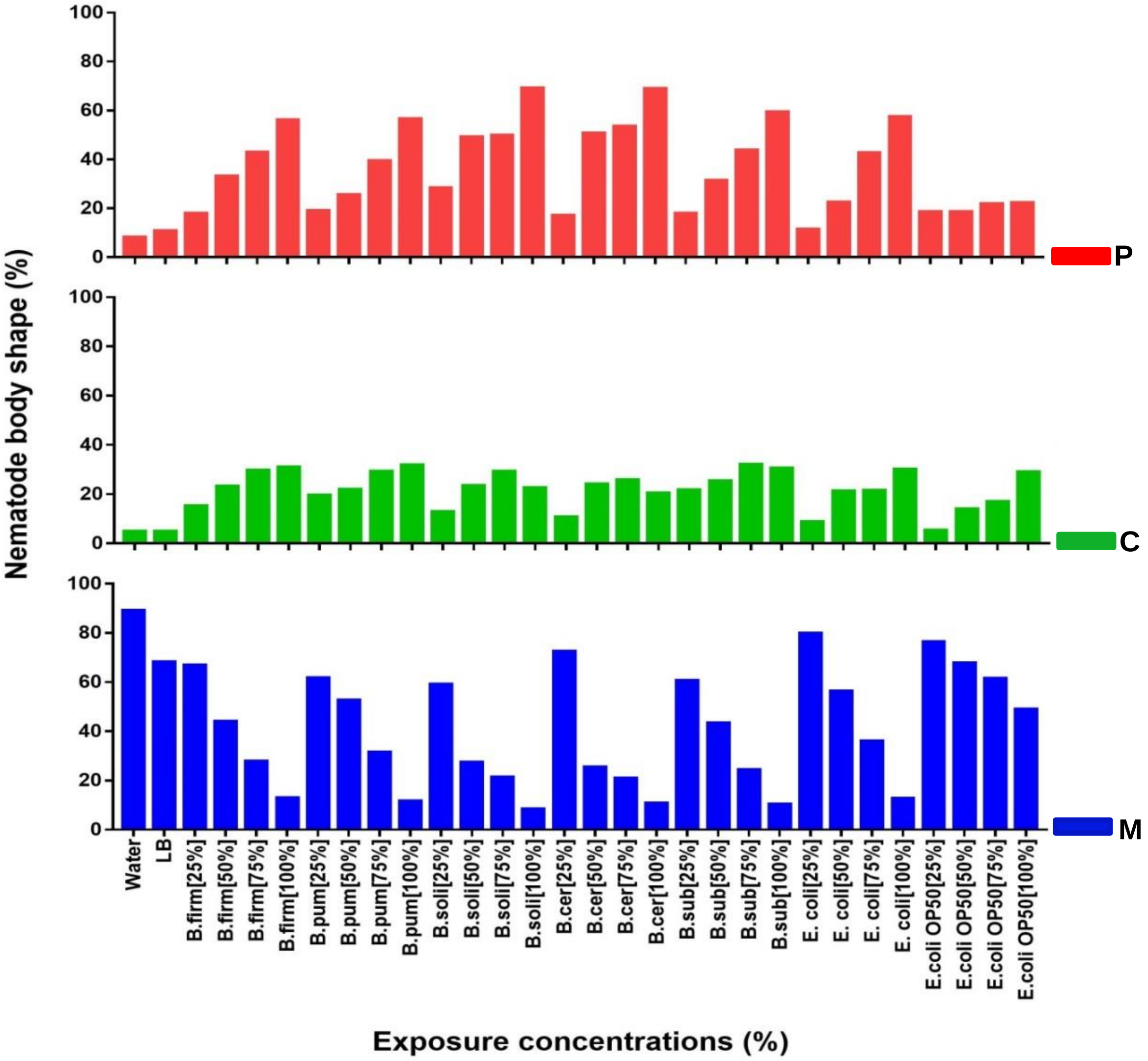

*Figure 6: Results of nematode bioassays to determine the effect of secondary metabolites produced by bacterial cultures cultivated in Luria-Bertani broth. M, C and P indicates the body shape of the nematodes [M: Motile, C: Curved and P: Paralysed (straight)]. Results obtained from sample replicates (n = 6).*

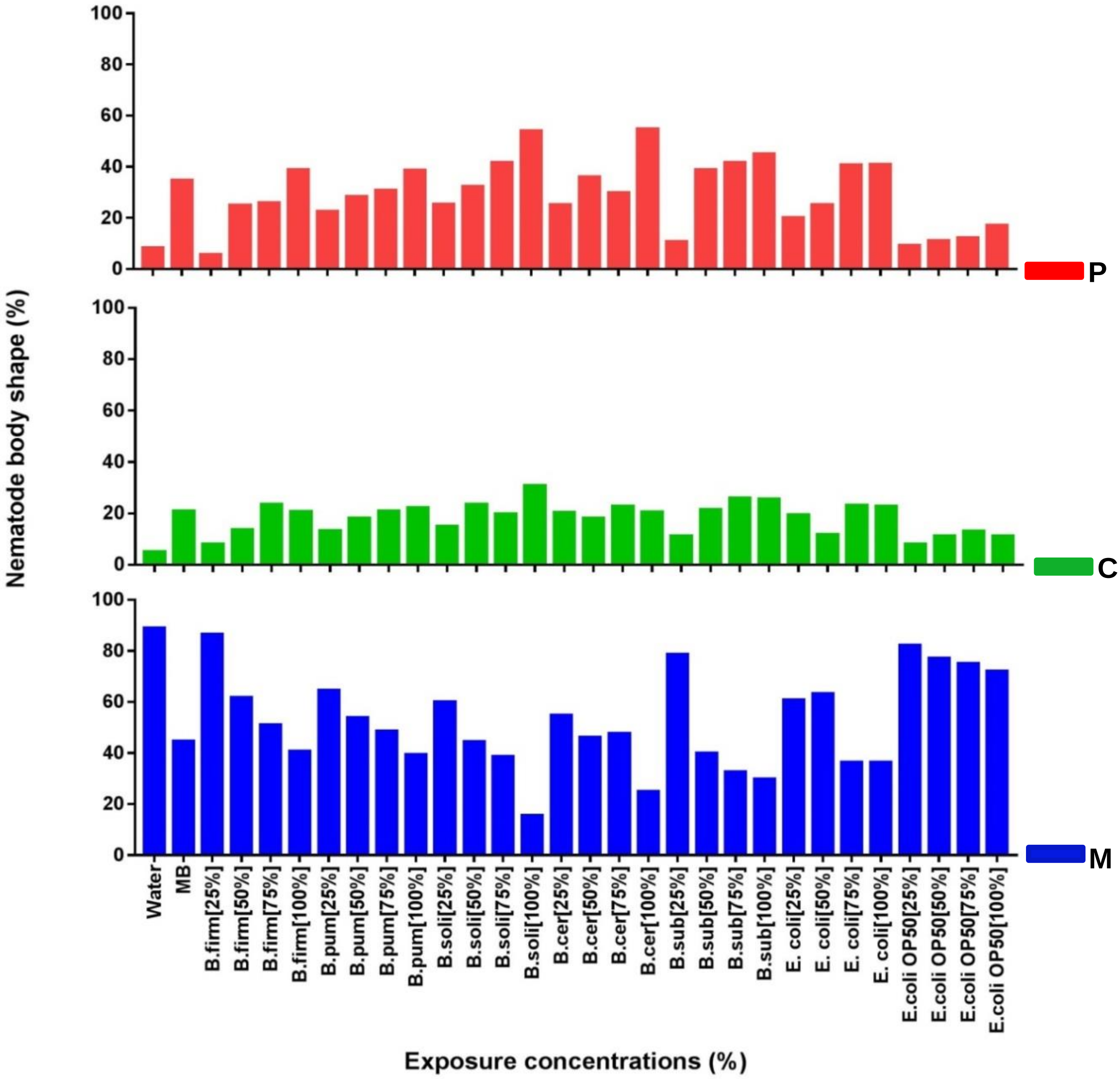

*Figure 7: Results of nematode bioassays to determine the effect of secondary metabolites produced by bacterial cultures cultivated in minimal broth. M, C and P indicates the body shape of the nematodes [M: Motile, C: Curved and P: Paralysed (straight)]. Results obtained from sample replicates (n = 6).*

Nematicidal assays using filtrates of bacterial spp. cultivated in MB (Figure 7) indicated that the highest levels of paralysis were 54.7% and 54% for the 100% filtrate concentrations of *B. cereus* and *B. soli* respectively. Paralysis caused by *B. subtilis* at 100% filtrate concentration was the only result not significantly (*p*<0.05) different compared to that of the control strain *E. coli* OP50 and the broth and water controls (Appendix B). Tukeys post-hoc test was used to determine whether the paralysis of *M. incognita* was statistically significant (*p*<0.05) between different concentrations of a single bacterial species cultivated in MB. This was done for each bacterial species. Significant paralysis of *M. incognita* was obtained at the 75 and 100% concentrations of the filtrates from *B. firmus*, *B. pumilus*, *B. cereus* and *B. soli* that were cultivated in MB (Appendix

B). The lowest percentage was that of *B. firmus* (5.7%) at a concentration of 25%. The assays in both LB broth and MB revealed that *E. coli* OP50 had the smallest decrease in nematode motility, even though the filtrate concentration increased.

The filtrates of *B. soli* and *B. cereus* had the highest nematicidal activity (Figures 6 and 7), however, there was lower nematicidal activity when cultivated in MB as opposed to LB broth. This observed difference might be caused by the composition of the selected broths, which can have an impact on the metabolites produced by the bacteria (Handtke *et al*., 2014). The secondary metabolites that bacterial spp. produce in the cell and then excrete, are subject to environmental changes. In more complex media such as LB broth, high nutrient availability might cause more rapid growth of bacteria compared to that of a minimal broth. In turn, this might result in an increase of the nematicidal metabolites of interest in the extracellular environment observed as higher nematicidal activity (Ileana-Andreea *et al.*, 2017).

## 4.4 Optimised nematode bioassays with bacterial filtrates

### 4.4.1 Growth curves

After initial nematode bioassays and metabolomics analysis, growth curves of the *Bacillus* strains and *E. coli* OP50 where determined to confirm whether the incubation period of 48 h, as indicated in literature of related investigations (Abada *et al*., 2018), is sufficient for all the strains to reach the stationary growth phase. This phase is identified as the period during which the bacterial strains show little deviation in the $OD_{600}$ values and there is no exponential growth (Figure 8 and 9). The stationary phase is the phase during which secondary metabolites are produced. Growth curves were determined by cultivating each strain in both LB broth and MB. The absorbance of each strain was measured at 600 nm ($OD_{600}$) using a BioTek PowerWave HT (Vermont, USA) at 1 h time intervals. The incubation period was extended from 48 h (Abada *et al*., 2018) to 114 h (~5 days) according to Arroyave-Toro *et al.* (2017). The growth curves that were obtained are shown in Figures 8 (LB broth) and 9 (MB) respectively.

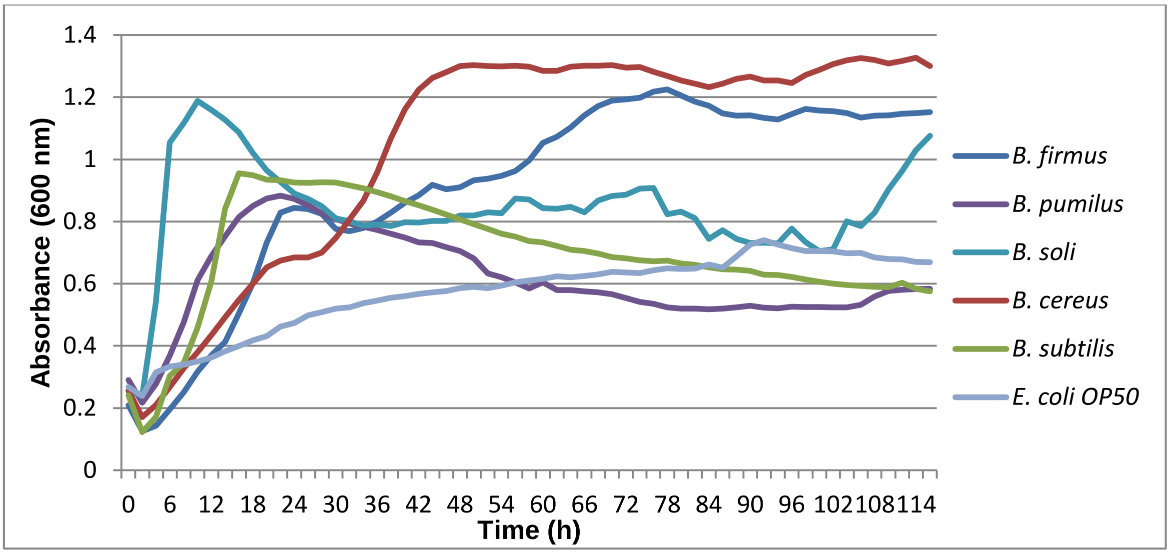

*Figure 8: Growth curve for bacterial strains cultivated in Luria-Bertani broth.*

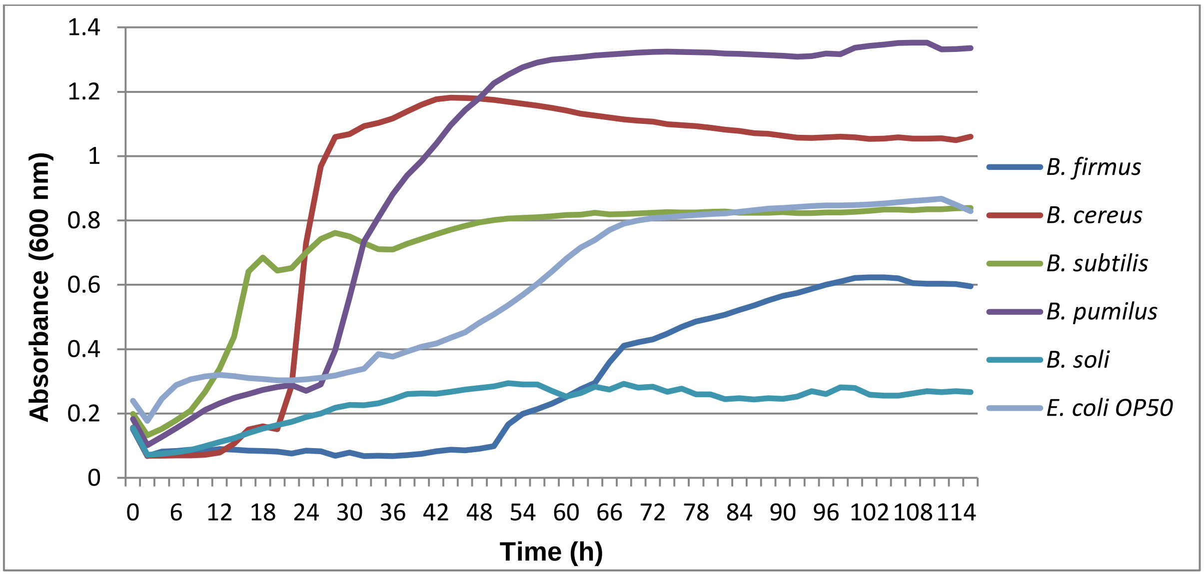

*Figure 9*: *Growth curve for bacterial strains cultivated in minimal broth.*

Results indicated that the initial incubation time of 48 h could not be applied to all bacterial strains to reach their stationary growth phase in each of the cultivation media. In the LB broth bacterial strains reached their stationary growth phase between 48 h and 72 h (Figure 8), in comparison to the approximate 96 h (Figure 9) of MB. These observations were valuable in the current study as the aim was to ensure that secondary metabolites (usually expressed in the stationary phase) were tested against the *M. incognita* J2. The bacterial strains were cultivated again in LB broth and MB but the incubation period was increased from 48 h to 72 h for LB broth, and to 96 h for

MB to ensure secondary metabolite production. Thereafter a second set of nematicidal bioassays were performed.

### 4.4.2 Nematode bioassays with bacterial filtrates

For the first (Figure 6) as well as the second set of bioassays (Figure 10), the *E. coli* OP50 control filtrates had the highest average percentage of *M. incognita* J2 motility. The highest levels of paralysis in LB broth after 114 h were observed for *B. firmus* (71.4%) and *B. pumilus* (73.6%) at 100% of the filtrate concentration (Figure 10), while *B. soli* and *B. cereus* had the highest levels of nematicidal activity in the initial bioassays (Figure 6). This may be attributed to the extended incubation period that was more optimal for production of secondary metabolites. Results from the growth curves (Figure 8) indicated that *B. soli* and *B. cereus* reached their stationary phase at about 48 h, well before *B. firmus* and *B. pumilus* at 72 h. However, when *B. firmus* and *B. pumilus* were allowed to reach their stationary growth phase, their nematicidal activity was found to be higher than that of *B. soli* and *B. cereus.*

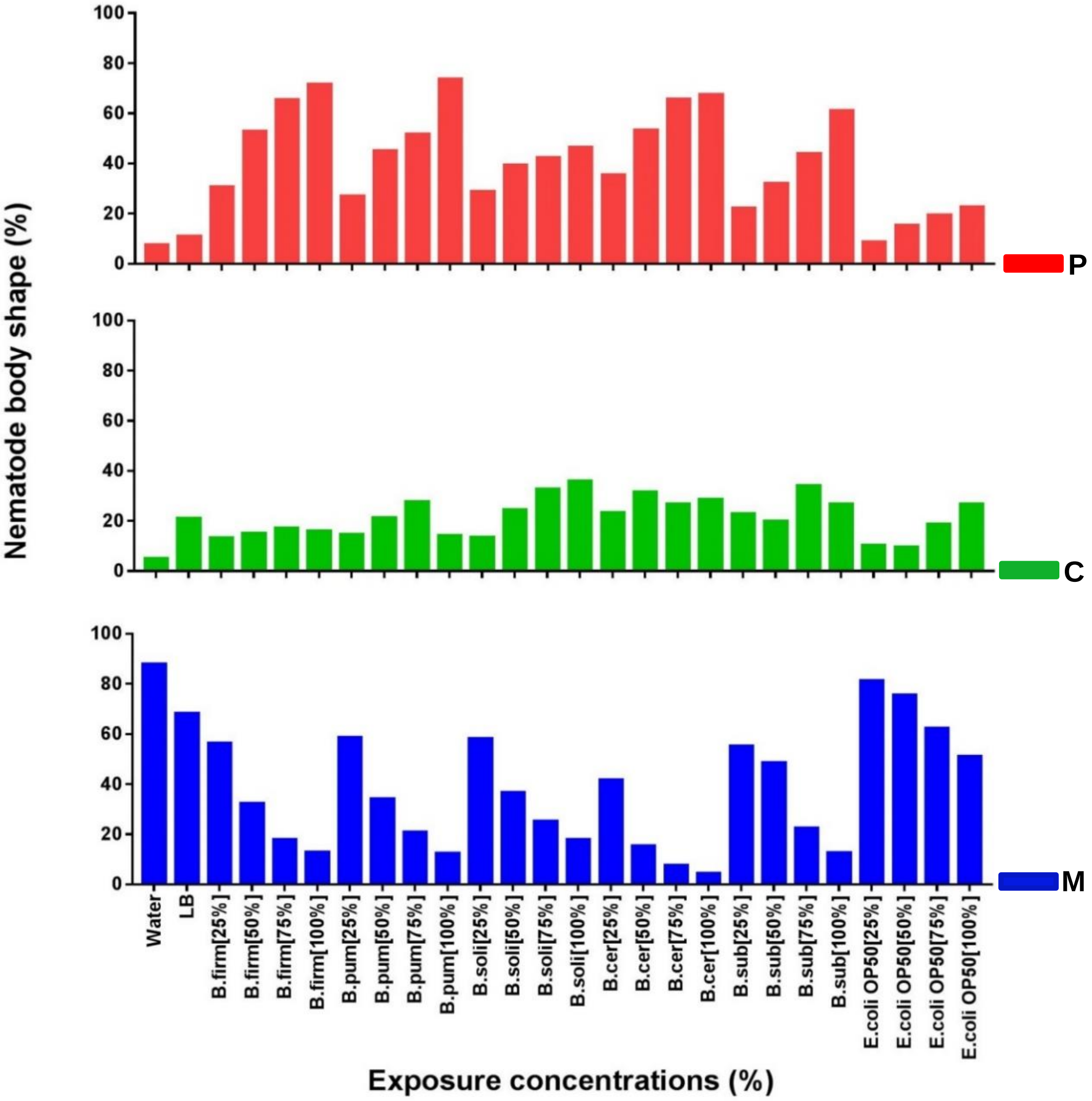

*Figure 10*: *Results of nematode bioassays to determine the effect of secondary metabolites produced by bacterial cultures cultivated in Luria-Bertani broth. M, C and P indicates the body shape of the nematodes [M: Motile, C: Curved and P: Paralysed (straight)]. Results obtained from sample replicates (n = 6).*

As for the LB broth, the first (Figure 7) and second set of bioassays (Figure 11) showed that the *E. coli* OP50 control filtrates had the highest average percentage of *M. incognita* J2 motility. The highest levels of paralysis in MB after 114 h were observed for *B. firmus* (63.7%) and *B. cereus* (69.8%) at 100% of the filtrate concentration (Figure 11), while *B. soli* and *B. cereus* had the highest levels of nematicidal activity in the initial bioassays (Figure 7). This again can be attributed to the extended incubation time that was more optimal for production of secondary metabolites. Results from the growth curves (Figure 9) indicated that *B. firmus* reached the stationary phase at about 108 h, well after all the other tested strains and in this its nematicidal activity was found to be higher than that of *B. soli* (Figure 10). The latter had the highest activity in the first set of assays where 48 h was used as the incubation time (Figure 7). These observations show the importance of cultivating individual bacterial spp. at optimised conditions in order to obtain the greatest yield of nematicidal products.

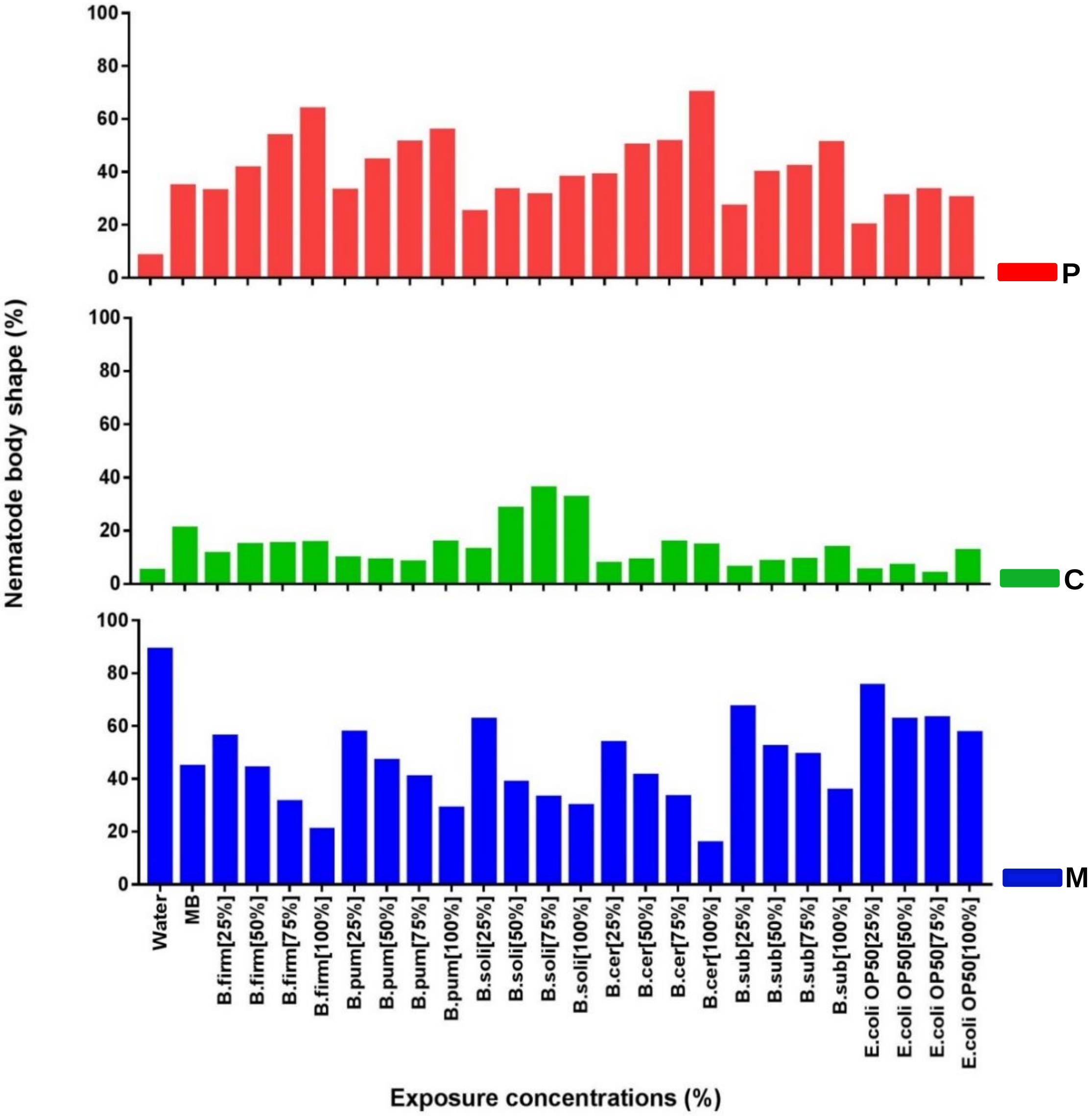

*Figure 11*: *Results of nematode bioassays to determine the effect of secondary metabolites produced by bacterial cultures cultivated in minimal broth. M, C and P indicates the body shape of the nematodes [M: Motile, C: Curved and P: Paralysed (straight)]. Results obtained from sample replicates (n = 6).*

In the current study, the observed paralysis of the J2 might be caused by production of different nematicidal toxins. This is in agreement with studies done by Terefe *et al*. (2009) and Xiong *et al*. (2015) that showed bacterial filtrates to have inhibitory effects on nematode motility. The nematicidal activity of *B. cereus* has also been observed in similar studies (Nagesh *et al.*, 2005; Xiang *et al.*, 2016), and it is known that *B. cereus* can produce several extracellular nematicidal toxins (Table 3) responsible for high levels of nematode paralysis. This study found that along with the filtrates of *B. cereus*, *B. firmus* and *B. pumilus*, the filtrates of *B. soli* were among the most effective in paralysing *M. incognita* J2. However, *B. soli* has not yet been studied in this capacity and there is no available literature on the nematicidal activity of this bacterium. Moreover, *B. soli* was initially included in this study as a control strain, however it proved to have levels of nematicidal activity comparable to that of the other *Bacillus* spp. (Figures 6, 7, 10 and 11).

Nematicidal activities of *B. firmus* filtrates against *M. incognita* were also observed in a study conducted by Xiong *et al.* (2015). They concluded that a strain of *B. firmus*, YBf-10, showed great potential as a source for nematicidal compounds while the nematicidal activity of this species was attributed to secondary metabolite production by Mendoza *et al*. (2008). Moreover, the activity of *B. firmus* is not only limited to J2 paralysis, but it also has ovicidal properties. When evaluating the effectiveness of BioNem, a bionematicide manufactured by AgroGreen, Mendoza *et al*. (2008) concluded that the *B. firmus* strain forming the active ingredient in the product produced toxins that had damaging effects on root-knot nematode eggs.

While studies have shown that *B. pumilus* is capable of stimulating plant growth (Lee & Kim, 2016), it can also produce extracellular hydrolytic enzymes, such as protease and chitinase (Table 3), that exhibit nematicidal activity. When reviewing the antagonistic potential of *B. pumilus* L1 against *M. arenaria*, Lee and Kim (2016) showed that hydrolytic enzymes caused damage to the cuticle of RKNs and to external structures of the nematode eggs. Such extracellular hydrolytic enzymes are of importance in bionematicide development (Nagesh *et al*., 2005; Ramezani Moghaddam *et al*., 2014) and might be present in the *B. pumilus* filtrates tested in this study.

Along with its plant growth promoting potential, Trojan horse mechanism (Table 3) and enzyme production, *B. subtilis* produces various secondary metabolites with a broad spectrum of activity, including suppression of phytopathogens. The most abundant of these secondary metabolites are cyclic lipopeptides (Table 3) (Nagórska *et al*., 2007; Ongena & Jacques, 2008; Yanfei *et al*., 2011; Zaghloul *et al*., 2015; Zheng *et al*., 2016). All of these nematicidal mechanisms identified in literature support the activity observed by *B. subtilis* filtrates in this study.

Various nematicidal activities provide rhizobacteria with unlimited potential for nematode biocontrol. The nematicidal activity demonstrated by the *Bacillus* filtrates in this study therefore support current literature which hypothesises that *Bacillus* secondary metabolites and hydrolytic enzymes are responsible for their antagonism towards nematodes. Their antagonism not only influences motility of the infective J2 stage of *M. incognita*, but can also damage the eggs. These are important physical aspects of the RKN life cycle (Figure 2) and impeding any step of the life cycle could lead to more effective control of reproduction and movement, thus limiting its damage to hosts (Xiong *et al.*, 2015).

# CHAPTER 5 RESULTS AND DISCUSSION: METABOLITE PROFILE ANALYSIS

## 5.1 Introduction

From the results obtained in the previous chapter, LB broth and MB were chosen as the appropriate cultivation mediums for the bacterial cultures. LB broth was chosen as a cultivation medium for two main reasons: (1) the LB broth had the lowest observed paralyses of *M. incognita* J2, and (2) it was the only cultivation medium that showed no negative effect on motility with increasing broth concentration. The MB was chosen due to its less complex media composition which interferes less during metabolomics analysis, as opposed to a more complex media. This chapter presents the results obtained from the metabolite profile analysis of the *Bacillus* spp. (*B. soli*, *B. firmus*, *B. cereus*, *B. subtilis*, and *B. pumilus*), and *E. coli* that had been cultivated in LB and MB broth. It was not possible within the constraints of the current investigation to repeat all metabolomics analyses with inclusion of *E. coli* OP50. However, this does not undermine the achievement of the set objectives for the study and will be investigated in a future project.

## 5.2 Liquid Chromatography-Mass Spectrometry (LC-MS) analyses

Preliminary results obtained from the LC-MS analysis of bacterial filtrates showed similar grouping of metabolite features, when compared to that of GC-MS analysis (results not shown). However, LC-MS results illustrated less distinctive grouping of the bacterial cultures and their metabolite features, in comparison to GC-MS results. The interference on the chromatographic separation and ionisation during analysis was problematic for both the complex LB and less complex MB (high salt content). The standard procedure with no sample clean-up for untargeted LC-MS metabolomics used by the analytical laboratory would need to be optimised for these types of media. For the present investigation, the LC-MS approach was therefore not pursued further as the analytical methods are outside the scope of this study. Therefore, it will not be discussed here. For future investigations, it would be recommended that the metabolomics laboratory optimise a method compatible with the media matrix.

### 5.3 Gas Chromatography-Mass Spectrometry (GC-MS) analyses

The filtrates of all bacterial cultures obtained from LB broth and MB were analysed by means of GC-MS. An interactive Principal Component Analysis (iPCA) was carried out on the data matrix of the different bacterial filtrates. The PCA is an unsupervised multivariate analysis that aims to find the directions that best explain the variance in a dataset and to enable visualisation of similarities between samples (Alonso *et al*., 2015). The iPCA visualisation summarises all the data into the first three principal components (PCs). Each data point represents a sample – in this case a bacterial filtrate and the closer together the points are on the plot, the more similar they are to each other (Xia and Wishart, 2016).

#### 5.3.1 Comparison of metabolite profiles from bacterial filtrates in Luria-Bertani broth

Figure 12 shows the iPCA carried out on the data of the bacterial cell-free filtrates obtained from the cultivation of *B. cereus*, *B. subtilis*, *B. firmus*, *B. pumilus*, *B. soli* and *E. coli* in LB broth. The grouping of data points was caused by the different metabolite features of each culture, with variances (%) shown in brackets. By excluding fresh and incubated LB broths from data before processing, only differences between the metabolite profiles of different bacterial spp. are visualised. In Figure 12, PC1 explained 26.7% of the total variance, whereas PC2 and PC3 explained 15.6% and 7.9% respectively. Profiles of *B. cereus*, *B. firmus*, *B. subtilis* and *B. soli* grouped closer together. Furthermore, *B. pumilus* grouped separately from the other *Bacillus* spp. The *E. coli* profiles grouped separate from the *Bacillus* spp. Among-flask variation was observed with the samples of *E. coli* flask 2 grouping further away from those of flask 1 and 3.

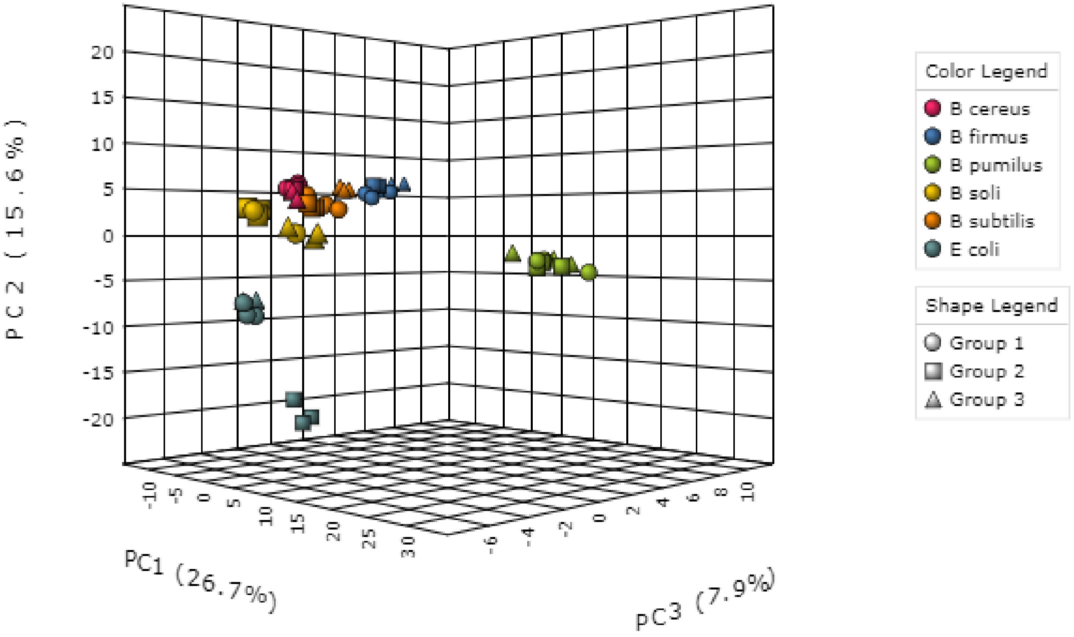

*Figure 12: 3-D iPCA plot indicating the distribution of the metabolite profiles obtained from untargeted metabolomics of all bacterial samples cultivated in Luria-Bertani broth. The three PC axes accounted for a total variance of 50.2%. Group 1-3 indicate sample replicates.*

In Figure 13 the iPCA carried out on the data of only the *Bacillus* spp. cultivated in LB broth is shown. This iPCA visualises the clustering of the metabolite profiles without the effect of the *E. coli* samples, so that bacterial metabolomes from the same genera could be compared. PC1 explained 37.5% of the total variance, whereas PC2 and PC3 explained 11.1% and 5.5% respectively. The metabolite profiles of *B. cereus* and *B. subtilis* are grouped together, indicating that they have more similar metabolite features compared to the other Bacillus spp. As with the LB broth samples, among-flask variation was also observed here, in this case for *B. cereus* and *B. subtilis.* This is of concern since it shows inconsistent inoculation or other methodological errors that occurred during the cultivation of the bacteria. If more replicate flasks were included, the outlier could be excluded from the statistical analysis. It is thus recommended that extra focus should be placed on methodological inconsistency and errors and that the number of flask replicates be increased in similar investigations in future.

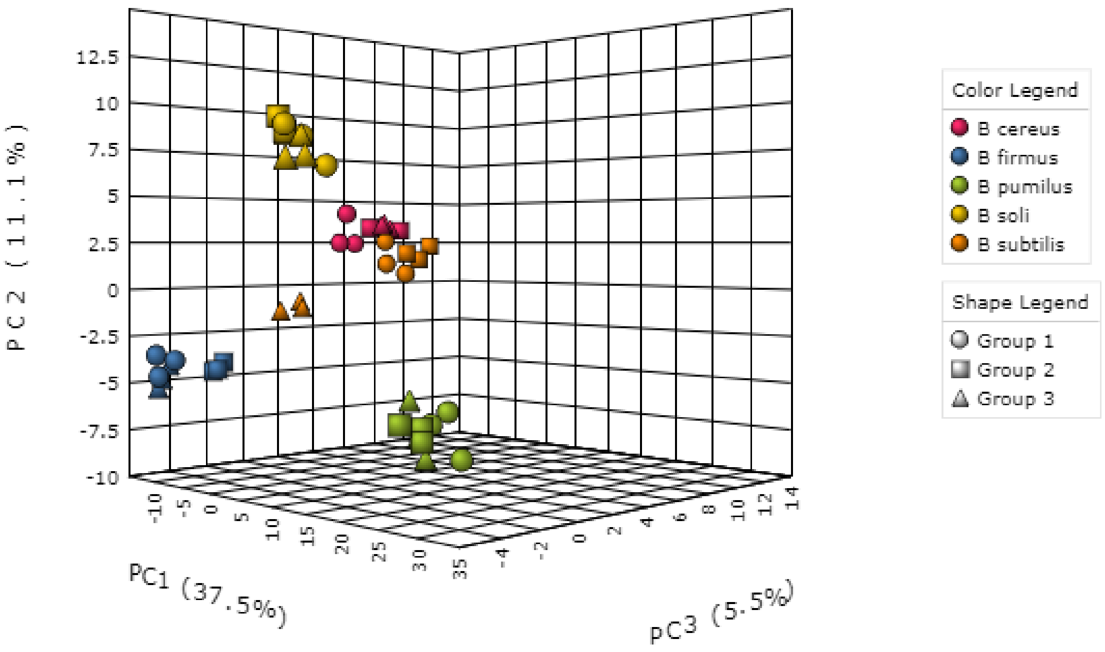

*Figure 13: 3-D iPCA plot indicating the distribution of the metabolite profiles obtained from untargeted metabolomics of all Bacillus samples cultivated in Luria-Bertani broth. The three PC axes accounted for a total variance of 54.1%. Group 1-3 indicate sample replicates.*

Since *B. pumilus* grouped separately (Figure 12 and Figure 13), it is evident that metabolite features produced by this spp. in LB broth are quite different from the metabolite features produced by *B. cereus*, *B. subtilis*, *B. soli* and *B. firmus*. Although all the *Bacillus* spp. in this study exhibited nematicidal activity, the separate grouping of *B. pumilus* could be attributed to the production of different features, as compared to *B. cereus*, *B. subtilis*, *B soli* and *B. firmus*. Alternatively, the separate grouping could be the result of observed media effects, such as different *Bacillus* spp. utilising media components differently.

### 5.3.2 Comparison of metabolite profiles from bacterial filtrates in minimal broth

Figure 14 shows the iPCA carried out on the metabolite profiles of the bacterial cell-free filtrates obtained from the cultivation of *B. cereus*, *B. subtilis*, *B. firmus*, *B. pumilus*, *B. soli* and *E. coli* in MB. PC1 explained 52.2% of the total variance, whereas PC2 and PC3 explained 12.5% and 4.9% respectively. When compared to the bacterial spp. cultivated in LB broth, those cultivated in MB provided more distinguishable separation among the different bacterial metabolite profiles and the total variance accounted for was higher (69.6% compared to 50.2% for the LB broth).

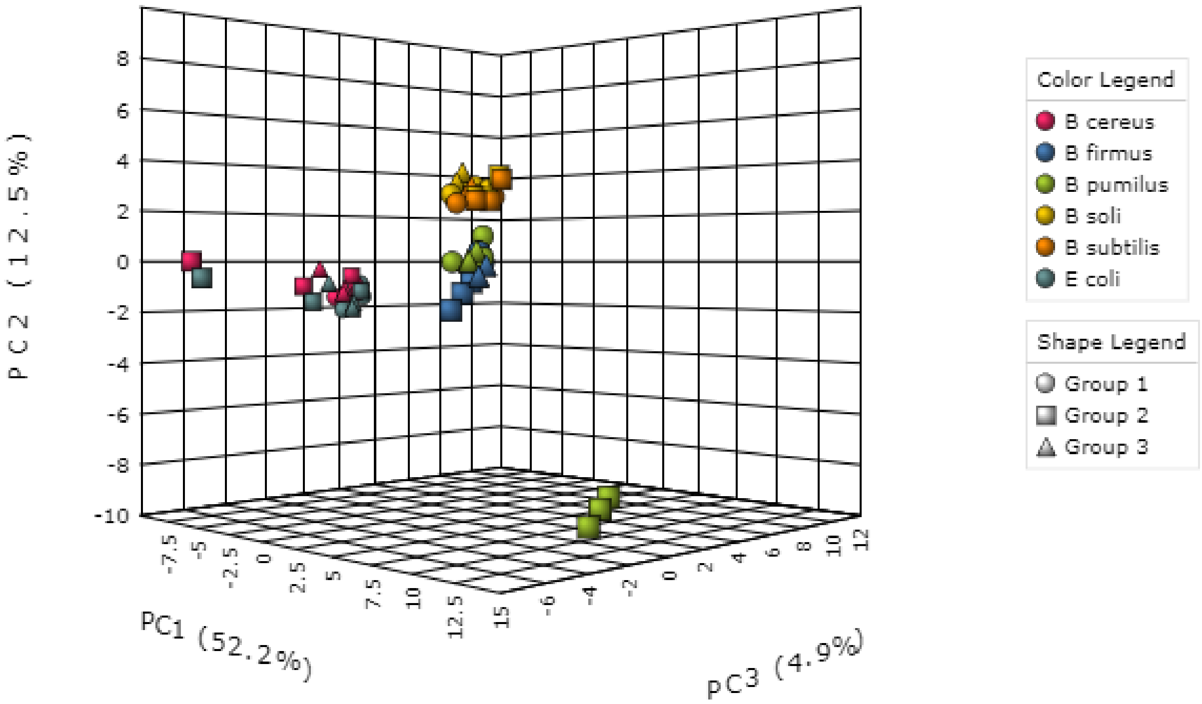

*Figure 14: 3-D iPCA plot indicating the distribution of the metabolite profiles obtained from untargeted metabolomics of all bacterial samples cultivated in minimal broth. The three PC axes accounted for a total variance of 69.6%. Group 1-3 indicate sample replicates.*

In contrast to the results seen in Figure 12, profiles of *B. soli*, *B. firmus*, *B. subtilis* and *B. pumilus* grouped closer together. Furthermore, profiles of *B. cereus* and *E. coli* grouped separately from the other *Bacillus* spp. The grouping of *B. cereus* and *E. coli* was not expected and should be investigated further since nematicidal activity was observed for *E. coli* during the nematode bioassays while it was initially included as a control.

In Figure 15 the iPCA carried out on the metabolite profiles of only the *Bacillus* spp. cultivated in MB is shown. Samples of *B. pumilus* flask 2 grouped further away from those of flask 1 and 3 (Figure 14 and 15), indicating among-flask variation in the samples. *Bacillus cereus*, *B. firmus* and *B. pumilus* profiles grouped together (Figure 15), indicating that they are more similar compared to *B. soli* and *B. subtilis*. PC1 explained 50.9% of the total variance, whereas PC2 and PC3 explained 14.9% and 5% respectively. In this iPCA it is again noted that the *Bacillus* spp.

cultivated in MB provided more distinguishable separation among the different metabolite profiles and the total variance accounted for was higher (70.8% compared to 54.1% for the LB broth).

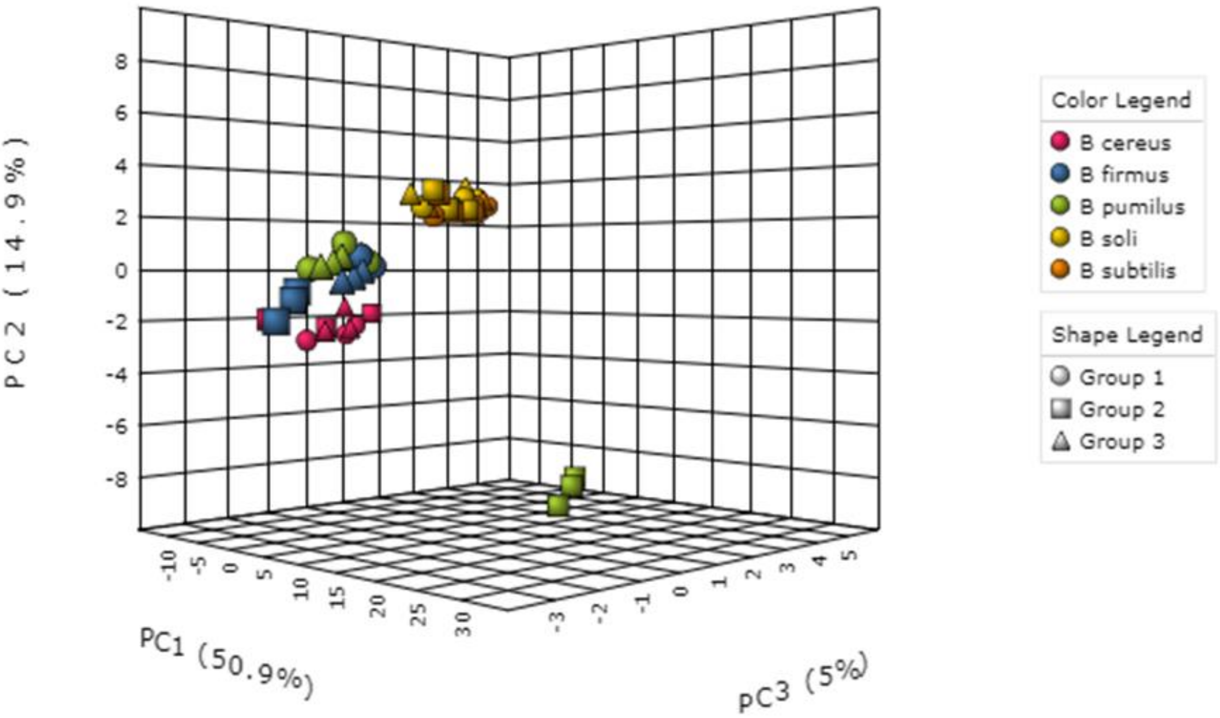

*Figure 15: 3-D iPCA plot indicating the distribution of the metabolite profiles obtained from untargeted metabolomics of all Bacillus samples cultivated in minimal broth. The three PC axes accounted for a total variance of 70.8%. Group 1-3 indicate sample replicates.*

In those cases, where there is more similarity between the metabolite profiles of *Bacillus* spp., it may be attributed to their phylogenetic and taxonomic association. Lu *et al*. (2014) found that the secondary metabolite profiles of bacterial spp. generally show correlation with their taxonomic similarities. In accordance with this, previous studies have shown that *B. firmus* and *B. cereus* (Yadav *et al*., 2015) show close evolutionary relatedness, while *B. pumilus* and *B. subtilis* (Hu *et al*., 2016) are genetically closer. This suggests that they might have similar metabolic relatedness. However, these specific groupings were less obvious in this study and were clearly influenced by the choice of cultivation medium, showing that taxonomic relatedness is not the only requirement for metabolic relatedness. When one compares the variation between *Bacillus* filtrates from the two media, it is noted that the filtrates had less variation when cultivated in MB (Figure 15) than in LB broth (Figure 13). This observation is crucial if one considers the possible effects choice of cultivation conditions will have on commercial production of biocontrol agents.

### 5.3.3 Unique metabolite features of bacterial species

Unique metabolite features are those features that were present in 100% of one experimental group, with one group in this study referring to a single *Bacillus* spp. (all replicates from all three flasks). Zero filtering was performed, meaning that if a feature was detected in all of the samples of one experimental group, the feature remained in the data matrix for further analysis. Those that did not meet this criterion were removed as they do not contain any statistical value (Venter, 2018). One-way ANOVA is a univariate analysis method that provides an initial overview of features that are potentially significant in discriminating the bacterial spp. under study. Figure 16 shows the one-way ANOVA of unique metabolite features produced by only the *Bacillus* spp. under investigation when cultivated in LB broth. Green plots indicate the features that were not significant ($p>0.05$), whilst the red plots indicate significant features ($p<0.05$) detected. A total of 390 unique metabolite features were detected across the five *Bacillus* spp. when cultivated in LB broth.

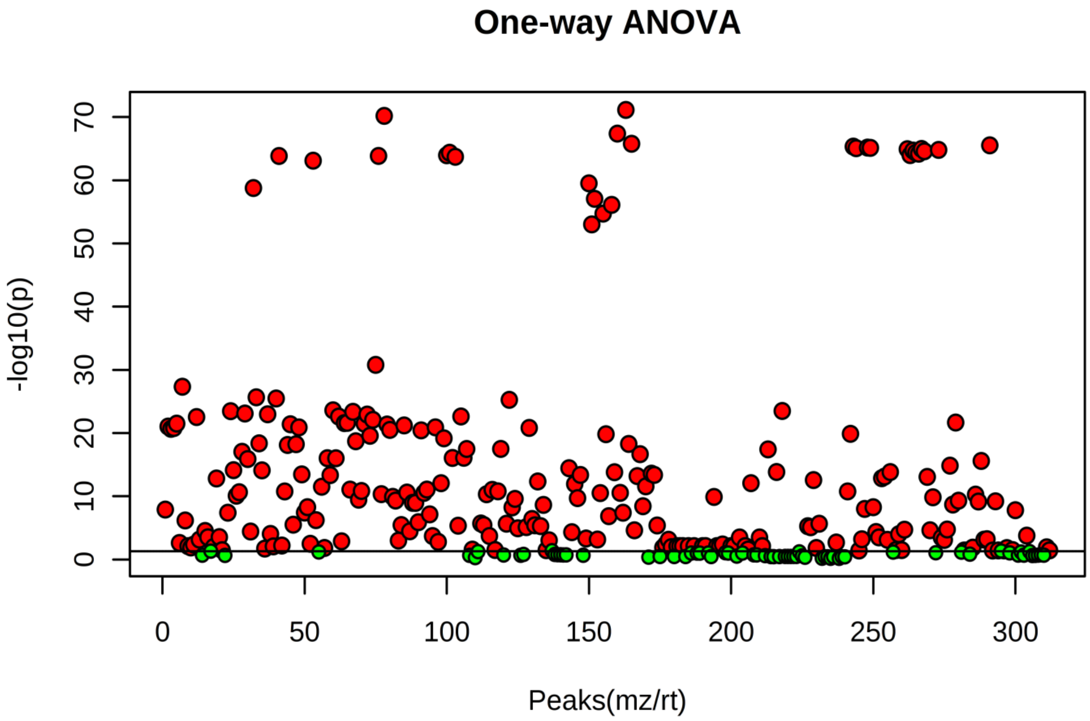

*Figure 16: Statistically significant features of Bacillus spp. cultivated in LB broth selected by One-way Analysis of Variance (ANOVA) of untargeted metabolomics, with a p value threshold of <0.05. Green plots indicate the features that were not significant (p>0.05), whilst the red plots indicate significant features (p<0.05) detected.*

Figure 17 shows the one-way ANOVA of unique metabolite features produced by only the *Bacillus* spp. under investigation when cultivated in minimal broth. Green plots indicate the features that were not significant (*p*>0.05), whilst the red plots indicate significant features (*p*<0.05) detected. A total of 58 unique metabolite features were detected across the five *Bacillus* spp. The lower number of unique features obtained from MB when compared to LB broth, is due to MB being a simpler medium but can also point towards the differences in metabolite production induced by the different cultivation media.

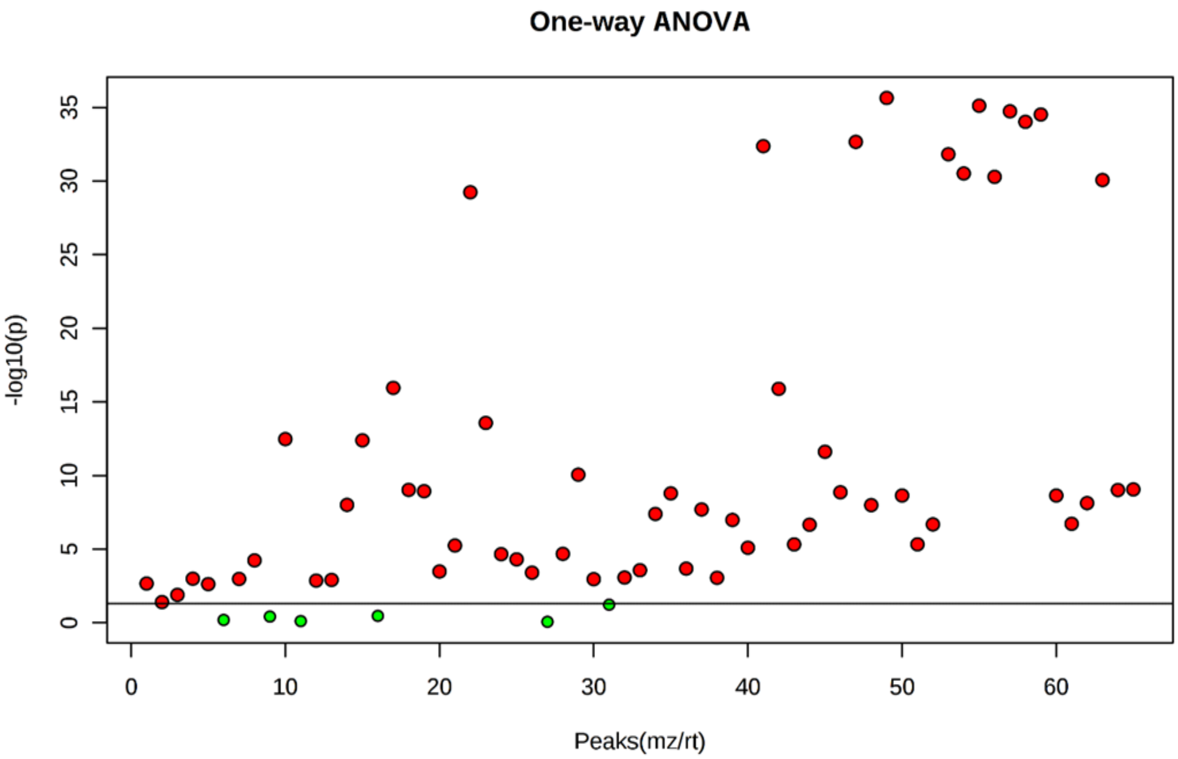

*Figure 17:. Statistically significant features of Bacillus spp. cultivated in MB selected by One-way Analysis of Variance (ANOVA) of untargeted metabolomics, with a p value threshold of <0.05. Green plots indicate the features that were not significant (p>0.05), whilst the red plots indicate significant features (p<0.05) detected.*

These unique metabolite features were identified based on the chromatographic peaks obtained from their mass to charge ratio (*m/z*) and specific retention time (*rt*), collectively referred to as peaks (mz/rt). Detected features which were not statistically significant (*p*>0.05) could be attributed to ingredients of the cultivation medium. These non-significant features are to be expected in all samples of the *Bacillus* spp. and thus not taken into consideration for additional investigations. However, detected features which were statistically significant (p<0.05) are of importance as they could possibly be metabolic compounds (metabolites) produced by the different *Bacillus* spp. that might contribute to their nematicidal activity.

### 5.4 Conclusion

Using a metabolomics approach to investigate bioactive molecules will eventually lead to a better understanding of nematicidal effects of secondary metabolites excreted by bacterial species. Depending on the aim of a study, different levels of identification can be obtained with metabolomics. These range from low-confidence identities, based only on accurate mass and isotope patterns, to the highest level of confidence where a reference standard and a retention time can be matched to the compound of interest (Venter, 2018).

The current investigation only went so far as low-level identification and it was already clear that methodological accuracy and sample repeatability can have significant effects on the metabolomics data obtained. If variability such as the among-flask variation observed in this study can be reduced, the numbers of unique features detected will be more reliable. Future investigations that attempt identification at higher confidence levels will be fruitless unless these basic steps of cultivation and sample preparation is not ideal.

# CHAPTER 6 CONCLUSION AND FUTURE RECOMMENDATIONS

## 6.1 General conclusion

The aim of this study was to compare the metabolic profiles of *Bacillus* spp. with known nematicidal activity, to bacteria without nematicidal activity. The study began with a literature overview of PPNs (with focus on the target nematode species used in this study, *M. incognita*) and relevant *Bacillus* spp., their cultivation conditions and the use of metabolomics methods to analyse their metabolites.

Different cultivation conditions, including media and incubation time were evaluated to identify the most appropriate conditions to ensure production of nematicidal metabolites by the different *Bacillus* species. Thereafter, *in vitro* tests were done using bacterial filtrates to determine their efficacy in immobilising *M. incognita* J2. Results obtained (Chapter 4) indicated that the cultivation media and conditions chosen can have a substantial effect on the production of nematicidal compounds. The bacterial strains exhibiting nematicidal activity were confirmed and incubation times were optimised for each species with Luria-Bertani (LB) broth and minimal broth (MB) respectively. Thereafter, metabolomics analysis was used to compare their metabolic profiles (Chapter 5). The profiles obtained, emphasised that the selection of cultivation conditions as well as the reproducibility of samples is crucial in metabolomics-based studies. Unless these aspects are satisfactorily addressed, the future goal of identification and production of specific bioactive molecules will not be achieved.

To achieve the aim of this study, four specific objectives were put forward at the start of the investigation. The major findings of these objectives were as follows:

### 1. Assessing and selecting appropriate culture media and conditions for nematode bioassays

The media evaluated in this study have been used in similar investigations, but these did not provide information on the effect of the cultivation media itself on nematode motility. This study proved that it is important to determine which cultivation media is suitable to conduct nematode bioassays as the media on its own can also have inhibiting effects on the motility of the nematodes. This will lead to false positive observations of paralysis of the target nematode. It is therefore important to test various cultivation media on the selected nematode species, as to ensure that a cultivation medium with minimal to no effects on the motility of the nematodes will be selected. Furthermore, the selected cultivation media should also be able to sustain the growth of the bacterial species that are being tested and ensure the production of metabolites of interest (such as nematicidal compounds). It was found that an incubation period of 48 h was insufficient for secondary metabolite production by *Bacillus* spp. and *E. coli* cultivated in different media and that different species had different growth curves. There was also a difference between the two media. For bacterial cultures cultivated in LB broth the incubation time had to be adjusted to at least 72 h to reach the stationary growth phase, while bacterial cultures cultivated in MB only reached their stationary phase of growth after 96 h or more.

The last and perhaps most difficult consideration when choosing cultivation media, is that it should adhere to the requirements discussed above while at the same time being appropriate for metabolomics analysis. Since the latter makes use of sensitive analytical equipment, compounds in the media can cause interference in metabolomics analysis. This should be avoided as it will have a negative effect on sensitivity and reproducibility.

From results obtained in this study, LB broth as a complex medium and MB as a simpler medium were chosen for cultivation of bacterial spp., *in vitro* nematode bioassays and metabolomics analysis of bacterial filtrates.

### 2. Testing *B. cereus*, *B. firmus*, *B. pumilus*, *B. subtilis*, *B. soli*, *and E. coli* for nematicidal activity against *M. incognita* J2*.*

All of the selected *Bacillus* spp., as well as *E. coli*, had nematicidal effects on the *M. incognita* J2. When cultivated in LB broth, *B. firmus* and *B. pumilus* showed the highest nematode paralysis in the optimised nematode bioassays. However, when cultivated in MB, *B. firmus* and *B. cereus* showed the highest nematode paralysis. This observation links to the first objective which showed that different cultivation conditions can affect the production of nematicidal compounds by the same bacterial spp. Also, the choice of a control bacterium should be carefully considered and seeing that *E. coli* also showed nematicidal activity, there might be many spp. besides *Bacillus* with potential as biocontrol agents. *E. coli* OP50 was found to have very little negative effects on nematode motility and should be used in future.

Although the results obtained in this study are in accordance with literature noting the nematicidal activity of *Bacillus* spp., more research is needed to determine the exact secondary metabolites and mechanisms of action that are responsible for the observed activity.

### 3. Evaluating the application of LC-MS and GC-MS for analysing complex (Luria-Bertani broth) and simpler (minimal broth) cultivation media and the bacterial metabolites produced in each

The use of a GC-MS analytical platform for untargeted metabolomics analysis proved to be more appropriate in this study. The sample preparation needed for GC-MS, although time consuming, is necessary as it enables the detection of features with polar functional groups. The use of the more complex cultivation media, LB broth, resulted in the detection of more statistically significant features produced by *Bacillus* spp., in comparison to the simpler MB. An LC-MS method was also evaluated in this study and showed more limitations than the GC-MS method. With LC-MS, less distinctive grouping of metabolite features was observed, making it difficult to visualise the metabolic variation between different bacterial spp. The type of LC-MS sample preparation and protocol used for untargeted metabolomics analysis requires more consideration and should be adapted in such way to ensure that optimal results will be obtained.

### 4. Profiling the bacterial metabolites from filtrates tested *M. incognita* J2.

Untargeted metabolomics on a GC-MS platform proved to be a suitable approach to profile the bacterial metabolites from filtrates tested against J2 of *M. incognita*. The metabolite profiles of the selected bacterial strains not only showed variation between different species, but also between the two different cultivation media selected for this study. When cultivated in LB broth, a total of 390 unique metabolite features were detected across the five *Bacillus* spp. When cultivated in MB, only 58 unique metabolite features were detected. The majority of these features were statistically significant ($p<0.05$). Although all of the bacterial species showed nematicidal activity (Chapter 4), results from the untargeted metabolomics analysis showed differences between the metabolite profiles of the various *Bacillus* spp. This could indicate different metabolites being responsible for observed nematicidal activity in the species under investigation.

Among-flask variation was also observed in this study and it is noted that this variation can influence the grouping of metabolite profiles visualised with interactive Prinicipal Component Analysis.

## 6.2 Recommendations

Two important questions that require further investigation were raised from this study. Firstly, what are the optimal cultivation media and conditions for different bacterial species with potential as biocontrol agents? Secondly, which metabolites are responsible for the observed nematicidal activities?

To ensure that the bacteria under investigation produce secondary metabolites (extracellular), they should be allowed to reach their stationary phase of growth; it is important to obtain a bacterial growth curve for each specific strain to be investigated beforehand. Since it is crucial that the culture media should be selected to ensure optimal cultivation conditions and the production of the metabolites of interest, this choice will be greatly influenced by the specific bacterium. Therefore, this should serve as a motivation for studies that will lead to the optimisation of analytical techniques that will be appropriate for the complexity of the matrix (culture media). This has already been done for various complex biological matrices such as urine and blood. In addition, the number of sample replicates obtained during cultivation of relevant bacteria should be increased. If there are more sample replicates, outliers can more easily be excluded from metabolomics data without compromising on data integrity.

Metabolomics is an important aspect of systems biology and has a broad range of applications in agricultural research, including the development of alternatives to agrochemicals. Future research about bioactive molecules or secondary metabolites of *Bacillus* spp. should include greenhouse experiments and field experiments, but only after successful laboratory studies. Standardised and universal procedures for bacterial cultivation, metabolic quenching, filtrate and microbial cell separation, metabolite extraction and nematode bioassays is required to comprehensively analyse bacterial metabolites, as these sample preparation processes can directly influence the metabolomics data obtained. Such standardisation will improve the reproducibility of the results and allow the comparison of data amongst different laboratories. Successful proof of concept studies in the laboratory will promote public, industry and governmental funding for research that in turn can contribute to the battle against PPNs and potentially eliminate harmful chemical nematicides from the global market.

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# APPENDIX A

*Table 5: Mean and standard error for nematode body shape after exposure to bacterial filtrates cultivated in Luria-Bertani broth. The M, C and P indicates the body shape of the nematodes [M: Motile. C: Curved and P: Paralysed (straight)]. Letters a and b: Tukey's test for mean separation where differences of p=0.05 indicates significant difference.*

| | | **Nematode body shape (%)** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **[Supernatant]** | | ***B. firmus*** | ***B. pumulis*** | ***B. cereus*** | ***B. subtulis*** | ***B. soli*** | ***E. coli*** | ***E. coli OP50*** |
| **25%** | **M** | 66.88 ± 1.89$^{c}$ | 61.59 ± 8.65$^{b}$ | 72.34 ± 9.89$^{b}$ | 60.49 ± 8.69$^{c}$ | 59.15 ± 3.77$^{c}$ | 79.87 ± 6.35$^{c}$ | 76.32 ± 15.66$^{a}$ |
| | **C** | 15.23 ± 1.22$^{b}$ | 19.56 ± 2.44$^{a}$ | 10.64 ± 1.82$^{a}$ | 21.60 ± 6.00$^{a}$ | 12.67 ± 1.88$^{c}$ | 8.81 ± 1.26$^{a}$ | 5.26 ± 1.36$^{a}$ |
| | **P** | 17.88 ± 1.70$^{b}$ | 18.84 ± 5.34$^{a}$ | 17.02 ± 4.79$^{b}$ | 17.90 ± 2.23$^{a}$ | 28.16 ± 2.08$^{b}$ | 11.32 ± 2.76$^{a}$ | 18.42 ± 2.96$^{a}$ |
| **50%** | **M** | 43.88 ± 4.23$^{b}$ | 52.55 ± 4.07$^{b}$ | 25.33 ± 6.50$^{a}$ | 43.37 ± 4.07$^{b,c}$ | 27.34 ± 1.88$^{a}$ | 56.29 ± 5.37$^{b,c}$ | 67.69 ± 9.51$^{a}$ |
| | **C** | 23.02 ± 2.13$^{a,b}$ | 21.89 ± 1.95$^{a}$ | 24.00 ± 3.27$^{a,b}$ | 25.30 ± 2.09$^{a}$ | 23.43 ± 1.71$^{a,b}$ | 21.19 ± 3.19$^{a,b}$ | 13.85 ± 2.92$^{a}$ |
| | **P** | 33.09 ± 2.87$^{a}$ | 25.54 ± 3.98$^{a,b}$ | 50.67 ± 4.81$^{a}$ | 31.33 ± 3.45$^{a,b}$ | 49.21 ± 3.36$^{a}$ | 22.52 ± 2.22$^{a}$ | 18.46 ± 2.66$^{a}$ |
| **75%** | **M** | 27.67 ± 5.65$^{a}$ | 31.42 ± 2.63$^{a}$ | 20.83 ± 3.88$^{a}$ | 24.31 ± 4.22$^{a,b}$ | 21.19 ± 3.50$^{a}$ | 35.90 ± 11.31$^{a,b}$ | 61.34 ± 9.78$^{a}$ |
| | **C** | 29.55 ± 4.61$^{a}$ | 29.28 ± 3.21$^{a}$ | 25.69 ± 5.09$^{b}$ | 31.94 ± 3.51$^{a}$ | 29.13 ± 1.32$^{b}$ | 21.37 ± 4.06$^{a,b}$ | 16.81 ± 3.83$^{a,b}$ |
| | **P** | 42.76 ± 3.18$^{a}$ | 39.28 ± 3.73$^{b,c}$ | 53.47 ± 7.17$^{a}$ | 43.75 ± 8.10$^{b,c}$ | 49.66 ± 3.66$^{a}$ | 42.74 ± 7.33$^{b}$ | 21.85 ± 4.99$^{a}$ |
| **100%** | **M** | 12.94 ± 1.93$^{a}$ | 11.59 ± 3.49$^{a}$ | 10.81 ± 1.71$^{a}$ | 10.34 ± 2.33$^{a}$ | 8.38 ± 1.55$^{b}$ | 12.73 ± 3.90$^{a}$ | 48.84 ± 5.97$^{a}$ |
| | **C** | 30.93 ± 2.34$^{a}$ | 31.88 ± 4.71$^{a}$ | 20.27 ± 2.56$^{a,b}$ | 30.34 ± 3.65$^{a}$ | 22.58 ± 1.55$^{a}$ | 30.00 ± 5.76$^{b}$ | 29.07 ± 5.53$^{b}$ |
| | **P** | 56.11 ± 3.69$^{c}$ | 56.52 ± 5.61$^{c}$ | 68.92 ± 7.33$^{a}$ | 59.31 ± 4.86$^{c}$ | 69.03 ± 3.52$^{c}$ | 57.27 ± 5.03$^{b}$ | 22.09 ± 3.79$^{a}$ |
| **dH$_2$0** | **M** | 88.97 ± 5.53$^{a}$ | | | **LB [100%]** | **M** | 68.18 ± 1.97$^{a}$ | |
| | **C** | 4.89 ± 1.52$^{a}$ | | | | **C** | 21.02 ± 4.17$^{a}$ | |
| | **P** | 8.16 ± 2.58$^{a}$ | | | | **P** | 10.80 ± 1.63$^{a}$ | |

# APPENDIX B

*Table 6: Mean and standard error for nematode body shape after exposure to bacterial filtrates cultivated in minimal broth. The M, C and P indicates the body shape of the nematodes [M: Motile. C: Curved and P: Paralysed (straight)]. Letters a and b: Tukey's test for mean separation where differences of p=0.05 indicates significant difference.*

**Nematode body shape (%)**

| **[Supernatant]** | | ***B. firmus*** | ***B. pumulis*** | ***B. cereus*** | ***B. subtulis*** | ***B. soli*** | ***E. coli*** | ***E. coli OP50*** |
|---|---|---|---|---|---|---|---|---|
| **25%** | **M** | 86.44 ± 1.91$^{c}$ | 64.48 ± 5.13$^{b}$ | 54.69 ± 3.68$^{a}$ | 78.52 ± 2.03$^{b}$ | 60.00 ± 5.17$^{b}$ | 60.65 ± 5.71$^{b}$ | 82 ± 1.89$^{a}$ |
| | **C** | 7.91 ± 0.71$^{a}$ | 13.11 ± 1.20$^{b}$ | 20.31 ± 2.39$^{a}$ | 11.08 ± 2.03$^{b}$ | 14.78 ± 2.09$^{a}$ | 19.35 ± 1.73$^{a}$ | 8 ± 2.59$^{a}$ |
| | **P** | 5.65 ± 1.68$^{b}$ | 22.40 ± 2.86$^{a}$ | 25.00 ± 1.14$^{a}$ | 10.60 ± 1.22$^{b}$ | 25.22 ± 1.29$^{a}$ | 20.00 ± 1.55$^{a}$ | 9 ± 1.13$^{a}$ |
| **50%** | **M** | 61.70 ± 3.25$^{b}$ | 53.67 ± 2.54$^{a,b}$ | 46.15 ± 5.53$^{a}$ | 39.89 ± 3.73$^{a}$ | 44.39 ± 4.98$^{a,b}$ | 63.19 ± 1.76$^{b}$ | 77 ± 2.41$^{a}$ |
| | **C** | 13.48 ± 2.56$^{a,b}$ | 18.08 ± 1.89$^{a,b}$ | 17.95 ± 1.28$^{a}$ | 21.31 ± 4.13$^{a,b}$ | 23.36 ± 2.25$^{a,b}$ | 11.66 ± 1.13$^{b}$ | 11 ± 1.10$^{a}$ |
| | **P** | 24.82 ± 2.56$^{a}$ | 28.25 ± 1.43$^{a,b}$ | 35.90 ± 2.75$^{b}$ | 38.80 ± 2.60$^{a}$ | 32.24 ± 3.30$^{a}$ | 25.15 ± 1.48$^{a}$ | 11 ± 2.35$^{a}$ |
| **75%** | **M** | 50.84 ± 2.36$^{a}$ | 48.55 ± 5.07$^{a,b}$ | 47.62 ± 5.02$^{a}$ | 32.49 ± 3.11$^{a}$ | 38.61 ± 5.31$^{a,b}$ | 36.25 ± 6.68$^{a}$ | 75 ± 2.63$^{a}$ |
| | **C** | 23.46 ± 2.45$^{c}$ | 20.81 ± 1.55$^{a}$ | 22.62 ± 1.19$^{a}$ | 25.89 ± 2.33$^{a}$ | 19.80 ± 2.73$^{a,b}$ | 23.13 ± 1.15$^{a}$ | 13 ± 1.36$^{a}$ |
| | **P** | 25.70 ± 2.55$^{a}$ | 30.64 ± 1.07$^{b}$ | 29.76 ± 3.01$^{a,b}$ | 41.62 ± 3.98$^{a}$ | 41.58 ± 7.93$^{a,b}$ | 40.63 ± 2.25$^{b}$ | 12 ± 3.12$^{a}$ |
| **100%** | **M** | 40.59 ± 3.12$^{a}$ | 39.18 ± 3.67$^{a}$ | 24.84 ± 3.42$^{b}$ | 29.59 ± 2.04a | 15.34 ± 2.29$^{c}$ | 36.36 ± 2.74$^{a}$ | 72 ± 3.28$^{a}$ |
| | **C** | 20.59 ± 1.68$^{b,c}$ | 22.22 ± 1.96$^{a}$ | 20.50 ± 2.10$^{a}$ | 25.51 ± 3.13$^{a}$ | 30.68 ± 3.94$^{b}$ | 22.73 ± 1.44$^{a}$ | 11 ± 2.34$^{a}$ |
| | **P** | 38.82 ± 1.82$^{c}$ | 38.60 ± 1.28$^{c}$ | 54.66 ± 1.84$^{c}$ | 44.90 ± 1.88$^{a}$ | 53.98 ± 4.44$^{b}$ | 40.91 ± 1.97$^{b}$ | 17 ± 1.59$^{a}$ |
| **dH$_2$0** | **M** | 88.97 ± 5.53$^{a}$ | | | **MB [100%]** | **M** | 44.51 ± 9.47$^{a}$ | |
| | **C** | 4.89 ± 1.52$^{a}$ | | | | **C** | 20.88 ± 5.56$^{a}$ | |
| | **P** | 8.16 ± 2.58$^{a}$ | | | | **P** | 34.62 ± 6.36$^{a}$ | |

# APPENDIX C TITLE PAGE OF PUBLISHED ARTICLE

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REVIEW ARTICLE

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## *Bacillus*-based bionematicides: development, modes of action and commercialisation

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**ABSTRACT**

Agricultural crops are severely damaged by root-knot nematodes causing extensive financial losses globally. Historically, agrochemicals have been the preferred method to combat these pests; however, threats to humans and the environment posed by these agrochemicals led to the need for developing new biocontrol agents. Importantly, the latter should adhere to biosafety regulations while being highly effective. Root-knot nematodes live in soil and thus the use of rhizobacteria such as *Bacillus* for biocontrol development have shown potential. Although various *Bacillus* species have been tested in this capacity, little is known about their secondary metabolites and the mechanisms of action responsible for their nematicidal activity. If these secondary metabolites can be qualitatively and quantitatively characterised, metabolic features could be synthetically engineered and used to combat root-knot nematodes. Although there is great potential for bionematicides, the commercialisation and development of such products can be difficult. This review summarises the importance of *Bacillus* species as natural antagonists of root-knot nematodes through the production of secondary metabolites. It provides an overview of the significance of root-knot nematodes in agriculture and the advances of chemical nematicides in recent years. The potential of *Bacillus* species as biocontrol agents, the known mechanisms of action responsible for the nematicidal activity demonstrated by *Bacillus* species, non-target effects of biocontrol agents and the commercialisation of *Bacillus*-based bionematicides are discussed.

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*Bacillus*; biocontrol; *Meloidogyne incognita*; plant growth-promoting rhizobacteria; root-knot nematode; secondary metabolites

## 1. Introduction

Root-knot nematodes (RKNs) such as *Meloidogyne* spp. severely damage the roots of agricultural crops (Gao et al., 2016) causing poor yield (Lee & Kim, 2016) that results in great financial losses annually (Barker & Koenning, 1998). Annual global crop losses associated with RKNs are estimated to be $78 billion (Lima, Correa, Nogueira, & Santos, 2017). Although agrochemicals have been used to combat these RKNs since the 1940s (Rosas-García, 2009), environmental concerns about the use of chemical toxins and their negative

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