

Characterisation of a biological soil culture and its effects on the biology of root-knot nematodes

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Abstract

Root-knot nematodes (*Meloidogyne* spp.) are the number one economically important and damaging nematode pest of agri- and horticultural crops. Withdrawal of synthetic nematicides from markets necessitated the exploitation of biological products with anti-nematodal characteristics. Therefore, crude extracts of a novel, soil-derived biological product (SoilBioMuti; SBM) that contain an array of micro-organisms was studied during this project in terms of i) its bacterial composition and ii) effects on the biology of the predominant root-knot nematode species *Meloidogyne incognita* infecting local maize crops. Identification of the bacterial genera contained in SoilBioMuti was done using plating as well as molecular (Next Generation Sequencing; NGS) techniques. Bacterial counts on two agar media (nutrient and MRS) were significantly higher in the fresh SBM compared to those in the 'cooled' SBM (exposed to 5 °C for 24 h before plating). The freshly-prepared SBM product contained approximately 99 % more bacteria than did the 'cooled' sample. According to NGS results, 45 bacterial genera were identified from the two freshly prepared stock SBM samples. Non-pathogenic genera constituted 49 % and beneficial bacterial approximately 50 % of the bacterial community of SBM and represented several genera that are known for their anti-nematodal effects. Novel knowledge on the adverse effect of 2.5 % SBM treatments has also been generated regarding the oxygen consumption of infective *M. incognita* second-stage juveniles (J2). According to data obtained, 100 J2 were optimal to use for determining the oxygen consumption rates (OCR). The OCR recorded for non-filtered (NF) SBM containing J2 were 42% compared to its counterpart treatment without J2. However, the OCR of J2 suspended in sterile tap water did not differ significantly from that of the NF SBM containing J2. The two filtered (F) SBM treatments had the lowest OCR and differed significantly from that of the NF SBM treatments and the tap-water control containing J2. *In vitro* evaluations on the effect of different SBM product concentrations (2.5, 3, 4 and 5 %) on J2 motility and *in vivo* testing of the reproduction abilities of *M. incognita* also produced novel information. For motility assays, all SBM concentrations (F and NF) significantly reduced J2 motility (below 10 %) throughout the 24 h experimental period. For reproduction assays, greenhouse data showed that all NF SBM product concentrations reduced the egg-laying female indices on roots of a susceptible tomato cultivar (Floradade). Although results from a first tunnel experiment yielded no conclusive data for 2.5 % SBM, a

follow-up experiment revealed 83 and 77 % reductions in nematode reproduction in maize roots that grew in soils treated with Wonder™ + hay and 5 % SBM + hay, respectively. The SBM treatments, however, had no significant effect on various plant-growth variables measured for both tunnel experiments. Although it can be concluded that the bacterial content of SBM has been determined to a certain extent and its adverse effects on the biology of *M. incognita* has been proven, knowledge on the specific bacteria (and/or other micro-organisms) that may act as the active substances is still elusive.

Keywords: Bacteria, maize, *Meloidogyne incognita*, micro-organisms, root-knot nematodes.

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Chapter 1: Introduction and literature review

1.1 Introduction

Increased awareness to preserve and maintain the soil quality of arable land to improve crop production, accentuates the importance of using eco-friendly strategies to protect crops against diseases and pests. The progressive increase in the global human population necessitates the successful production of agri- and horticultural crops (from hereon referred to as crops only) that serve as food. A wide range of diseases and pests, particularly plant-parasitic nematodes, threatens crop production worldwide. Plant-parasitic nematodes are omnipresent in agricultural soils where they cause damage to a wide range of crops (Jones *et al.*, 2013). Root-knot nematodes (*Meloidogyne* spp.) are listed as the economically most important nematode pest of numerous crops worldwide and are also the most abundant in local maize-based cropping systems where they inflict substantial yield losses. The predominant root-knot nematode species in local maize (*Zea mays* L.) production areas are *Meloidogyne incognita* (Kofoed and White, 1919), Chitwood, 1949, followed by *Meloidogyne javanica* (Treub, 1885), Chitwood, 1949. For the purpose of this study, *M. incognita* was hence used as the test organism.

By means of this dissertation, the author initially enlightens the reader about the broad taxonomy of plant-parasitic nematodes as well as the trophic groups such pests belong to. Emphasis is further also placed on basic knowledge of *Meloidogyne* spp., referring to their biology and morphology, distribution and management with focus on eco-friendly products to reduce population densities of these pests. The technical part of the dissertation encompasses firstly the identification of the bacterial community contained by a novel product, referred to as SoilBioMuti (SBM) that is proposed to have anti-nematodal (nematicidal or nematostatical) properties. This was done by deoxyribonucleic acid (DNA) identification of three SBM batches (produced during different times) that were recommended by the owner of the product and used unofficially by local producers for its growth-promoting and anti-nematodal characteristics (Annex 1). Secondly the effect of SBM, filtered (F) and non-filtered (NF), on the oxygen consumption of second-stage juveniles (J2) of *M. incognita* was determined using fibre-optic sensor technology. In the third place, *in vitro* evaluation of the effect of SBM on the motility of *M. incognita* J2 over a period of

24 h was determined followed by *in vitro* determination of the effect of the product on the reproduction of this root-knot nematode species. The latter was done in glasshouse and tunnel experiments.

1.2 Literature review

1.2.1 Maize

With regard to world food production, maize, also called corn, ranks as one of the top three most cultivated crops. Only wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) are produced in larger quantities than maize (FAO, 2016). The nutritional value of maize kernels is high, containing ± 72 % starch, 10 % protein and 4 % fat, supplying energy of 365 Kcal/100 g. The crop is grown worldwide, with the United States of America (USA), China, and Brazil topping the list as the dominant maize-producing countries, producing some 563 of the 717 million metric ton (MT)/year. Maize is cultivated in tropical and subtropical, but also temperate regions (Ranum *et al.*, 2014). Rainfall in areas where maize is cultivated under irrigation mostly range from 450-600 mm per annum, and from 600-900 mm per annum in areas where the crop is cultivated under dry-land conditions (Sprague and Dudley, 1988; Tekwa and Bwade, 2011).

In developing countries, including SA, maize serves as staple food for some 200 million people (Anonymous, 2016a). White maize is mainly cultivated for human consumption and yellow maize for animal fodder (SAGIS, 2016). Maize is grown during the summer season in South Africa (SA) under a range of climatic conditions, with a range of hybrids being bred to perform optimally (Anonymous, 2016a). Most people regard maize as a breakfast cereal. However, in a processed form it is also used as biofuel, ethanol and starch (Anonymous, 2016a; Ranum *et al.*, 2014).

1.2.1.1 Origin and classification

Maize was introduced to SA in 1655, shortly after the Dutch colonists arrived (Saunders, 1930). The origin of maize was in the Mesoamerican region, probably in the Mexican highlands 7 000 years ago, from where it spread to other parts of the world during the 15th century (Paliwal, 2000; Farnham *et al.*, 2003; Ranum *et al.*, 2014). Domestication of maize hence began at least 6 000 years ago (Piperno and Flannery, 2001; Matsuoka *et al.*, 2002). The genus *Zea* is classified under the family

Poaceae (OECD, 2003; USDA, 2005). Currently, there are five species included in the genus *Zea* (Ellneskog-Staam *et al.*, 2007).

1.2.1.2 Basic anatomy, morphology and growth and development

The typical maize plant (Fig. 1.1.) is a seasonal and tall (1–4 m) annual grass monocotyledon, bearing a single, cylindrical and solid erect stem ranging from <0,6 - >5,0 m (depending on the genotype) which consists of nodes and internodes. Maize is a monoecious plant, bearing both male and female flowers as separate inflorescences. Male flowers are located in the tassel and female flowers in the ear.

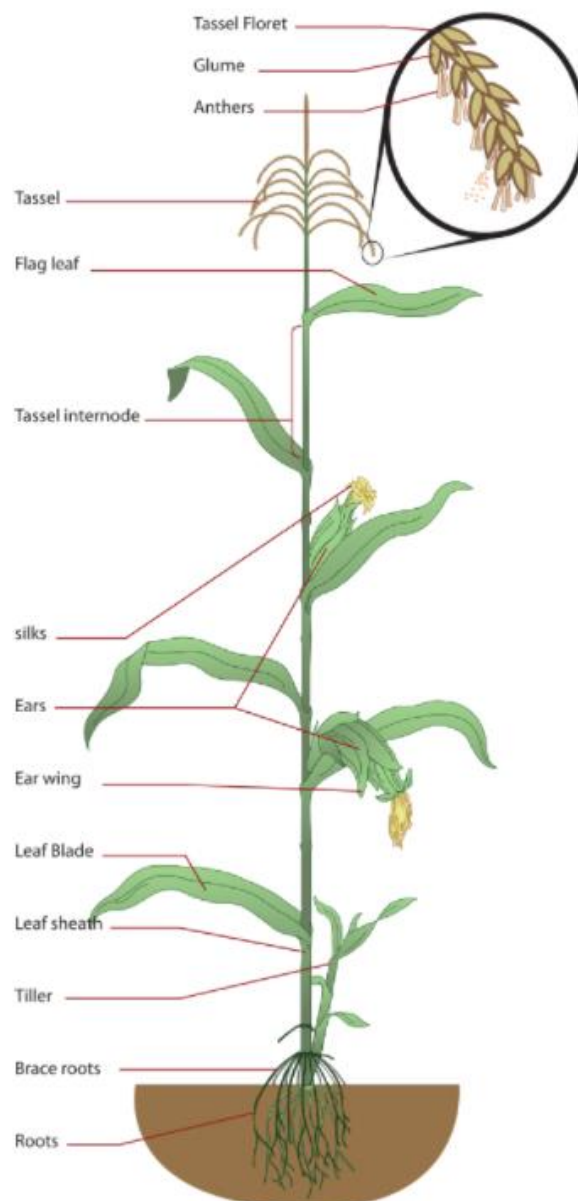


Fig. 1.1. The basic anatomy and morphology of a maize plant Illustration: ([https://www.google.co.za/search?q=image + of+a+maize+ plant&espv](https://www.google.co.za/search?q=image+of+a+maize+plant&espv)).

The growth stages of maize plants are tabulated in Table 1.1.

Table 1.1. The growth stages of maize with concise details of the characteristics of each stage (Anonymous, 2016a).

Growth stage	Characteristics
Planting to seedling emergence	Germination, the growth point and the entire stem are about 25-40 mm below the soil surface. Seed emergence, 10 days under warm, moist conditions and 14 days under cool or dry conditions. Optimum temperature ranges for germination is between 20-30 °C, while optimum moisture content of the soil should be approximately 60 % of soil capacity.
Four leaves completely unfolded	The maximum number of leaves and lateral shoots are predetermined. New leaves unfold approximately every third day. The growth points are still below the soil surface, while aerial parts are limited to the leaf sheath and blades. Initiation of tasselling occurs at this stage.
Eight leaves completely unfolded	Leaf area increases 5-10 times, stem mass increases 50-100 times. Ear initiation has commenced and tillers begin to develop from nodes below the soil surface. The growth point is approximately 5.0-7,5 cm above the soil surface.
Twelve leaves completely unfolded	Tassel in growth point begins to develop. Lateral shoots bearing cobs develop from 6th-8th nodes above soil surface. Potential number of seed buds has been determined.
Sixteen leaves completely unfolded	Stem lengthens, while the tassel is almost fully developed. Silks begin to develop and lengthen from the base of the upper ear.
Silk appearance and pollen shedding	All leaves are completely unfolded; tassel have been visible for two to three days. The lateral shoot, bearing the main ear, as well as bracts have almost reached maturity. Demand for nutrients and water is high.
Green mealie stage	Ear, lateral shoot and bracts are fully developed. Starch accumulates in endosperm.
Soft dough stage	Grain mass continues to increase. Sugars are converted into starch.
Hard dough stage	Sugars in kernel disappear. Starch accumulates in crown of kernel and extends downwards.
Physiological maturity	Kernels have reached maximum dry mass. Layer of black cells develops at kernel base. Grains are physiologically mature. Moisture content must be reduced.
Drying of kernels (biological maturity)	Grains have reached physiological maturity and must dry out to reach biological maturity. Under favourable conditions, drying takes place at approximately 5 % per week up to the 20 % level, after which drying occurs slower.

1.2.1.3 Adaptation and production potential

Maize is not grown in areas where the mean daily temperature is less than 19 °C in SA, or where the mean temperature of the summer months is less than 23 °C. The critical temperature that will adversely affect yield is approximately 32 °C. Frost can damage maize at all growth stages and a frost-free period of 120-140 days is required to prevent damage (Du Plessis, 2003).

Maize production in SA is largely rain dependent as 80 % of maize is cultivated on dry land, while only 20 % is irrigated (SAGIS, 2016). South Africa is largely dependent on seasonal rain for crops to be produced, but is prone to extreme climatic conditions e.g. the occurrence of El Niño (Walker and Schulze, 2007), which often results in a poor national yield (Martin *et al.*, 2000). Water requirements for maize plants to grow optimally are referred to in Paragraph 1.2.1.

The main local maize production areas are situated in seven of the nine provinces, viz. the Free State, Mpumalanga, North-West, Gauteng, KwaZulu-Natal, Limpopo and the Northern Cape (Fig. 1.2). The total hectares (ha) planted during the 2015/16 growing season accumulated to approximately 1.9 million, with approximately 7.2 million metric ton (MT) being produced (SAGIS, 2016; FAO, 2016). The latter season was, however, characterised by extreme drought. Therefore, means for the period 2010/11 to 2014/15 were calculated to put maize production in perspective and were just over 12 million Mt from approximately 2.5 million ha (GrainSA, 2016).

1.2.1.4 Cultural and establishment practices

In SA conservation agriculture (CA) is practised on some fields where maize is grown (Fowler, 2000) and is defined as “any tillage system that maintains at least 30 % of the soil surface covered by residue” (Lal, 1997). Another prerequisite is that reduced till, stubble mulch till, strip till and/or no-till crop rotation, including legumes, should be practised.

The extent to which CA has been adopted by local producers is, however, quite limited and is restricted to a small number of summer grain producers (<0.5 %) in the Free State and North-West provinces. However, winter grain producers in the Western and Southern Cape (>70 %), and grain and sugarcane producers in KwaZulu-Natal (50-60 %) are practising CA on a much larger scale (S. Engelbrecht, GrainSA, Pretoria, 2016, personal communication).

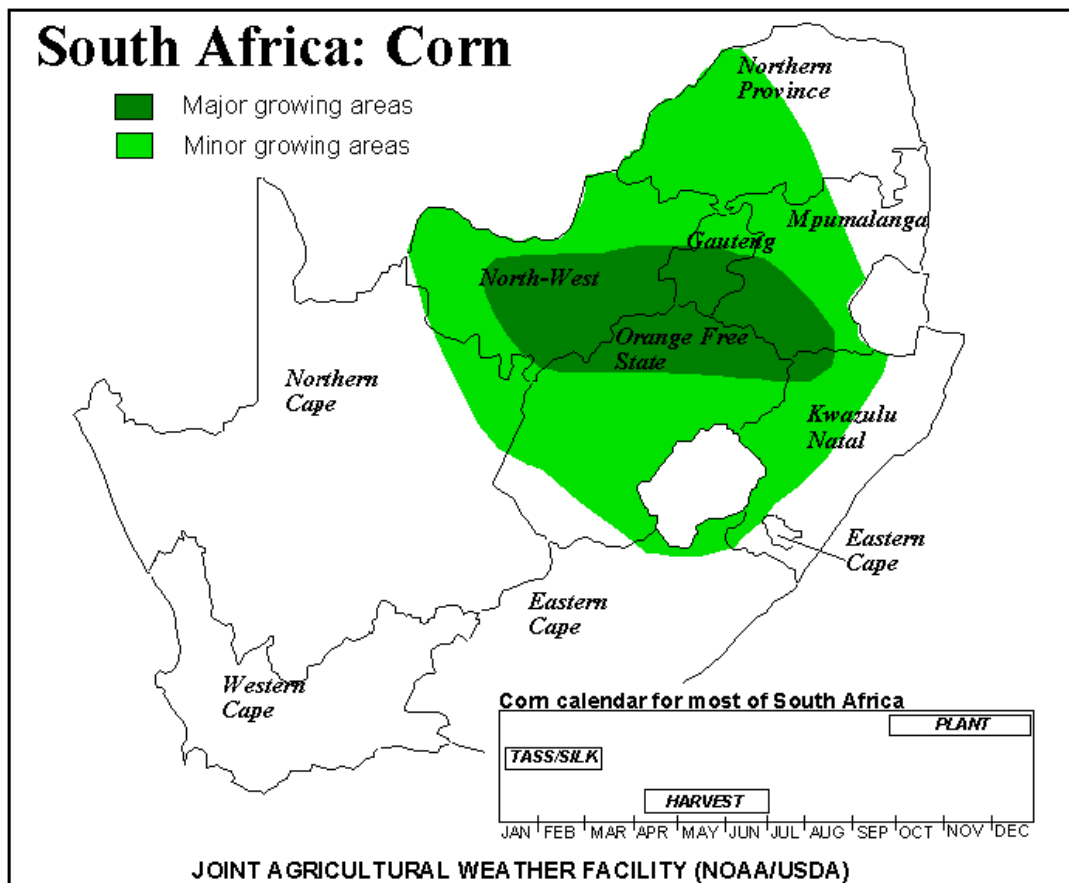


Fig. 1.2. The production areas, as indicated in light (minor production areas) and dark green (major production areas) shades, where maize are grown in South Africa (<http://www.spectrumcommodities.com/education/commodity/maps/corn/safcrn>).

The majority of maize production in SA is, however, subjected to intense and frequent ploughing practices, referred to as conventional tillage (Giliomee, 1999; Du Plessis, 2003). The main aim of this practice is to control weeds, reduce wind and water erosion and mix organic material left behind from the previous crop into the soil to improve soil structure and preparation of seedbeds (Du Toit, 1997).

Planting of maize can commence as soon as groundwater and soil temperature are suitable for seed germination. When a minimum air temperature of 10-15 °C occurs for seven successive days, seed germination should proceed normally. Planting depth for maize varies from 5-10 cm, depending on the soil type and planting date. Row widths under dryland maize cultivation can vary from 0,91-2,1 or 2,3 m, depending on mechanical equipment available and type of soil tillage system used (Anonymous 2016a).

1.2.1.5 Cultivar choice, fertiliser requirements and harvesting

Correct cultivar choice, can make a great contribution to risk reduction and should constitute an important part of production planning. Cultivars differ from one another with regard to a variety of characteristics, with each cultivar having its own adaptability and yield potential. The differences between cultivars allow producers to fully utilise such traits and optimally grow specific cultivars adapted to specific environmental conditions. Cultivars also differ in their susceptibility to several fungal and bacterial diseases (Du Plessis, 2003) and root-knot nematode species (Ngobeni *et al.*, 2010). Therefore, cultivars with the best levels of resistance or tolerance to a disease or pest e.g. root-knot nematodes, should be selected for planting to minimise crop losses where such a disease/pest prevails.

Fertilisers are generally applied according to standard recommendations, using suitable fertiliser mixtures for optimal maize cultivation. These usually include nitrogen (N), phosphorus (P) and potassium (K) mixtures. However, in some soils deficiencies of other micro-elements e.g. zinc (Zn) may also occur and needs to be tested for (Du Plessis, 2003).

Commercial maize is harvested mechanically, while developing producers usually harvested maize ears by hand. Once moisture levels of maize ears are in the order of 12-14 %, it can be harvested and delivered to a silo (Anonymous, 2016a).

1.2.1.6 Production constraints

Except for restricted rainfall as discussed earlier, the presence of weeds in all maize production areas as well as diseases and pests pose important constraints for local maize production, reducing the yield potential of such crops (Du Plessis, 2003). Annual yield losses of approximately 10 % occur as a result of weed infestations in maize crops. The presence of weeds during harvesting may slow the process, pollute grain, transmit odours to grain and hence cause downgrading, or incur additional costs for removal of weed seeds. Certain seeds, such as those of the thorn apple (*Datura ferox* L.), may be poisonous when consumed by animals or humans (Anonymous, 2016a).

Except for weeds and various bacterial and fungal diseases, plant-parasitic nematodes represent another economically important constraint for maize producers (Mc Donald and Nicol, 2005). According to the latter authors, more than 60 plant-parasitic nematode species have been associated with maize in different parts of the world. The most important plant-parasitic nematode genera demonstrated to be important limiting factors in world maize production include root-knot (*Meloidogyne* spp.) lesion- (*Pratylenchus* spp.) and cyst- (*Heterodera* spp.) nematodes. In SA, *Meloidogyne* and *Pratylenchus* spp. have been listed as the economically most important nematode pests of maize (Keetch, 1989; Riekert, 1996a and b; Riekert and Henshaw, 1998; Anonymous, 2013). Various other nematode genera e.g. *Nanidorus*, *Paratrichodorus*, *Rotylenchulus*, *Scutellonema*, *Tylenchorhynchus* and others have also been associated with maize crops in SA (Louw, 1982; Keetch and Buckley, 1984; Kleynhans *et al.*, 1996), but their pathogenicity is not necessarily known and their distribution usually limited.

Since *Meloidogyne* spp. is the most abundant and economically important nematode pest genus associated with local maize crops, the next part of this chapter will focus on the classification, biology, damage potential and management of this genus.

1.3 Nematode pests with special reference to *Meloidogyne* spp.

Root-knot nematodes have been listed as the number-one nematode pest crops globally (Jones *et al.*, 2013). *Meloidogyne* is a cosmopolitan genus with a wide geographical distribution and host range and infect both cultivated and non-cultivated plants in different agro-ecological regions of the world (Nicol *et al.*, 2011). Root-knot nematodes are hence one of the most destructive nematode pests that damage crops. Species found in warm/tropical areas are referred to as thermophiles, while those occurring in colder parts of the world are characterised as cryophiles (Jones *et al.*, 2013). Up to 2013, 98 *Meloidogyne* spp. had been identified worldwide with the four most damaging species being the thermophilic *Meloidogyne arenaria* (Kleynhans *et al.*, 1996), *M. incognita* and *M. javanica* and the cryophilic *Meloidogyne hapla* (Chitwood, 1949) (Jones *et al.*, 2013). In South African maize-production areas, *M. incognita* and *M. javanica* are the predominant species, responsible for maize yield losses (Riekert 1996a and b; Riekert and Henshaw,

1998), while *Meloidogyne arenaria* (Neal, 1889) also occur in some production areas (Kleynhans *et al.*, 1996; Agenbag, 2016).

1.3.1 Classification

Nematodes are classified into two major classes, namely Chromadorea and Enoplea (Siddiqi, 2000; De Ley and Blaxter, 2002) (Fig. 1.3.) and can either be free-living/non-parasitic or parasites of plants, humans and animals (Maggenti, 1981). The class Chromadorea includes both plant-parasitic and non-parasitic nematodes under the order Rhabditida (also known as order Tylenchida) (De Ley and Blaxter, 2002). *Meloidogyne* spp. are classified under the order Rhabditida. Two plant-parasitic nematode families, namely Longidoridae and Trichodoridae are classified under the class Enoplea and orders Dorylaimida and Triplonchida (De Ley and Blaxter, 2002). The rest of the nematode families, classified under the latter orders, are non-parasitic nematodes (Siddiqi, 2000). No reference to non-parasitic nematodes is, however, made for the purpose of this study.

1.3.1.1 Basic biology and morphology, with reference to *Meloidogyne*

Nematodes are microscopic, multicellular organisms and are omnipresent in oceans and terrestrial ecosystems as well as freshwater bodies under extreme environmental conditions such as at Antarctica (Decraemer and Hunt, 2013).

Nematodes are mostly vermiform and range from 0.2-1 mm in length (Hunt *et al.*, 2005) and 15-35 μm in width (Agrios, 1997). However, some plant-parasitic nematodes such as individuals belonging to the Longidoridae can be as long as 12 mm (Luc *et al.*, 1990; Decraemer and Hunt, 2013). In some plant-parasitic nematode genera, the females lose their vermiform shape and become pyriform, globose or lemon-shaped while the males remain vermiform (Luc *et al.*, 1990; Decraemer and Hunt, 2013). This phenomenon is known as sexual dimorphism and is present in genera such as *Meloidogyne* and others, viz. *Cactodera*, *Globodera* cyst nematodes, *Heterodera* cyst nematodes, *Rotylenchulus* reniform nematodes and *Tylenchulus* citrus nematodes (Luc *et al.*, 1990).

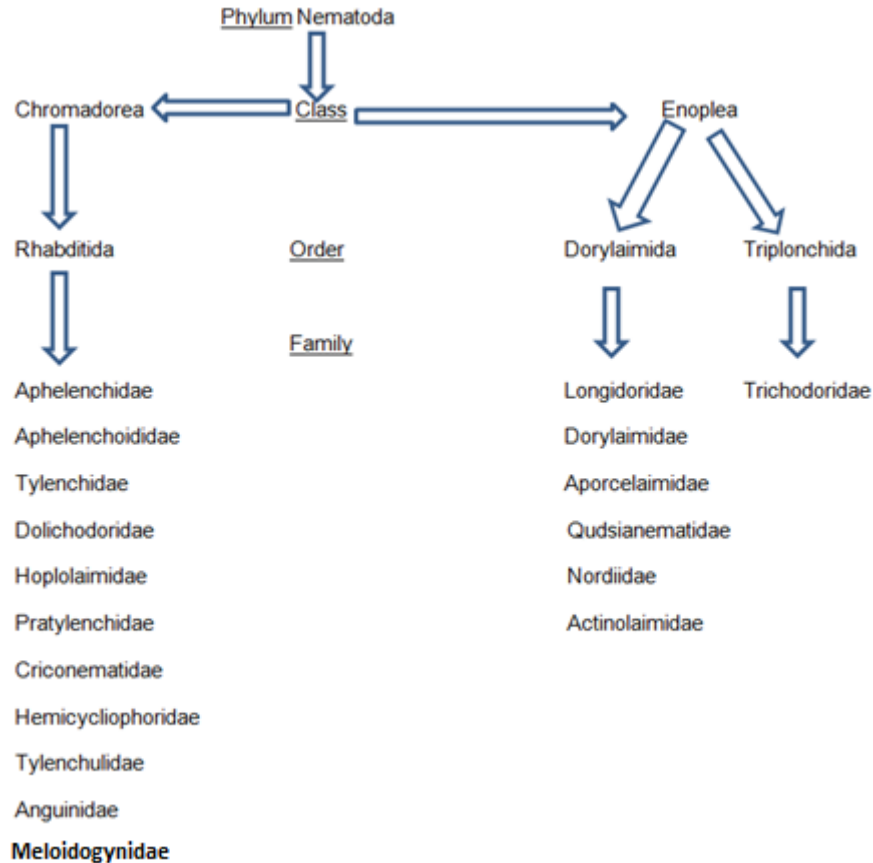


Fig. 1.3. The classification of plant-parasitic nematodes, with the family Meloidogynidae indicated in bold under the order Rhabditidae (Decraemer and Hunt, 2013).

The body structure of nematodes is relatively simple (Decraemer and Hunt, 2013). A typical nematode body comprises of an external cylinder, body wall and internal cylindrical digestive system which is separated by a pseudocoelomic cavity (Decraemer and Hunt, 2013). This pseudocoelomic cavity is filled with fluid which is under pressure and plays a major role in supporting the body shape of nematodes. This cavity also contains a number of cells and other organs, including the reproductive system (Decraemer and Hunt, 2013).

The body of nematodes is more or less translucent, covered by a colourless cuticle and consists of four basic systems, namely the digestive, musculature (only longitudinal), reproductive and nervous systems (Decraemer and Hunt, 2013). All plant-parasitic nematodes are equipped with either a hollow stomato- or odonto or solid onchio stylet, which is used to penetrate the plant and withdraw nutrients from

cells. *Meloidogyne* specimens contain a hollow stomatostylet (Agrios, 1997; Decreeamer and Hunt, 2013).

Plant-parasitic nematodes basically are divided into two trophic groups, namely i) ecto- and ii) endoparasites (Yeates, 1998; Decreeamer and Hunt, 2013). *Meloidogyne* spp. are sedentary, endoparasitic nematodes whose bodies remain embedded in the tissue of the host plant. The swollen second- (J2), third- (J3) and fourth- (J4) stage juveniles, and female become stationary in one area of the root/other belowground plant part and feed on specialised giant cells (Decreeamer and Hunt, 2013).

1.3.1.2 Life cycle

Meloidogyne spp., as other plant-parasitic nematodes, usually have four juvenile stages (J1, J2, J3 and J4) between the egg- and adult phase, with intervening moults which allows increase in their body size both in width and length (Luc *et al.*, 1990). Eggs of *Meloidogyne* spp. are produced by mature females as egg-masses that are protected by a gelatinous matrix (Decreeamer and Hunt, 2013). Second-stage juveniles (Fig. 1.4A) generally hatch from the eggs once environmental conditions are optimal and are the infective stage of root-knot nematodes. Feeding of *Meloidogyne* spp. J2 involves contact of their lips with the surface of the epidermal root cells of for example a maize plant, with the stylet being moved forwards and backwards while enzymes are released by oesophageal glands to enable penetration of the cell wall (Perry *et al.*, 2013). After penetration, *Meloidogyne* spp. J2 migrate intercellularly between cortical cells to the vascular cylinder (Perry *et al.*, 2013). Here the J2 develop to J2, J3 and J4 and ultimately to females (Fig.1.4B.) or males during adverse conditions (Fig. 1.5.) (Agrios, 1997). The life cycle of root-knot nematodes depends on soil temperature and the species, but is usually between 20-30 days for the termophils (Heyns, 1971; Decreeamer and Hunt, 2013). *Meloidogyne* spp. are defined as 'r' strategists since they represent small nematodes with relatively short life cycles and high numbers of offspring (Bongers and Bongers, 1998). The feeding J2 and females induce structural and physiological changes in the plant cells, referred to as giant cells, they feed on. These cells are metabolically active, have dense cytoplasm and contain increased numbers of cell structures to enable the feeding female to obtain enough nutrients for growth and reproduction (Perry *et al.*, 2013).

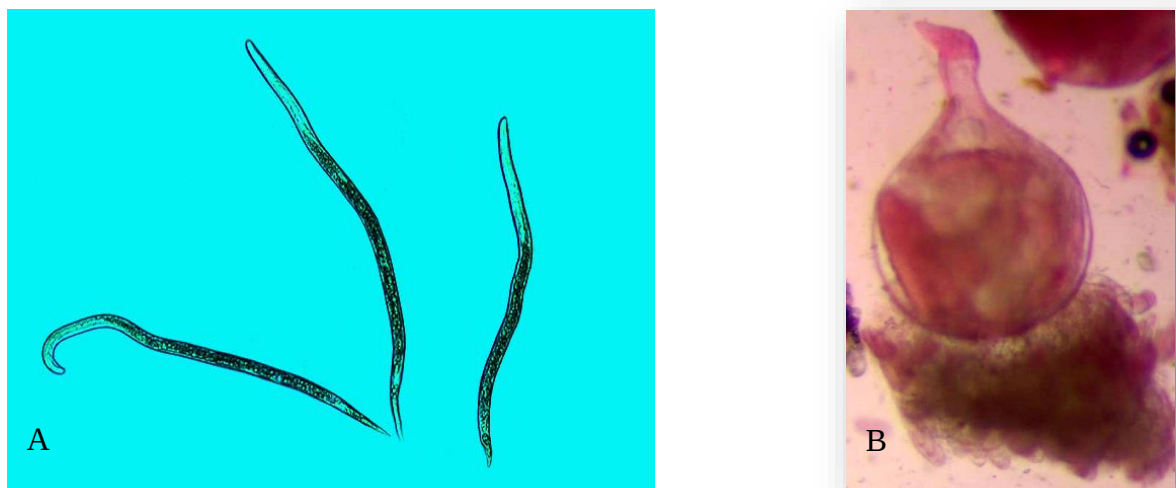


Fig 1.4. A and B. *Meloidogyne* spp. second stage juveniles J2 (A) and an obese female (B) (A: Nemapix, Vol 2, Eisenback and Ulrich Zunke and B: Photo: Driekie Fourie, Agricultural Research Council –Grain Crops Institute).



Fig. 1.5. A vermiform *Meloidogyne* sp. male (Photo: Suria Bekker, North-West University).

1.3.1.3 Reproduction strategies

The reproductive systems of root-knot nematodes are well developed, with one or two ovaries and a uterus that terminates in a vulva in females (Decreamer and Hunt, 2013). Male nematodes have testes, seminal vesicles, copulatory spicules and in some species a bursa and/or supplements to facilitate copulation (Decreamer and Hunt, 2013).

Root knot nematodes have three types of reproduction mechanisms, namely: i) amphimixis, during which the sperm from a male fertilises oocytes in females and meiosis subsequently occurs; ii) facultative meiotic parthenogenesis, in which amphimixis occurs in the presence of males but, in their absence meiosis occurs and

ii) obligate mitotic parthenogenesis, where males are not involved (Chitwood and Perry, 2009).

1.3.1.4 Symptoms and damage potential

Reduction in plant growth and yield loss are the most general effects as a result of parasitism by root-knot nematodes (Jones *et al.*, 2013). Above- and below-ground symptoms may vary according to the parasitic nature of the specific nematode species, its relationship with its host and the age and physiological condition of the host (Manzanilla-López *et al.*, 2004). Furthermore, factors such as damage by other pests and diseases, nutrient deficiency, drought, excessive rainfall, and others may make it difficult to distinguish from symptoms caused by root-knot nematodes (Jones *et al.*, 2013)

Above-ground symptoms of crop plants infected with root-knot nematodes may not be visible but are usually seen as stunted, yellowish, wilted, and/or plants that senesce early as well as poor growth, reduced yields or poor quality. In root-knot nematode infested maize fields, patches of poor growing plants generally occur (Fig. 1.6.). Below-ground symptoms due to infection by root-knot nematodes generally represent the formation of knots/galls that are visible on infected roots of crops (Fig. 1.7.), but can be absent on maize roots. It is of utmost importance to identify nematode-pest symptoms correctly in order to assist decision making with regard to management strategies.



Fig. 1.6. Above-ground symptoms of root-knot nematode infected maize plants as patches of poor growth in a field (Photo: Driekie Fourie, North-West University).



Fig. 1.7. Below-ground symptoms of root-knot nematode infection on roots of maize plants, showing thickened root tips, minimal and abnormal hair roots and atypical architecture of roots Photo: Suria Bekker, North-West University).

1.3.1.5 Economic importance

The economically most important root-knot nematode species of maize in SA are *M. incognita*, *M. javanica* and *M. arenaria* (Kleynhans *et al.*, 1996; Riekert, 1996a and b; Riekert and Henshaw, 1998; Agenbag, 2016). This is based on i) indirect observations of improved crop growth and yield after nematicides were applied to root knot-infected maize crops, ii) extraction of eggs and J2 from samples obtained for diagnostic analyses, using an adapted NaOCl technique (Riekert, 1995) and iii)

nematode surveys in maize production areas were *Meloidogyne* spp. were the most abundant. Although *Meloidogyne* spp. has been recorded to cause an estimated annual loss of \$157 billion globally (Abad *et al.*, 2008), the impact of this genus on crop production is still grossly underestimated. In many crop producing regions in Africa, there has been no comprehensive assessment that focuses specifically on the economic impact of *Meloidogyne* spp. (Coyne *et al.*, 2006). The earliest official estimate of nematode-associated yield losses of approximately 12 % for local maize crops was by Keetch in 1989. This figure, however, referred to plant-parasitic nematodes collectively and not to a specific genus and/or species. Riekert (1996a and b) and (Riekert and Henshaw 1998) thereafter reported maize yield losses of up to 60% as a result of root-knot nematode parasitism. Such losses are indicative of the damage caused by mixed populations of *M. incognita* and *M. javanica* that are the predominant species in sandy-soils in production areas of the North-West and Free State provinces.

Due to the microscopic nature of root-knot nematodes and the fact that they attack below-ground roots and plant parts, some producers are still sceptical about the extent of damage these pests cause to maize. This is because maize is a so-called 'low-cash' crop due to the low income per Mt of grain (GrainSA, 2016) in relation to other crops such as potato or other vegetables, legume and oilseed crops e.g. groundnut, soybean, sunflower (Anonymous, 2016a). Any production inputs into maize cultivation that cannot be related to an increase in yield would hence be considered a risk. Therefore, nematode control, and particularly the application of a nematicide, falls into this category and is particularly applicable to rain-fed maize production (Riekert, 1996b).

1.4 Control strategies

The main nematode control strategies used by maize producers in SA, *viz.* chemical control and crop rotation are briefly discussed below. The use of genetic host plant resistance is also concisely elaborated on. A number of other management strategies used to combat nematode pests of maize in SA, has been described extensively by Mc Donald and Nicol (2005).

1.4.1 Chemical control

Nematicides still remains the first choice of many producers when they consider the control of plant-parasitic nematodes (Gowen *et al.*, 2007). Nematicides are primarily applied to reduce root damage caused by nematode pests and ultimately to increase productivity (Tobin *et al.*, 2008). However, European legislation (Reg. CE 396/2005; 1095/2007; 33/2008, 299/2008 and 1107/2009) (<http://www.eur-lex.europa.eu/legal-content/>) has enforced extensive revision and restriction on the use of many commercial pesticides on agricultural crops. Reasons for the withdrawal of nematicides from world markets are: i) their highly toxicity and negative impact on the environment, animals and humans, ii) the long persistence of toxic substances of nematicides in ecosystems (terrestrial and aquatic) and iii) high costs of such products (Ferraz and de Freitas, 2004; Haydock *et al.*, 2013).

In South Africa, two nematicides, containing the active substances aldicarb and endosulfan, which were registered for use on maize for decades, have been withdrawn from the market (Anonymous 2012; Verdoorn, 2012). According to Van Zyl (2013), there are currently only two traditional active, synthetically-derived substances registered against nematode pests on maize in SA. These include carbofuran and terbufos. ‘Softer’ nematicides that are also registered on maize in SA (Van Zyl, 2013) is furfural, a by-product of sugar, and abamectin a natural fermentation product from the soil micro-organism *Streptomyces avermitilis* (Coyne *et al.*, 2009).

1.4.2 Crop rotation

Crop rotation is one of the oldest and most widely used methods to reduce root-knot nematode pests and diseases in crops (Coyne *et al.*, 2009; Duncan and Moens, 2013). However, for *Meloidogyne* using crop rotation as a means of control is challenging since these nematode pests have a wide host range (Riekert, 1996b; Fourie *et al.*, 2011; Duncan and Moens, 2013). Crop rotation will hence only be effective when poor-host or resistant cultivars are used.

1.4.3 Host plant resistance

Host-plant resistance is a cost-effective and eco-friendly strategy for reducing root-knot nematode population densities (Starr *et al.*, 2013). Resistance can be defined

as the ability of a host-plant to inhibit nematode development as well as its reproduction relative to that of a susceptible host (Sikora *et al.*, 2005). A number of studies have been done regarding genetic host plant resistance of maize against root-knot nematodes (Ngobeni *et al.*, 2010; Gathigia, 2010; Gao *et al.*, 2008; Khan, 2008). In SA (Ngobeni *et al.* 2010) identified several maize hybrids and open-pollinated varieties with resistance to both *M. incognita* and *M. javanica*. However, screening activities should be done annually since new hybrids enter the market continuously.

Since this study focused on the exploitation and evaluation of an unregistered biologically-derived product, referred to as SoilBioMuti (SBM), to reduce population densities of *M. incognita*, the remainder of this chapter deals with information that is available for such products with regard to the management of *Meloidogyne*.

1.5 Biological control of *Meloidogyne*

The term biological control in the classical sense is defined as ‘the action of parasites, predators or pathogens in maintaining another organism's population density at a lower average than would occur in their absence’ (De Bach, 1964).

Research is progressively aimed at investigating and exploiting eco-friendly nematode management strategies, of which one is the use of biological agents e.g. various bacteria and fungi (Terefe *et al.*, 2009; Viaene *et al.*, 2013) (Table 1.2.). The application of antagonistic micro-organisms/compounds produced by such organisms, provides an opportunity to minimise damage caused by nematode pests. Referring to *Meloidogyne* spp. in particular for the purpose of this study. Micro-organisms are also competitors of nematode pests for habitat and nutrients, thus decreasing nematode-pest population densities and the subsequent damage such pests can cause (Selim *et al.*, 2010). Moreover, biological control generally does not pose problems in terms of residual and environmental effects (Terefe *et al.*, 2009) and has the potential to be used as an effective, long-term nematode management strategy (Stirling, 1991).

Tabel 1.2. Examples of micro-organisms reported used as biocontrol agents of nematodes.

Micro-organism	Method of control	Specific organisms
Predatory mite	Feed on nematodes	<i>Tyrophagus putrescentiae</i> (Anwar, 1994)
Endoparasitic fungi	Often obligate parasites and have a limited saprophytic phase	<i>Nematoctonus</i> , <i>Rehmeria coniospora</i> (Townsend <i>et al.</i> , 1998)
Predacious fungi	Capture and kill nematodes	<i>Arthrobotrys</i> spp. (Slepetiene <i>et al.</i> , 1993; Vouyoukalou, 1993; Dias and Ferraz, 1994)
Opportunistic fungi	Colonise nematode reproductive structures and have the ability to deleteriously affect them.	<i>Paecilomyces lilacinus</i> and <i>Verticillium chlamydosporium</i> (Morgan-Jones <i>et al.</i> , 1983; Freire and Bridge, 1985; Jatala, 1986)
Parasitic bacteria	Obligate parasites of nematodes and has a very wide host range	<i>Pasteuria penetrans</i> (Sayre, 1980; Stirling, 1991), <i>Pseudomonas denitrificans</i> (Adams and Eichenmuller, 1963)
Non-parasitic rhizobacteria	Have the ability to colonise roots aggressively. Most rhizobacteria that are known to be detrimental to plant-parasitic nematodes act by means of metabolic-by-products, enzymes and toxins rather by parasitism.	<i>Agrobacterium</i> (Hallman <i>et al.</i> , 2009), <i>Alcaligenes</i> , <i>Bacillus</i> (Sikora and Hoffmann-Hergarten, 1993) <i>Clostridium</i> , <i>Desulfovibrio</i> , <i>Pseudomonas</i> (Siddiqui and Shaukat, 2002), <i>Serratia</i> and <i>Streptomyces</i> (Tian <i>et al.</i> , 2006).

Biological control of nematode pests is optimal when the product/agent is applied before planting of a crop to allow its i) establishment and proliferation in the soil e.g. fungi or ii) produce nematotoxic metabolites e.g. bacteria (Anastasiadis *et al.*, 2008).

For the purpose of this study, the focus will be on bacteria as biocontrol agents of *Meloidogyne* spp. only. The reasons for this approach is that i) the target nematode for this study was *M. incognita* (race 2) and ii) only the bacterial content of the naturally-derived product that was tested was analysed.

1.5.1 Bacteria with anti-nematodal characteristics

Since the rhizosphere provides the first line of defence to roots against nematode attack, rhizosphere bacteria are considered ideal and superior biological control agents (Saraf *et al.*, 2014) In addition, bacteria are generally the most effective micro-organisms used for their anti-nematodal properties and are abundant in the root rhizosphere where plant-parasitic nematodes generally occur (Neipp and Becker, 1999; Siddiqui and Mahmood, 1999; Viaene *et al.*, 2013). Rhizosphere

bacteria have, for example, the ability to rapidly spread in the rhizosphere, colonise potential nematode infection sites on plant roots/tubers/other below-ground parts and influence behaviour of nematode pests. These characteristics make rhizosphere bacteria useful for nematode management (Hasky-Günther *et al.*, 1998).

The most common bacterial genera identified as possible biocontrol agents of nematode pests, particularly *Meloidogyne* spp., are *Actinomyces*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Clostridium*, *Desulfovibrio*, *Flavobacterium*, *Lactobacillus*, *Pasteuria*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Streptomyces* (Johnston, 1958; Zavaleta-Mejia, 1985; Sikora *et al.*, 1989; Dicklow *et al.*, 1993; Siddiqui and Mahmood, 1999; Tian *et al.* 2006; Mendoza *et al.*, 2008; El-Hadad *et al.*, 2011). Most of these bacteria are regarded as plant growth promoting rhizobacteria (PGPRs) that colonise the rhizosphere of plants aggressively (Siddiqui and Mahmood, 1999). Most PGPRs produce secondary metabolic by-products, e.g. toxins, enzymes and/or antibiotics that is suggested to inhibit the motility, hatching and penetration of nematode infective stages (e.g. J2 of *Meloidogyne* spp.) and reduce their reproduction and survival rates (Siddiqui and Mahmood, 1999; Dong and Zhang, 2006). Secondary metabolites include the production of siderophores that chelate iron ions, antibiotics and hydrogen cyanide, all of which contribute to the reduction of pathogenic micro-organisms in the rhizosphere. Such metabolites lead to establishment of an optimal environment that is suitable for root growth (Hashem and Abo-Elyousr, 2011). Various bacterial genera and species used as biocontrol agents against *Meloidogyne* spp. are summarised in Table 1.3. Several lactic-acid bacteria have also been reported for their adverse effects on *Meloidogyne* spp., i.e. *Lysobacter capsici* (Lee *et al.*, 2014) and *Lactobacillus farragnis* (Seo *et al.*, 2012) amongst others.

Tabel 1.3. Bacterial species that exhibit anti-nematodal effects against *Meloidogyne* spp.

Bacterium	Reference	Bacterium	Reference
<i>Agrobacterium radiobacter</i>	Sikora <i>et al.</i> (1989)	<i>Pasteuria penetrans</i>	Sayre (1980); Stirling, 1991)
<i>Alcaligenes faecalis</i>	Siddiqui and Mahmood (1992)	<i>Pseudomonas aureofaciens</i>	Westcott and Kluepiel (1992)
<i>Bacillus thuringiensis</i>	Ignoffo and Dropkin (1977)	<i>Pseudomonas mindocina</i>	Siddiqui and Husain (1991)
<i>Bacillus subtilis</i>	Sikora (1988)	<i>Pseudomonas fluorescens</i>	Weidenborner and Kunz (1993)
<i>Bacillus cereus</i>	Oka <i>et al.</i> (1993)	<i>Pseudomonas solanacearum</i>	Kermarrec <i>et al.</i> (1994)
<i>Bacillus pumilus</i>	Gokte and Swarup (1988)	<i>Serratia marcescens</i>	Zavaleta-Mejia (1985)
<i>Bacillus lincheniformis</i>	Siddiqui and Mahmood (1992)	<i>Streptomyces</i> sp. isolate CR-43)	Dicklow <i>et al.</i> (1993)
<i>Clostridium butyricum</i>	Johnston (1958)		

A short summary of only *Pasteuria*, *Bacillus* and *Pseudomonas* is given below, referring to their effect on *Meloidogyne* spp. and their mode of action as biocontrol agents. Extensive research on these bacterial genera has been done to evaluate and verify their adverse effects on root-knot nematodes.

Pasteuria spp. are mycelial and endospore-forming bacteria, that act as parasites of plant-parasitic nematodes (Stirling, 1991). These bacteria were initially described as protozoa, placed under *Bacillus* but has recently been reclassified as *Pasteuria* (Tian *et al.*, 2006). *Pasteuria penetrans* is one of the most studied micro-organisms in terms of its anti-nematodal characteristics. It is an obligate bacterial parasite of plant-parasitic nematodes, particularly *Meloidogyne* spp. This bacterium interferes with the migration of *Meloidogyne* spp. J2 towards the roots of a host plant and also inhibits nematode reproduction (Vagelas *et al.*, 2012). Except for their adverse effect on *Meloidogyne* spp., *P. penetrans* also adversely affect the ability of females of *Pratylenchus tornei* Sher and Allen, 1953 to reproduce. The same scenario has been reported for *Pasteuria nishizawae* for females of *Heterodera* and *Globodera* spp. (Tian *et al.*, 2006). The life cycle of *Pasteuria* begins when endospores present in the soil attach to the cuticle of *Meloidogyne* J2. Attachment is achieved through the binding of carbohydrate ligands on the surface of the endospores that binds to lectin-like receptors on the J2 cuticle (Tian *et al.*, 2006). Germination and penetration of the bacteria only commences once the J2 is inside the plant root/tuber/other below-

ground plant part. Penetration by the bacterium occurs by means of a germ tube, after which microcolonies are formed which proliferate and sporulate inside the bodies of *Meloidogyne* spp. females. Up to two million spores can be present in the female nematode body. The *Pasteuria*-infected root-knot nematode females still function normally since their reproduction is not inhibited or terminated (Siddiqui and Mahmood, 1999; Stirling, 1991, Vagelas *et al.*, 2012). Plant tissue infected with *Pasteuria* decomposes in the soil and the endospores are released to continue their life cycle and infect nematode pests present in the soil. Advantages of using *Pasteuria* as a biocontrol agent represent i) its ability to reduce host-plant infectivity by root-knot nematode J2, ii) inhibition or prevention of the reproduction of *Meloidogyne* spp. females and iii) the ability of endospores to persist and tolerate extreme environmental conditions in soil due to their biochemical and physiological properties.

The genus *Bacillus* represents another well-known bacterium that has been reported to promote plant growth and provide effective biological control against various plant diseases and nematode pests, particularly *Meloidogyne* spp. (Mendoza *et al.*, 2008; Hallman *et al.*, 2009; Singh and Siddiqui, 2010). *Bacillus* spp. evaluated and reported to adversely affect *Meloidogyne* spp. in terms of their biology and/or reproduction include *Bacillus amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. firmus*, *B. megaterium* and *B. subtilis*, (Sikora and Hoffmann-Hergarten, 1993; Terefe *et al.*, 2009). Several studies reported that *Bacillus* spp. were effective in paralyzing *Meloidogyne* spp. J2 with subsequent reductions in female reproduction rates and population densities (Giannakou *et al.*, 2004; Mendoza *et al.*, 2008; Terefe *et al.*, 2009; El-Hadad *et al.*, 2011). When the PGPR *B. cereus* S18 was, for example, applied 10 days before root-knot nematode inoculation it led to significant reductions in gall index and number of galls (Mahdy, 2002). Results showed variable rates of control of *Meloidogyne* spp. by *B. cereus* S18. This bacterial strain had little to no biocontrol activity against *M. arenaria*, while it substantially reduced populations of *M. incognita* and *M. javanica* (Mahdy, 2002). *Bacillus firmus* on the other hand reduced J2 of *M. incognita* by 98-100 % and gall formation by 91 % under *in vitro* conditions in a tomato glasshouse experiment. Also, final *M. incognita* populations were reduced by 76 % and egg numbers by 45 % (Terefe *et al.*, 2009).

Control of root-knot nematodes has also been achieved by *Pseudomonas*, through the production of 2,4-diacetylphloroglucinol (DAPG) (Siddiqui and Shaukat, 2002). *Pseudomonas* protects plants against plant-parasitic nematodes by promoting plant growth, competing with nematodes for essential nutrients and eliciting induced systemic resistance (Siddiqui and Mahmood, 1999). Induced systemic resistance is hence achieved by thickening the physical and mechanical strength of the cell walls of a host plant by accumulation of newly formed callose and phenolic compounds (Tian *et al.*, 2006). Antagonistic bacteria like *Pseudomonas aeruginosa* and other *Pseudomonas* spp. have also been reported to be effective as biological agents against *Meloidogyne* spp. These bacteria are even more effective when combined with organic amendments, which offer readily available nutrients for root-knot nematode survival and the growth of antagonistic bacteria (Giannakou *et al.*, 2004). Hashem and Abo-Elyousr (2011) reported that *P. fluorescens* reduced *M. incognita* J2 by 45%, subsequently reducing disease severity significantly on tomato crops under glasshouse conditions. (Ali *et al.* 2002) treated mungbean with *P. aeruginosa* and this way reduced *M. javanica* populations and subsequent gall formation. *Pseudomonas putida* also reduced *M. incognita* population densities, galls, egg masses and the eggs per egg mass on tomato roots under glasshouse conditions (Hashem and Abo-Elyousr, 2011). Trials done in Germany showed that a combination of PGPRs such as *Rhizobium* and *Pseudomonas straita* furthermore proved to be effective in reducing *M. incognita* reproduction in tomato (Garcia-Gutiérrez, *et al.*, 2013).

1.5.2 Registered and commercially-available biological control products

Various biological control products that contain PGPRs as the active substance have been developed and are used successfully to reduce nematode pest populations. (Radwan *et al.*, 2012) reported that four biological control products reduced the incidence and damage severity of *M. incognita*, in some cases up to 90% under glasshouse conditions. These products were i) Bioarc™ (active substance, *Bacillus megaterium*), ii) Biozeid® (active substance, *Trichoderma album*), iii) Algaefol® (active substance, *Ascophyllum nodosum*) and iv) Plant Gard® (active substance, *Trichoderma harzianum*). Application of all these products resulted in a reduction of root galling caused by *M. incognita* compared to control treatments. Reduction in root galling as a result of *B. megaterium* was 89 %, *Trichoderma album* 88 %, *Ascophyllum nodosum* 87 %, and *Trichoderma harzianum* 86 %.

Ascophyllum nodosum 87 % and *T. harzianum* 70 %. In another study, applications of the biological control agent Bionem™ (active substance, *Bacillus firmus*) reduced *Meloidogyne* sp. densities (Anastasiadis *et al.*, 2008). The above-mentioned results provided substantial proof that the said commercially available biological control products have the potential to replace chemical nematicides. The combined use of more than one bionematicide, however, often yielded better results than application of a single product (Van der Putten *et al.*, 2006), with no harmful effects on the environment (Giannakou *et al.*, 2004). A single application usually results in the establishment of the bacterial agent in the soil, while repeated applications contribute towards its proliferation and generally optimise the long-term control of nematode pests (Anastasiadis *et al.*, 2008). The efficacy of commercially-available biocontrol products against root-knot nematodes was also enhanced when used in combination with soil solarisation.

Biological agents registered for use on crops in SA are listed in Table 1.4. These products are registered with the Department of Agriculture and Fisheries (DAFF) under Act No 36 of 1947 and are considered to comply with the classical definition of biological control (see Paragraph 1.6), as most of them are antagonists of nematode pests. Nonetheless, other biological products that have anti-nematodal characteristics are marketed commercially as soil inoculants since they promote plant growth rather than acting directly against nematode pests. Most of these products are also not registered with DAFF under Act No 36 and sold unofficially to producers with no scientific proof of their anti-nematodal characteristics being available.

Table 1.4. A list of currently used or registered biocontrol products for the control of plant-parasitic nematodes in South Africa (Gerber, 2010; Van Zyl, 2013; Anonymous, 2016b).

Product name and micro-organism it contains	Active substance	Owner company:	Crop
Poncho Votivo® (bacterium)	<i>Bacillus firmus</i>	Bayer Pty Ltd.	Maize
Romulus® (fungus)	<i>Trichoderma harzianum</i>	Dagutat Biolab Bk	Carrot
Spartacus® (fungus)	<i>Beauveria bassiana</i>	Dagutat Biolab Bk	Carrot

It must, however, be borne in mind that biological control is not always as effective as indicated above. For example, using natural enemies of plant-parasitic nematodes in tropical areas has been unsatisfactory because of the reduced adaptations of such biological agents to climates of other regions and their inadequate host specificity to nematode pests in such areas (Van der Putten *et al.*, 2006). Also, factors known to influence the efficacy of biocontrol agents include challenges associated with mass production of such live antagonists, soil pH, moisture, interactions with other organisms and others (Van der Putten *et al.*, 2006). In annual row crops grown in SA, the harsh environmental and soil conditions are factors that may limit the use of biocontrol agents of nematode pests. Establishment of such agents, as well as their proliferation in soils are crucial. It is suggested that the success rate of such nematode antagonists may be higher should endemic micro-organisms be exploited and used for this purpose (H Fourie, North-West University, Potchefstroom, 2016, personal communication).

The need, however, exists to add to the range of products and strategies to manage plant-parasitic nematodes under local environmental conditions, in this case particularly root-knot nematodes that infect maize. This will enable producers to choose from a wider spectrum of pest management tools and combat *Meloidogyne* spp. The cost of such products in particular plays an important role since their use has to be economically justifiable under rain-fed conditions which are the areas where approximately 80 % of South Africa's maize production areas are located. Products with anti-nematodal characteristics are hence continuously being investigated and their adverse impact on plant-parasitic nematode complexes determined.

A novel, soil-derived product with such potential was developed and produced by the late Mr Nico Snyman. Extraction of microbes from virgin soils in Zambia and South Africa and informal culturing thereof, according to a fixed procedure and sound principles using molasses as the main ingredient representing 30 % of the total volume of the product (see Annex 1), is the basic procedure followed to produce SBM. The product furthermore contains 3 % micro-organisms and 57 % water Annex 1). In the material safety data sheet (Annex 1) no health hazard is indicated should the product be swallowed, inhaled or come into contact with the skin of humans. Another property of SBM is that it is said to be biodegradable and hence poses no problem to the environment. This product is also said to exhibit above-average growth-promoting effects for several crops, including maize, and is unofficially recommended at a 2.5 % dosage rate according to results from unofficial trials that were done by maize producers across SA.

The growth-promoting characteristic of SBM was demonstrated during 2015 in a study by an Honours student Mr. Erard Erasmus under the guidance of Dr Jacques Berner (Plant Physiologist of the North-West University). This is demonstrated in Fig. 1.8., showing the distinct difference in colour and height of maize plants treated with SBM, and SBM + a standard N:P:K fertiliser product in relation to an untreated control. The inclusion of hay is, however, according to the late owner of the product, a prerequisite for SBM to function optimally in terms of its crop growth stimulating properties (N Snyman, Rustenburg, 2014, personal communication).



Fig. 1.8. The distinct differences in colour and length of maize plants treated with SoilBioMuti SBM) and SBM + a standard N:P:K fertiliser product compared to plants treated with either the standard fertiliser product and the untreated control (Photo: Erard Erasmus, North-West University).

1.6 Aim and objectives

The main aim of this study was to characterise the microbes present in SBM and evaluate the product *in vitro* and *in vivo* to determine its effect on the biology and physiology of the root-knot nematode species *M. incognita*. The specific objectives were to:

- i) characterise the bacteria present in SBM using traditional plating as well as molecular-based approaches,
- ii) determine the effect of SBM on the oxygen consumption of J2,
- iii) determine the effect of different concentrations of SBM on the motility of *M. incognita* J2 *in vitro*,
- iv) determine the effect of different concentrations of SBM on the reproduction of *M. incognita* in *in vivo* glasshouse experiments,
- v) determine the effect of a 2.5 and 5 % SBM concentration in combination with hay on the reproduction of *M. incognita* *in vivo* in two tunnel experiments under prevailing environmental conditions and
- vi) determine the bacterial profile in soils to which a 5 % SBM and other treatments were applied in one of the tunnel experiments see objective iv).

1.7 References

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Chapter 2: Determining the bacterial content of a novel, biologically-derived product SoilBioMuti (SBM) that exhibits anti-nematodal characteristics

2.1 Introduction

The progressive withdrawal of synthetically-derived Class I nematicides is leaving producers with fewer options to efficiently manage plant-parasitic nematodes under environmental conditions that prevail in South Africa (Fourie *et al.*, 2016). This is demonstrated by withdrawal of two chemical nematicides, registered on maize in South Africa, with active substances aldicarb and endosulfan (Anonymous 2012; Verdoorn 2012). This is, however, a worldwide trend with more and more synthetically-derived nematicides being withdrawn from markets, leaving producers with fewer options to control and keep nematode pest populations below damage threshold values (Collange *et al.*, 2011). Concern is hence raised about the future of agricultural production systems in which chemicals are used predominantly to protect crops against diseases and pests, particularly plant-parasitic nematodes. Consumers, environmentalists, legislators and farmers suggested over the last two decades that farming practices will have to change considerably to achieve a significant reduction in pesticide usage (Higa and Parr, 1994). This scenario is experienced worldwide, also for crops other than maize and prompted increased research towards investigating and exploiting alternative, eco-friendly nematode management strategies.

Alternative management strategies represent the use of biological agents (e.g. various bacteria, fungi and other micro-organisms), the use of less or non-toxic plant-derived products and well planned crop rotation systems (Terefe *et al.*, 2009; Viaene *et al.*, 2013). The application of antagonistic micro-organisms or compounds produced by such organisms in particular provides an opportunity to minimise crop damage caused by nematode pests. Bacteria are generally the most effective micro-organisms used for their anti-nematodal (nematicidal/nematostatical) properties and are abundant in the root rhizosphere where the infective stages of plant-parasitic nematodes generally occur (Viaene *et al.*, 2013). One such bacterium genus is *Bacillus* that has been reported to promote plant growth and provide effective biological control against various plant diseases and nematode pests such as *Meloidogyne* spp. (Mendoza *et al.*, 2008; Hallman *et al.*, 2009; Singh and Siddiqui

2010). For example, several studies reported that *Bacillus* spp. were effective in paralysing second-stage juveniles (J2) of *Meloidogyne* spp., with subsequent reductions in the reproduction rates and population densities of such pests (Giannakou *et al.*, 2004; Mendoza *et al.*, 2008; Terefe *et al.*, 2009; El-Hadad *et al.*, 2011). Other bacteria species with anti-nematodal effects are *Actinomycetes*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Burkholderia*, *Chromobacterium*, *Clostridium*, *Desulfovibrio*, *Flavobacterium*, *Macrophomina*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Streptomyces* (Rodriguez-Kabana *et al.* 1965; Hollis and Rodriguez-Kabana, 1967; Siddiqui and Mahmood, 1999; Tian *et al.* 2006; Akhtar and Siddiqui, 2008; Hallman *et al.*, 2009; Viaene *et al.*, 2013; Jansen-Girgan *et al.*, 2016).

Products with anti-nematodal characteristics are continuously being investigated and their adverse impact on plant-parasitic nematode complexes determined. A novel, soil-derived product with such potential has been developed, and produced by the late Mr Nico Snyman[†]. Extraction of microbes from virgin soils and informal culturing thereof, according to a fixed procedure and sound principles using molasses as the main ingredient, is the basic procedure followed to produce SoilBiomuti (SBM). This product has above-average growth-promoting effects on several crops, including maize and is unofficially recommended for commercial use by local producers at a 2.5% dosage rate. The inclusion of hay is, however, according to the owner of the product, a prerequisite for SBM to function optimally in terms of its crop growth stimulating properties (N Snyman, 2014, Rustenburg, personal communication).

The main objective of this part of the study was hence to characterise the bacterial complex at genus level present in SBM using traditional plating as well as a molecular-based approaches, *viz.* next generation sequencing (NGS).

2.2 Material and methods

The aim of this activity was to characterise the bacteria present in SBM using traditional plating as well as molecular-based approaches. The origin of the SBM product is from virgin veld obtained in Zambia. This microbial extract is reared using a fixed procedure and sound principles with molasses as the main ingredient, representing 30 % of the total volume of the product (see Annex 1). Three product

batches that were produced over time were supplied by the late owner (Mr Nico Snyman[†]) to the author of this dissertation for the studies to be done during this research.

2.2.1 Identification of microbes present in SoilBioMuti (SBM)

2.2.1.1 Standard agar-plating technique

Standard aseptic microbiological techniques were applied. A serial dilution series (10^{-1} to 10^{-5}) of two SBM samples was prepared using distilled water. These two represented i) a freshly prepared SBM sample (A) that was incubated for 24 h at 5 °C in a refrigerator and ii) a freshly prepared SBM sample (B). Viable cell counts were obtained using the aseptic spread-plate technique. Different microbial growth media, designed to be selective for heterotrophic bacteria (Actinomycetes) were used for enumerating bacterial cells. These bacterial populations were subjected to their physiological ability to grow on each of the selective media. General heterotrophic counts were done on non-selective nutrient agar (NA) [16 g/l nutrient broth, 15 g/l agar] (Biolab, Midrand, South Africa). Lactic acid bacteria were enumerated using MRS agar (Biolab, Midrand, South Africa) according to manufacturer's recommendations. This specific agar medium is named after the first letters of the surnames of de Man, Rogosa and Sharpe who developed it in 1960. All media were sterilised by autoclaving at 121 °C for 15 min. Three replicates were included for each treatment and bacterial count data were subject to t-test analyses (Statistica for Windows, Version 13) (www.statsoft.co.za).

2.2.1.2 Molecular identification of microbes

2.2.1.2.1 Genomic deoxyribonucleic acid (DNA) isolation

Direct DNA extractions from three freshly prepared SBM stock solutions, obtained from the owner Mr Snyman[†], were done using the Machery-Nagel Nucleospin Soil kit (Macherey Nagel, Germany). Samples were aliquoted in 50 ml conical tubes and centrifuged (11 000x g) for 5 min. Two hundred and fifty mg of sample material of each SBM batch were used in accordance to the manufacturer's instructions. The Machery-Nagel Nucleospin Soil kit has a choice of two lysis buffers and an additional enhancer that can be used in combination with these buffers. All possible lysis conditions were tested during the optimisation process with lysis buffer SL1 with added enhancer SX delivering the best results. Isolated DNA quantity and quality

(i.e. A_{260}/A_{280} and A_{260}/A_{230}) were determined using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA).

2.2.2.2 Barcode amplification

2.2.2.2.1 Deoxyribonucleic acid (DNA) test amplification

All DNA extracted from the three SBM samples was initially screened for amplification using polymerase chain reaction (PCR) primer set 338f/518r (Table 2.1) in a TC-Plus thermal cycler (Techne, UK). The reaction was carried out in 50 μ l volumes and contained 25 μ l double strength MyTaq PCR mastermix (Bioline, UK), 100 pmol of each primer, 1 μ l isolated genomic DNA and 22 μ l PCR-grade water (Bioline, UK). Cycling conditions for the amplification were set at 95 °C for 300 s (initialisation and denaturing) followed by 63 °C for 45 s (annealing), 72 °C for 60 s in 35 cycles (extension), and a final extension step at 72 °C for 10 min.

2.2.2.2.2 Visual confirmation of amplicons

Amplicons were visualised using horizontal electrophoresis (5 μ l PCR product mixed with 1 μ l of 6x Orange loading dye, Thermo Fischer Scientific, USA), in a 1.5 % (w/v) agarose gel prepared by 1x TAE buffer (Bio-Rad, UK). Gels were electrophoresed for 45 min at 80V in a BioRad Wide MiniSub Cell (BioRad, UK). The agarose gels were stained with Ethidium bromide (EtBr) and gels were visualised under UV light. Images were captured using a Gel Doc XR+ molecular imager (BioRad, UK) with Image Lab Software (Version 3.0).

2.2.2.3 Barcoding polymerase chain reactions (PCRs)

All barcode polymerase chain reactions (PCRs) were carried out as stipulated within the Illumina 16S Metagenomic library preparation guide, as can be found at (http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) using the universal primers (Table 1) for 16S rDNA V3-V4 region (Klindworth *et al.*, 2013) and ITS1 and ITS2 regions (White *et al.*, 1990). All primers were tagged with the Nextera overhang adapters (16S Metagenomics library preparation guide, Illumina) and synthesised by Integrated DNA Technologies (IDT, IA, USA). The overhang adapters allowed subsequent PCR indexing with the Nextera XT indexing

system, Illumina adapter addition and compatibility with the Illumina sequencing primers, of the amplified products.

Table 2.1. List of primer sequences used for ITS and 16S ribosomal ribonucleic acid (rRNA) amplifications.

Primer Names	Targeting Region	Genomic region Primer Reference	Full sequences of the genomic region primers with their Illumina overhang adapters ¹ (5' to 3' orientation)
338f	16S	Lane (1991)	ACTCCTACGGGAGGCAGCAG
518r	16S	Lane (1991)	ATTACCGCGGCTGCTGG
16S_Nxt_For	16S V3-V4	Klindworth <i>et al.</i> (2013)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG
16S_Nxt_Rev	16S V3-V4	Klindworth <i>et al.</i> (2013)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG ACTACHVGGGTATCTAATCC
ITS1_Nxt_For	ITS1	White <i>et al.</i> (1990)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTC CGTAGGTGAACCTGCGG
ITS2_Nxt_Rev	ITS1	White <i>et al.</i> (1990)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG CTGCGTTCTTCATCGATGC
ITS3_Nxt_For	ITS2	White <i>et al.</i> (1990)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGC ATCGATGAAGAACGCAGC
ITS4_Nxt_Rev	ITS2	White <i>et al.</i> (1990)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGT CCTCCGCTTATTGATATGC

¹Illumina overhang tags, provided in *italic*, are given from the Illumina's *16S metagenomic library preparation guide*. Illumina tags required for next generation sequencing indexing and library preparation are also given. Illumina tags are given in *italics* and genome targeting primer sequences from references are in **bold**. (<http://www.illumina.com/content/dam/illumina-Support/documents/documentation/chemistrydocumentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf>).

All extracted DNA samples were quantified with the Qubit® dsDNA Assay Kit (Invitrogen, Life Technologies) on a Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, USA) in accordance to the protocols provided by the manufacturer. The first stage barcoding PCRs used 12.5 ng of this quantified DNA that was normalized to 5 ng/μl prior to PCR. Barcode amplifications reactions contained 0.5 μM final concentration of each forward and reverse primer, 200 μM dNTPs, 1× Phusion® High-Fidelity Buffer and 0.02 U/μl Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA) in a final reaction volume of 50 μl, adjusted with Milli-Q® H₂O (Merck Millipore, KGaA, Darmstadt, Germany). Reaction conditions consisted of an initial denaturing step at 98 °C for 3 min, which

was followed by 30 cycles of denaturation (98 °C for 30 s), primer annealing (55 °C for 30 s) and extension (72 °C for 30 s). The reaction was concluded with a final elongation step of 5 min at 72 °C. The same conditions were used for all three barcoded regions, but primer annealing temperatures were increased to 60 °C for ITS amplifications to obtain single amplicons. All barcode amplicons were visualised on 2 % agarose gels with conditions as stated earlier. All amplicon PCRs that did not successfully amplify or that produced the wrong size bands or multiple bands, were repeated. Successful barcode amplification reactions were cleaned with QIAamp® MinElute™ PCR Purification Kit (Qiagen) as recommended by the manufacturer's protocol and quantified using the Qubit® 2.0 Fluorometer and reagents, before submission for next generation sequencing.

2.2.2.4 Next Generation Sequencing (NGS)

A total of 5 µl cleaned amplicon DNA was used for indexing PCR and library construction using the indexes from the Nextera XT (Illumina, Inc. San Diego, CA, USA) kit. The 'Illumina Metagenomics Library Preparation Guide' was followed to obtain the sequencing libraries from the amplicons. All samples were quantified using the Qubit® 2.0 Fluorometer and reagents. Individual samples were normalised and multiplexed into a single sample for NGS. Next generation sequencing of the multiplexed sample(s) were performed with the MiSeq Reagent Kit V3 (2 × 300 bp paired end, Illumina, Inc.) on a MiSeq desktop sequencer (Illumina, Inc.). All sequencing was done at the Agricultural Research Council's Biotechnology Platform in Pretoria, South Africa.

2.2.2.5 Bioinformatics and sample analyses

All the primary analyses, including image analysis, base calling and initial sequencing reports were done by means of MiSeq instruments. All the de-multiplexing of indexed samples was done using the MiSeq Reporter V2.5.1 software with separation of samples occurring on perfect index matches only. Paired-end reads were uploaded into 'CLC Genomics workbench 8.5' (CLC-bio, Inqaba Biotech) and quality reports were generated (data not shown). Sample quality reports were inspected and only samples that had Q20 values or higher, contained appropriate length sequences, had sufficient reads and were of good quality, were uploaded to the MG-RAST server V3.6 (<http://metagenomics.anl.gov/>; Meyer *et al.*, 2008) for

analyses. Samples failing quality control were re-sequenced, after which failed samples were discarded.

The standard MG-RAST pipeline that included QC clipping, was run. MG-RAST uses the 'Sylvia, Greengenes' and 'RBD' databases for taxon identification. Read files from the MiSeq were combined prior to uploading of samples where samples were sequenced more than once in different runs. Individual samples were analysed as single reads or combined reads, with the combined analyses conducted on reads merged using the online MG-RAST overlapping tool.

2.3 Results

2.3.1 Identification of microbes present in SoilBioMuti (SBM)

2.3.1.1 Standard agar-plating technique

Table 2.2. The number of heterotrophic bacteria from two SoilBioMuti (SBM) samples done on two different agar media to determine the bacteria present in 'cooled' products exposed to 5 °C for 24 h (A) and in freshly prepared SBM product (B).

Agar medium	A: Cooled SBM*	B: Fresh SBM*	t-value	P value
MRS	2 067±763	273 333±53 125	-10.21	0.0001
Nutrient	26 333±5 558	2 533 333±169 967	-29.48	0.0001

*Replicates included per treatment were n = 3.

Bacterial counts on both agar media were significantly ($P \leq 0.05$) higher in the fresh SBM (Sample B) compared to those in the 'cooled' SBM (Sample A) that was exposed to 5 °C for 24 h before the plating assay (Table 2.2). The freshly-prepared SBM product (Sample B) had approximately 99 % higher numbers of bacteria than that of sample A when plated on nutrient as well as MRS agar. The use of MRS agar is aimed at promoting the proliferation of lactic-acid producing bacteria and indicated high numbers of such bacterial groups present in the freshly-prepared SBM sample (B) in particular (Anonymous, 2016a).

2.3.1.2 Molecular identification of bacterial genera

Genomic DNA was successfully extracted from SBM products and was subjected to NGS assays (see Paragraph 2.2.1.2.1). Bacteria were identified to genus level during this particular study.

Forty-five genera of bacteria were identified from the two SBM samples subjected to NGS (Fig. 2.1; only legends of genera represented by either red and/or bright green apply to SBM samples). The reason for this is that due to the high cost of running NGS samples for identification, samples from other research projects (colour coded with blue and olive green blocks) were included in this NGS analyses to make it more cost-effective.

Numerous species of most of these genera exist and hence the genera identified were divided in non-pathogenic/beneficial, plant-pathogenic, human pathogens and miscellaneous (limited/dubious knowledge available) based on knowledge available for these genera in general.

2.3.1.2.1 Non-pathogenic bacteria

The genera of bacteria generally known as non-pathogenic and identified as contained within two SBM samples constituted 49 % of the complex identified (Fig. 2.1.). These include *Acetobacter*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Bradyrhizobium*, *Burkholderia*, *Frankia*, *Glutacetobacter*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Microbispora*, *Micromonospora*, *Paenibacillus*, *Pediococcus*, *Phyllobacterium*, *Rhodococcus* (also reported as a pathogen), *Streptosporangium*, *Streptomyces* and *Weissella*.

2.3.1.2.2 Plant-pathogenic bacteria

Bacterial genera identified in the SBM samples that are known as plant pathogens represented 9 % of the bacterial complex identified (Fig. 2.1). These genera constituted *Erwinia*, *Leifsonia*, *Pseudomonas* and *Pectrobacterium*.

2.3.1.2.3 Animal and/or human bacterial pathogens

Thirty-six percent of the genera identified from SBM samples are listed as either pathogens of animals and/or humans and include *Actinomyces*, *Arcobacter*,

Burkholderia, *Clostridium*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Nocardia*, *Nocardiosis*, *Paracoccus*, *Pseudomonas* (plant pathogenic species also exist), *Rhodococcus* (few species are pathogenic), *Rothia* (non-pathogenic species also exist), *Sphingobium*, *Sphingomonas*, *Staphylococcus* (Fig. 2.1).

2.3.1.2.4 Miscellaneous

Grouped under 'miscellaneous' genera were approximately 13 % of the genera identified from the bacteria complex contained in the SBM samples (Fig. 2.1), including *Cellulosimicrobium*, *Deinococcus*, *Frigoribacterium*, *Kribella*, *Lactobacillus* and *Oscillatoria*.

It is important to bear in mind that the percentages ascribed to each of these bacterial groups above do not total 100 when added together. This is because species of genera such as *Burkholderia* and *Rhodococcus* are regarded as beneficial but can also be a pathogen of animals, while some *Pseudomonas* species represent both plant and animal pathogens.

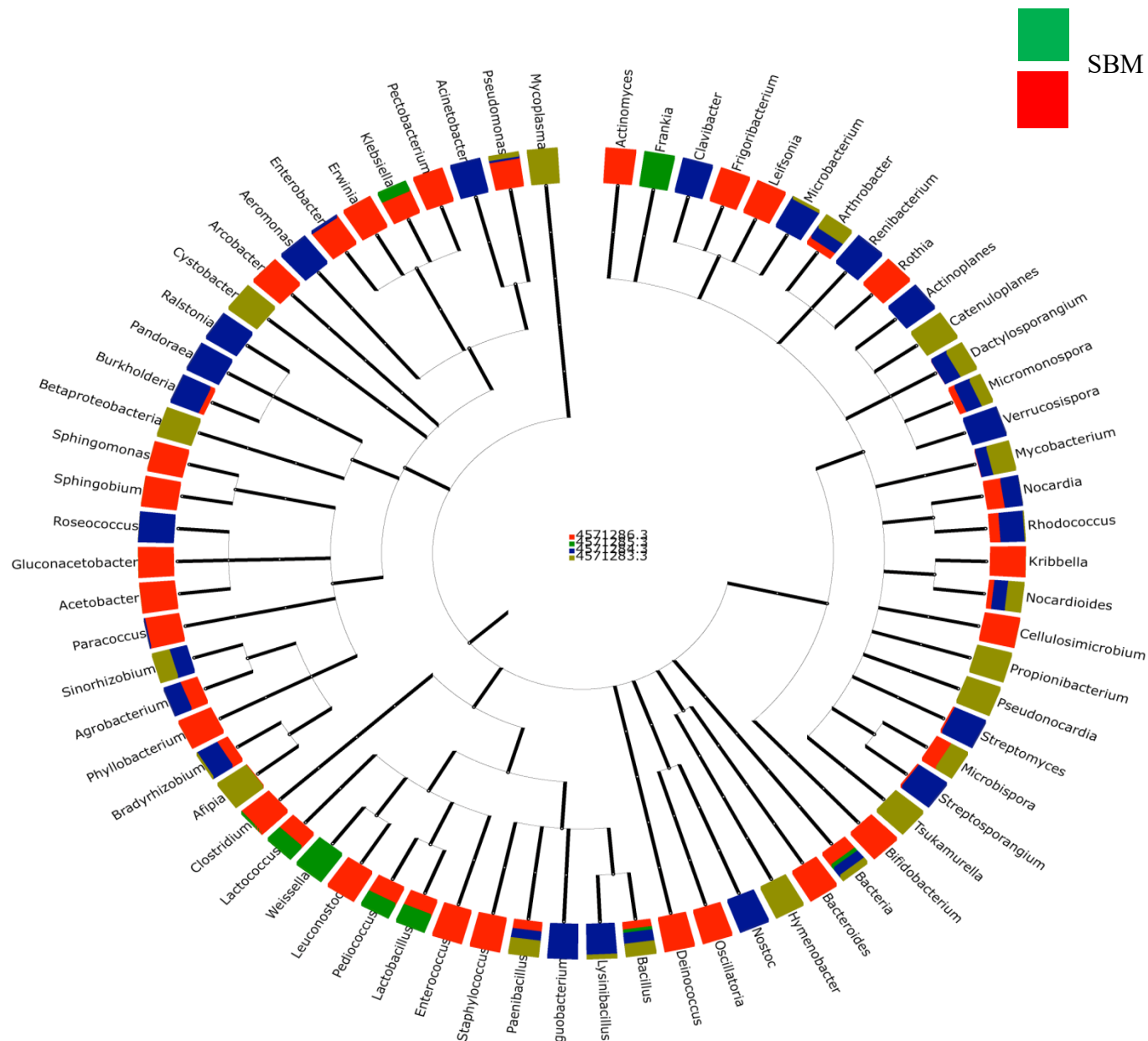


Fig. 2.1. Genera of bacteria that were characterised from SoilBioMuit (SBM) samples using next generation sequencing with only legends of genera indicated in red and/or green (partially or totally) were identified from samples (samples other than SBM, which are represented by colours other than red and green, were analysed during the same assay to enable cost-effectiveness of the apparatus used).

2.4 Discussion

Characterisation of SBM using the traditional plating technique revealed that lactic-acid producing bacteria in particular were most abundant in freshly prepared SBM samples cultured on MRS medium. Another outcome of this study was that molecular analyses, using NGS, showed that freshly prepared SBM samples contained 45 bacterial genera of which 49% represented beneficial bacteria. This represents valuable and useful information on the integrity of this novel, soil-derived product. These results hence confirm that the product contains a high percentage of organisms known for their beneficial characteristics regarding plant growth as well as pest control. From a theoretical standpoint, the anti-nematodal and plant growth stimulating effects, reported by the creator of SBM, are thus supported to a certain degree.

The significantly higher abundance ($\pm 99\%$) of bacterial cells, when plated on two agar media, in freshly prepared compared to 'cooled' SBM samples (exposed to 5 °C for 24 h before the plating assay) indicated that the bacteria present in SBM are temperature sensitive. Bacterial genera, such as *Bacillus* that was identified in the SoilBioMuti products tested, are optimal in terms of their function at temperatures of 25 °C, but can remain functioning at temperatures of up to 35 °C (Dongyai *et al.*, 2010). Hence products containing such organisms should be stored at temperatures that range between the latter values to ensure that the organisms stay active. Nedwell (1999) showed a direct link between a micro-organism affinity and temperature and that when temperature dropped below the optimum growth temperature for the specific organism, the organism's efficiency also decreased. This effect was recorded until a temperature was reached, at which point the organism could no longer maintain its optimal growth rate, resulting in inhibition of its metabolism. Therefore, SBM should not be stored in cold conditions, but rather at a room temperature of 20 °C-25 °C.

The significantly higher numbers of lactic-acid producing bacterial cells cultivated on MRS agar plates for freshly-prepared SBM, compared to those of the 'cooled SBM' samples was interesting. This is most probably due to the higher temperature at which the freshly-prepared SBM was stored that is closer to optimal temperature for the survival and development of the organisms it contains and encouraged the growth

and proliferation of the lactic-acid producing bacteria (Anonymous, 2016a). This has been documented for bacteria such as *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* that can produce considerable amounts of lactic acid (Anonymous, 2016a). The first three of these genera as well as *Lactococcus* and *Weissella* are well-known for their lactic-acid production (Arima and Ogunlowo, 2014) and have been identified from the SBM products tested. Several studies reported the adverse effects of lactic-acid-producing bacteria on *Meloidogyne* spp., e.g. Lee *et al.* (2014) showed that filtrates from *Lysobacter capsici* YS1215, containing 25 µl/ml of lactic acid, only allowed a 5.9% J2 hatching rate of *Meloidogyne incognita* (Kofoid and White, 1919), Chitwood, 1949, five days after incubation compared to a 44.5% J2 hatch rate of the sterile, control water treatment. According to Seo *et al.* (2012), a product that contained a mixture of the lactic-acid producing bacterium *Lactobacillus farraginis* as well as two *Bacillus* spp. (*B. cereus*, and *Bacillus thuringiensis*) resulted in a decrease of 41-46% in *M. incognita* egg mass numbers on roots of melon (*Cucumis melo* L.), compared to the untreated control, 60 days after onset of the experiment. The dominance of lactic-acid bacterial genera in SBM may hence be an indication that these genera may contribute substantially towards the anti-nematodal properties of this product as is demonstrated in Chapters 3 and 4.

Other beneficial bacterial genera identified, e.g. *Bacillus*, *Burkholderia* and *Paenibacillus* are also known for their adverse effects on the biology and reproduction of *Meloidogyne* (Mendoza *et al.*, 2008; Hallman *et al.*, 2009; Seo *et al.*, 2012; Viaene *et al.*, 2013). The genus *Bacillus* has been reported to promote plant growth and provide effective biological control against various plant diseases and nematode pests, particularly against *Meloidogyne* spp. (Mendoza *et al.*, 2008; Hallman *et al.*, 2009; Singh & Siddiqui, 2010). Species of *Bacillus* therefore evaluated and reported adversely affect either *Meloidogyne* spp. in terms of their biology and/or reproduction include: *Bacillus amyloliquefaciens*, *B. megaterium*, *B. circulans*, *B. subtilis*, *B. firmus*, and *B. cereus* (Sikora and Hoffmann-Hergarten, 1993, Terefe *et al.*, 2009). Several studies reported that *Bacillus* spp. were effective in paralysing *Meloidogyne* spp. J2 with subsequent reductions in the reproduction rates and population densities of such pests (Giannakou *et al.*, 2004; Mendoza *et al.*, 2008; Terefe *et al.*, 2009; El-Hadad *et al.*, 2011). A strain of *B. cereus* indicated little to no biocontrol activity on *M. arenaria*, though it did show significant control of *M.*

incognita and *M. javanica* (Mahdy, 2002). *Bacillus firmus* on the other hand was reported to reduce J2 hatching of *M. incognita* by 98-100% and gall formation by 91% in a greenhouse experiment. Also, final *M. incognita* populations were reduced by 76% and egg numbers by 45% (Terefe *et al.*, 2009). In a study by Jansen-Girgan *et al.* (2016) the cell-free filtrates of a standard *B. firmus* strain (ATCC8247) significantly reduced *M. incognita* J2 motility 3, 6 and 24 h after J2 being suspended therein *in vitro*. *Burkholderia* also suppresses many soil-borne plant pathogens (Daubaras *et al.*, 1996; Mueller *et al.*, 1997). Bell pepper (*Capsicum annuum* L.) plants treated with formulations of *Burkholderia cepacia* (strains Bc-2 and Bc-F) had significantly lower *M. incognita* eggs and J2 numbers per gram root compared to the untreated controls (Meyer *et al.*, 2001). This bacterium is particularly recognized for its abilities to promote plant growth (Bevivino *et al.*, 1998) and enhancement of crop yields (Chiarini *et al.*, 1998; Tabacchioni *et al.*, 1993). For *Paenibacillus*, various concentrations (10-30%) of a bacterial culture filtrate of *Paenibacillus elgii*, strain HOA73, significantly reduced *in vitro* hatching of *M. incognita* J2 and resulted in substantial mortality (Nguyen *et al.*, 2013). Addition of this bacterial culture also significantly reduced the galling, egg mass numbers and *M. incognita* population densities as well as fresh tomato shoot weight by 62.1, 69.8, 53.0 and 17.9%, respectively, compared to the control seven weeks after nematode inoculation. Two strains of *Paenibacillus polymyxa* (GBR-462 and GBR-508) and one of *P. lentimorbus* (GBR-158) reduced *M. incognita* root gall formation between 64-88% on roots of tomato also infested by *F. oxysporum* f. sp. *lycopersici*, compared to the untreated control (Son *et al.*, 2009).

Other beneficial bacteria identified in fresh SBM samples represented several genera. However, some of these genera can also be pathogenic as indicated below. For example, *Acetobacter*, which is known for its acetic-acid producing abilities (Lu *et al.*, 1999); *Agrobacterium*, known to cause galls in plants by means of horizontal gene transfer and is commonly used as an experimental organism in research (Gohlke and Deeken, 2014); *Arthrobacter* that degrades harmful substances in soils (McGuinness and Dowling, 2009); *Bacteroides* that represents the normal flora present in the oral, respiratory, intestinal, and urogenital cavities of humans (Aas *et al.*, 2005); *Bifidobacterium* that represents ubiquitous inhabitants of the gastrointestinal tract, vagina and mouth of mammals, including humans (Aas *et al.*,

2005); *Bradyrhizobium*, *Frankia* and *Glutomonacotobacter* for their nitrogen-fixing characteristics (Shridhar, 2012); *Leuconostoc* being acid-tolerant (McDonald *et al.*, 1990); *Microbispora* that is active against human cancer cells (Savi *et al.*, 2015); *Micromonospora* and *Streptosporangium* for its antibiotic-production characteristics (Zitouni *et al.*, 2004); *Phyllobacterium* (grouped in Proteobacteria together with *Agrobacterium* and *Rhizobium*) for having great adaptive capacity to environmental conditions and their ability to communicate with plant tissue, and also known for plant growth promoting abilities (Mantelin *et al.*, 2006); *Pseudomonas* spp. for their antagonism against *Meloidogyne*, e.g. *P. fluorescens* against *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 (Raaijmakers *et al.*, 2002), and *Streptomyces* known as natural decomposers of organic matter in soils and for its lactic-acid producing abilities (Aislabie and Deslippe 2013).

Except for the beneficial bacteria present in the SBM samples, the presence of specific pathogenic bacteria was also detected using NGS. The mere presence of animal-, human- and plant-pathogenic bacterial genera in SBM is a matter of concern and should also be taken cognisance of. These included *Erwinia* of which several species are known to inflict blights, cankers, die back, leaf spots, wilts, discoloration of plant tissues, stalk rot, crown rot, stem rot or fruit collapse (Starr and Chatterjee, 1972); *Leifsonia* that causes ratoon stunting disease of sugarcane (Monteiro-Vitorello *et al.*, 2004); *Pectrobacterium* that has been previously characterised as part of the genus *Erwinia* and *Pseudomonas* that is notorious for causing diseases in plants (Anonymous, 2016b) and *Rhodococcus*, which is commonly found in dry and dusty soils and can be important for diseases of domesticated animals such as horses and goats (Prescott, 1991; Verville *et al.*, 1994).

The bottom line is that the bacterial genus/genera and the species present in a product, specifically the one/those that act as the active substances in terms of its anti-nematodal effect(s), should be identified before such a product can be registered for commercial use to combat nematode pests. This is according to Act No 36 of 1947 of the Department of Agriculture and Fisheries (DAFF). Nonetheless, identification of the bacterial genera contained within SBM as a result of this study added significant value to the knowledge base of scientists. However, the mode of

action of the active substances contained within the product concerning their effect on *Meloidogyne* will only be understood once such organisms are identified to species level. This is because differences exist between species of the same genus regarding their efficacy and function as biological control agents. Differences can also exist between multiple strains of the same species. Due to this, using micro-organisms as biological control has been challenging. Another important aspect that emanated from this study is that SBM should be freshly prepared, and not cooled, to ensure the optimum abundance of bacteria contained within it.

2.5 Conclusion

Almost 50% of the bacterial genera contained within SBM has been identified as being beneficial, with lactic-acid producing bacteria being the most predominant according to the traditional plate assays. A number of plant-promoting bacteria as well as those with anti-nematodal characteristics were also identified in the product, suggesting that the product has plant growth promoting as well as nematode control abilities. It must, however, be said that a number of pathogenic bacterial genera were also identified with the potential to infect and caused disease in animals, humans and plants.

2.6 References

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Chapter 3: The effects of SoilBoiMuti on the oxygen consumption of second-stage juveniles (J2) of *Meloidogyne incognita*

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3.1 Introduction

Literature on the respiratory physiology of nematodes is scarce and fragmented (Perry and Moens, 2013). However, valuable baseline information for terrestrial (Blair, 1955; Bhatt and Rhode, 1969; Klekowski *et al.*, 1972; Nordmeyer and Dickson, 1989; Ferris *et al.*, 1995; Suda *et al.*, 2005; Van Aardt *et al.*, 2010; Fourie *et al.*, 2014), freshwater (Schiemer and Duncan, 1974) and marine (Atkinson, 1973) nematodes in terms of their oxygen consumption has been recorded. Information related to the physiology of nematodes, e.g. data concerning their oxygen consumption rates (OCR), enables researchers to conduct fundamental and applied research. Information on the OCR of plant-parasitic nematodes, for example allows determination of the adverse effects of products with anti-nematodal properties on the respiratory biology of such pests (Nordmeyer and Dickson, 1989; Fourie *et al.*, 2014). A decrease in the oxygen consumption of nematode individuals that have been exposed to an anti-nematodal product compared with those suspended in only tap water, is for example direct evidence of the adverse effects of such a product on the nematode's respiration.

The Winkler-chemical analysis method (Blair, 1955), Cartesian-diver (Bhatt and Rohde, 1969; Klekowski *et al.*, 1972; Schiemer and Duncan, 1974; Reversat, 1977; Golterman, 1983; Van Aardt *et al.*, 2010) and different types of manometric methods (Gilson, 1963; Umbreit *et al.*, 1972) have been used in the past to measure the OCR of small aquatic animals, including nematodes. A major disadvantage of the Winkler, Cartesian-diver and the manometric methods is that a single measurement using such protocols takes between 20-30 minutes to complete. With the advancement of

technology to measure OCR, polarographic oxygen sensors (POS) (Clark, 1956; Atkinson, 1973, Nordmeyer and Dickson, 1989; Fourie *et al.*, 2014), rate of CO₂ evolution by infra-red (IR) gas analysis (Ferris *et al.*, 1995) and fibre-optic sensors (FOS) (Suda *et al.*, 2005, Dancy *et al.*, 2013) became available and have been used. The POS and IR gas analysis methods require less than 5 min (Umbreit *et al.*, 1972) per determination and the FOS (Seahorse Bioscience, USA) less than 20 min for the simultaneous determination of the OCR of nematodes in 72 or 96 wells. This protocol has been used by (Dancy *et al.*, 2013) to measure the OCR of 25-150 *Caenorhabditis elegans* individuals per well.

Using the latest FOS technology to measure the OCR of plant-parasitic nematodes represents a first, baseline study to determine the effects of anti-nematodal products on the physiology of the test nematode species, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949. The progressive withdrawal of synthetically-derived Class I nematicides results in fewer options for producers to successfully manage plant-parasitic nematodes. Two nematicides, with the active substances aldicarb and endosulfan, were registered for use on maize in South Africa (and elsewhere in the world), but has been withdrawn from the market (Anonymous 2012; Verdoorn 2012). Hence, research towards investigating and exploiting more eco-friendly nematode management strategies was prompted and include the use of biological agents (e.g. bacterial and fungal species), non-toxic plant-derived products and well planned crop rotation systems (Hallman *et al.*, 2009; Terefe *et al.*, 2009; Viaene *et al.*, 2013). SoilBioMuti (SBM), a novel, unregistered water-based product that contains soil micro-biota (amongst others fungi, yeast, Rhizobium and humus-making bacteria and mycorrhiza) (Annex 1; Fourie *et al.*, 2016) is unofficially recommended at 2.5% concentration to restore poor soils and also acts as a plant-growth enhancer. Hence, the objective of this study was to determine the effect of this product on the OCR of second-stage juveniles (J2) of *M. incognita* using FOS.

3.2 Materials and Methods

3.2.1 Mass rearing of *Meloidogyne incognita*

For this and other activities of this study (see Chapter 4) a local population of *M. incognita* was reared *in vivo* in roots of the root-knot nematode susceptible tomato (*Solanum lycopersion* L.) cultivar Floradade in a greenhouse using the protocol of Fourie *et al.* (2012). This in short entailed that the *M. incognita* race 2 population were obtained from root-knot-nematode-infected hulls from groundnut (Vaalharts Irrigation Scheme in the Northern Cape Province; 27.95° S, 24.85° E). The identification of this species was done by both morphological (Taylor and Sasser, 1978) and molecular (sequence derived amplified region – polymerase chain reaction' SCAR-PCR) (Zijlstra *et al.*, 2000) techniques, as well as the North Carolina Differential Host Range Test (Taylor and Sasser, 1978). Single egg masses from this population was removed from infected hulls and inoculated on roots of individual seedlings of the root-knot nematode susceptible tomato cultivar Moneymaker (Singh and Khurma, 2007) and maintained in a greenhouse at an ambient temperature regime of 19±1°C minimum (night) and 26±1°C maximum (day), with a 14:10LD photoperiod. The soil in the 20-l capacity pots in which these seedlings were grown was fumigated three weeks before transplanting of seedlings with Telone II fumigated soil (active substance 1,3 dichloropropene, 1.110 g/l) and was also pasteurised for 72 h at 60 °C, using a commercial pasteuriser. Eggs and second-stage juveniles (J2) of this *M. incognita* population were extracted (using the adapted NaOCl method of Riekert, 1995) from tomato roots approximately 90 days after the onset of the rearing process and used for the hatching of J2 as explained below.

Eggs and J2 were extracted from roots of 30-40-day-old tomato plants using the adapted NaOCl technique (Riekert, 1995) and suspended in aerated sterile, tap water on a 25-µm-mesh sieve at 26 °C (Moura *et al.*, 1993). The water contained total dissolved solids at 240.6 µg l⁻¹, calcium at 430 µg l⁻¹, sodium at 224.3 µg l⁻¹, potassium at 4.8 µg l⁻¹, iodine at 48.5 µg l⁻¹ and chlorine at 24.0 µg l⁻¹, with an electrical conductivity (EC) of 365 µS.cm⁻¹ and pH 7.75.

After 24 h, hatched J2 that moved through the 25-µm aperture sieve were collected on a 20-µm aperture sieve and used for OCR measurements. The fresh mass per *M. incognita* (0.3635 µg) was determined according to the protocol of Andràssy (1956)

and the body volume calculated (Fourie *et al.*, 2014). This was done using the average diameter and length of 10 J2, which were obtained by using a Nikon DS-FI1 light microscope furnished with NIS Elements software (Version 3.07).

3.2.2 Fiber-optic oxygen sensor (FOS) measurements

The FOS analyser was used to measure *M. incognita* J2 OCR data from 10 different population batches of J2 of *M. incognita* that hatched from eggs obtained during the five-month period that this study was conducted. The Seahorse XFe96 analyser (Seahorse Bioscience) was used for this purpose. This analyser made use of a cell-culture micro-plate (in which the J2 were pipetted) and a sensor cartridge, which contains one probe and four injection ports for each well. The Seahorse XFe96 analyser uses the fluorescence of a chemical complex in a sol-gel to measure the partial pressure of oxygen. After probe calibration of the FOS, the utility plate was ejected and the cell-culture micro-plate containing the J2 inserted and analysed (Rogers *et al.*, 2011). Since the temperature of the FOS instrument was controlled by adjusting the room temperature, the temperature for the experiments with this apparatus ranged between 25.6 and 27.6°C.

3.2.2.1 Determining the number of second-stage juveniles (J2) to use for oxygen consumption measurements

The first step of this study was to determine how many J2 should be used per well for OCR measurements since no literature in this regard for *Meloidogyne* spp. is available. The number of J2 was determined by pipetting six, 10 μ l aliquots from the freshly hatched 24-h-old J2 stock solution into a De Grisse counting dish (De Grisse, 1963). Counts were done using a stereomicroscope (40 $\times$ magnification) to calculate the mean number of J2 per 10 μ l. The J2 counts were then extrapolated to ensure that five, 25, 50, 75 and 100 J2 were transferred to the designated wells of the 96-well cell-culture micro-plate. Each well was topped up with sterile tap water to a maximum volume of 225 μ l. Six replicates were done for each J2 batch for each experiment (initial and repeat), totaling 12 replicates when the data from the two experiments were pooled. Data for each experiment were first separately analysed using Analysis of Variance (ANOVA) (Statistica 12 for Windows; <http://www.Statsoft.com>). Means were separated by the Tukey HSD Test ($P \leq 0.05$). Ultimately, data for the two experiments were subjected to Factorial Analyses of

Variance (Statistica). No significant ($P \leq 0.05$) interaction existed between the data for each of the different J2 numbers treatments and pooled data were hence used.

3.2.2.2 The effect of SoilBioMuti (SBM) on the oxygen consumption rate (OCR) of second-stage juveniles (J2)

According to data obtained from the activity referred to in Paragraph 3.2.2.1, 100 24-h-old J2 were suspended per well for 24 h in a temperature-regulated cabinet at 25 ± 1 °C in the following treatments before OCR measurements were made:

- i) tap water
- ii) 2.5% non-filtered (NF) SBM + J2
- iii) 2.5% filtered (F) SBM + J2
- iv) 2.5% SBM (NF) without J2
- v) 2.5% SBM (F) without J2

The F product was obtained by filtering the stock SBM product through a 0.22 µm filter (Sartorius Stedim Biotech, Germany) to remove the bacterial cells and other micro-organisms. Five replicates were done for each treatment for each experiment (initial and repeat), totaling 10 replicates when two experiment's data were pooled. Data for each experiment were first separately analysed using Analysis of Variance (ANOVA) (Statistica, Version 13 (<http://www.statsoft.com>)). Means were separated by the Tukey HSD Test ($P \leq 0.05$). Ultimately, data for the two experiments were subjected to Factorial Analyses of Variance. No significant ($P \leq 0.05$) interaction existed between the data for each of treatments and pooled data were hence used.

3.3 Results

3.3.1 Determining the number of second-stage juveniles (J2) to use for oxygen consumption measurements

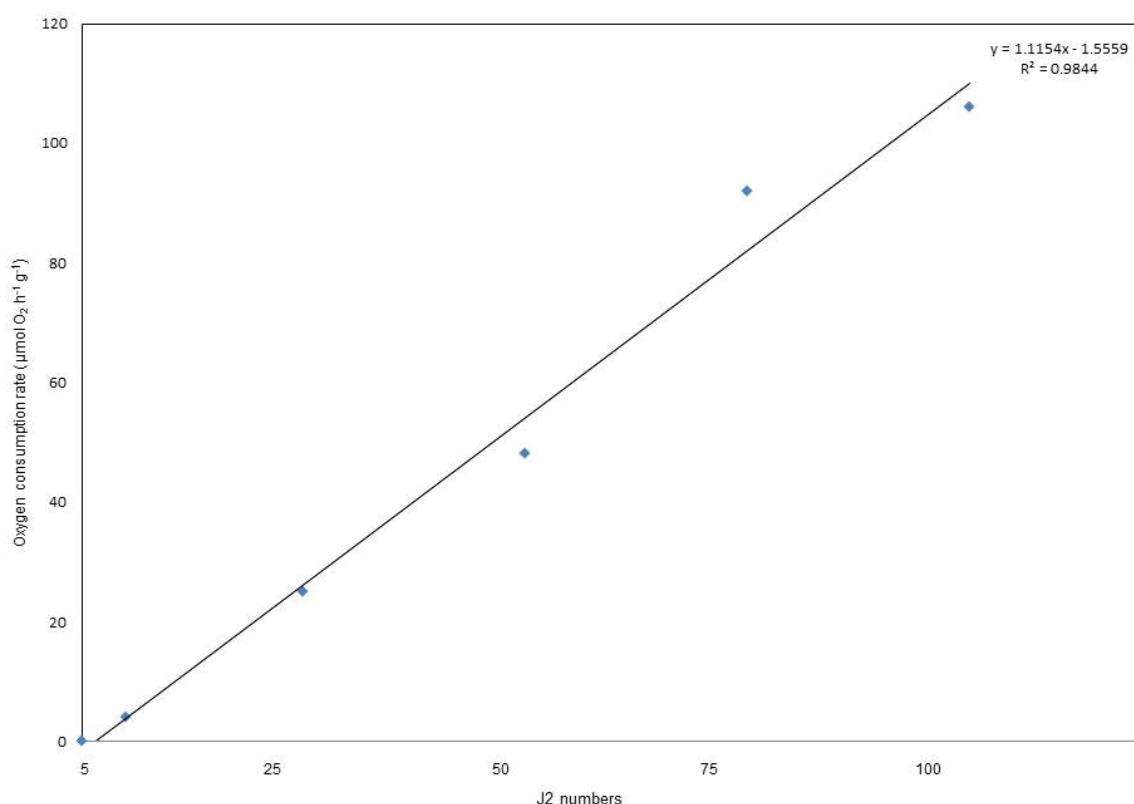


Fig. 3.1 The oxygen consumption rate (OCR) of different numbers of *Meloidogyne incognita* second-stage juveniles (J2) suspended in sterile tap water as measured by the Seahorse fibre-optic oxygen sensor (FOS) analyser.

The OCR of *M. incognita* J2 increased linearly with increasing J2 numbers (Fig. 3.1). Five J2/well had the lowest mean OCR, viz. 10.9 μmol O₂/h/g live mass. The highest OCR values were recorded for the 100 J2/well, viz. 103.8 μmol O₂/h/g live mass. Significant differences ($P \leq 0.05$) existed among the treatments. The OCR of 0, 5 and 25 J2/well was significantly ($P \leq 0.05$) lower compared to that of the OCR of 75 and 100 J2/well. The OCR of 50 J2/well did not differ significantly from the other treatments.

3.3.2 The effect of SoilBioMuti (SBM) on the oxygen consumption rate (OCR) of second-stage juveniles (J2)

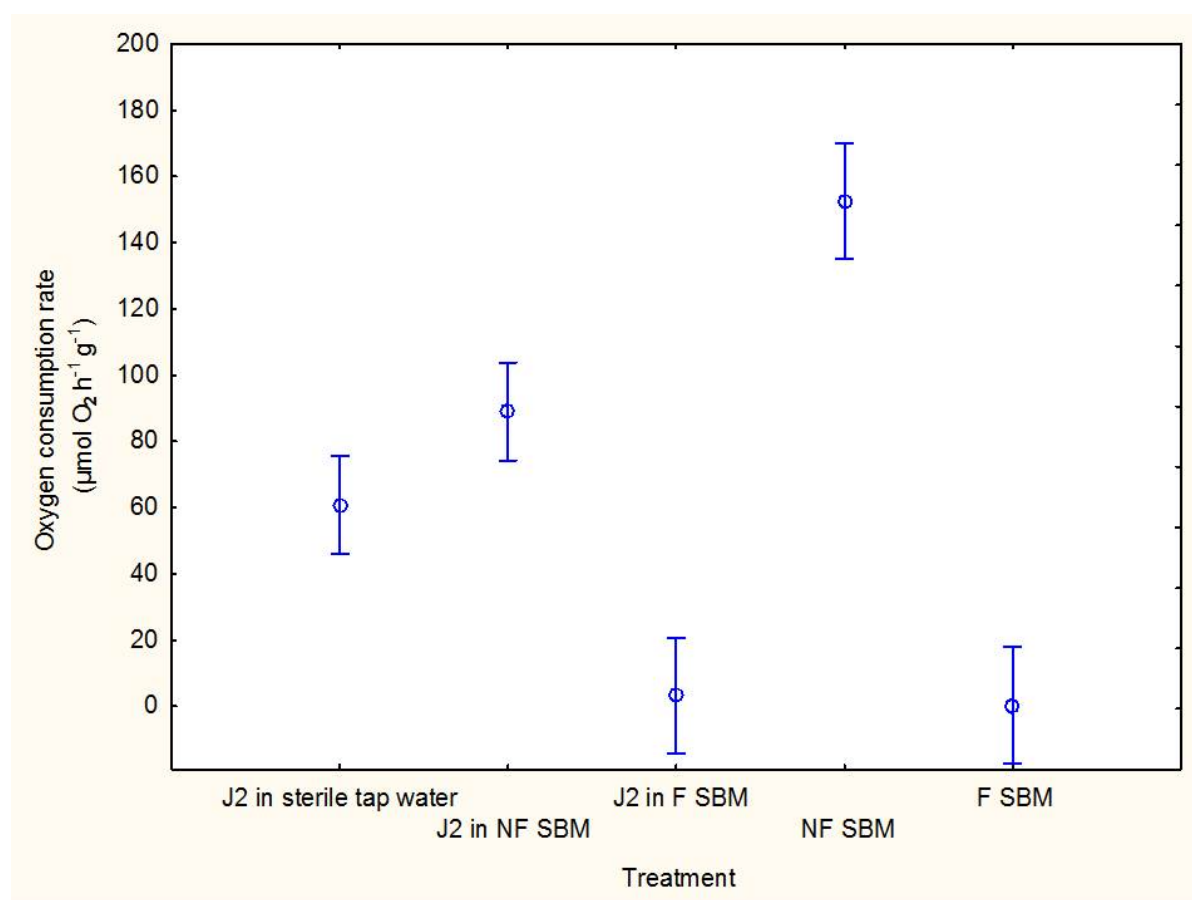


Fig. 3.2. The oxygen consumption rate (OCR) of *Meloidogyne incognita* second-stage juveniles (J2) suspended for 24 h in sterile water, non-filtered (NF) and filtered (F) SoilBioMuti (SBM), and the OCR of micro-organisms present in SoilBioMuti (current effect: $F = 52.478$, $P = 0.0001$. Vertical bars denote 0.95 confidence intervals).

The OCR measured for NF SBM containing J2 were significantly ($P \leq 0.001$) lower (42%) compared to that of the NF SBM without J2 (Fig. 3.2.). However, the OCR of J2 in sterile tap water did not differ significantly from that of the NF SBM containing J2. The two F SBM treatments had the lowest OCR and differed significantly from that of the NF SBM treatments and the treatment represented by J2 suspended in sterile tap water.

3.4 Discussion

The linear increase in OCR with higher numbers of *M. incognita* J2 was recorded during this study was in agreement with those obtained by Dancy *et al.* (2013) for bigger nematodes (*Caenorhabditis elegans*). Accordingly, the number of J2 used to determine their OCR using the FOS analyser is recommended to be between 25 and 100. Although the OCR of as few as five J2 was and could be measured, the mean value was low during this study and may not be optimal for experimental purposes. It is hence proposed that 100 *Meloidogyne* J2/well will be adequate to obtain accurate results and this number was hence used to determine the OCR of J2 suspended in SBM.

A NF SBM stock solution, unofficially used for its anti-nematodal characteristics (amongst others) (Annex 1; Van Aardt *et al.*, 2016), proved to be effective in reducing the OCR of *M. incognita* J2 during this study. The 42 % reduction in J2 OCR suspended in NF SBM compared to that of NF SBM without J2 was most probably caused by the secondary compounds excreted by the micro-organisms that are contained within the product. Traditional (agar-plating) and new-generation, molecular sequencing led to the identification of numerous bacteria being present in SBM (see Chapter 2; Paragraph 2.3.1.2.; Fig. 2.1.). According to these data generated, the bacterial composition of SBM represented various bacteria genera (e.g. *Bacillus*, *Burkholderia*, *Enterobacter*, *Lactobacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces* and others) that is known as antagonists of plant-parasitic nematodes (Guyon *et al.*, 2003; Hallman *et al.*, 2009; Joo *et al.*, 2012). The low OCR measurements obtained for the F SBM treatment without J2, can be explained since the bacterial cells (and other micro-organisms) were removed through microfiltration. Also, the similarly low OCR value of the F SBM product containing the J2 is most probably the result of an adverse effect of the secondary metabolites, enzymes and/or toxins produced by the bacteria and/or other micro-organisms contained within the SBM product. The adverse effects of secondary metabolites of, for example, *Bacillus* spp. on *Meloidogyne* spp. J2 motility has been demonstrated by various researchers (Mendoza *et al.*, 2008; Padgham and Sikora, 2007). The antagonistic effect of lactic-acid producing bacteria to *Meloidogyne* spp. has also been reported (Seo *et al.*, 2012; Takei *et al.*, 2008; Lee *et al.*, 2014;). The stock SBM products tested in this study contained high numbers (273 333 cells/250

mg for fresh SBM on MRS agar and 2533 333 cells/250 mg on nutrient agar) of this bacterial group (see Chapter 2; Paragraph 3.2.1; Fig. 2.1.) and hence this group of bacteria may possibly be the dominant one responsible for suppressing the OCR of *M. incognita* as was found in this study. It is, however, suggested that a SBM concentration higher than the 2.5 % tested in this study, be evaluated for its efficacy in reducing the OCR of J2 to optimize the use of this novel product.

Inhibition of the OCR of plant-parasitic nematode genera other than *Meloidogyne*, as well as that of free-living nematodes has been documented when exposed to nematicides (Bhatt and Rhode, 1970; Ritzrow and Kämpfe, 1971; Reversat, 1977). However, literature on OCR measurements of *Meloidogyne* spp. to determine the effect of biological agents of only this study and a dated study by Nordmeyer and Dickson (1989) exist according to the knowledge of the author of this dissertation. The latter authors used polarographic oxygen sensor (POS) technology, equipped with a Clarke Electrode, and recorded a 61.3% reduction in the oxygen uptake of *M. incognita* J2 suspended for 24 h in 0.5 µg/ml Avermectin B2 (a secondary metabolite of the soil bacteria *Streptomyces avermetilis*). This was significantly higher compared to the 4.3 and 24.7% reductions of the oxygen consumption of J2 that were exposed to carbofuran and aldicarb (both a dosage rate of 5 µg/ml), but not than those suspended in ethoprop, fenamiphos and oxamyl (dosage rate of 5 µg/ml). Interesting, however, is that although Avermectin B2 resulted in total paralyses of J2, it did not completely inhibit their oxygen consumption. Oxygen data generated during the study of Nordmeyer and Dickson (1989) hence complement those obtained during this study for SBM in that the adverse effects of bacterial agents on *M. incognita* respiration may be similar or even superior than that of synthetically-derived nematicides.

The oxygen consumption of plant-parasitic nematodes is a valuable and useful measurement of their biological activity, since inhibition of their respiratory functions has a major effect on their energy metabolism (Kämpfe, 1978) as was demonstrated for *Meloidogyne* spp. J2 in this present study and the one by Nordmeyer and Dickson (1989). Therefore, results generated during this study represent novel information regarding the number of J2 to be used for OCR measurements of *M. incognita*. Value is hence added to limited knowledge available for this specific field

of nematological research. However, although the OCR of *M. incognita* J2 was reduced after suspension in SBM, the specific mode(s) of action of the bacterial agents and/or other micro-organisms present in the product is not known. Also, the effect of SBM on the reproduction of this species has to be evaluated to enable useful recommendations relating the use of this product for its anti-nematodal characteristics. The latter has been done *in vitro* and *in vivo* as explained in Chapter 4.

3.5 Conclusion

Both F and NF 2.5% SBM products had a negative effect on the respiration of *M. incognita* J2. This indicates that a decrease in the metabolism of these root knot nematodes specimens occurred when exposed to SBM. Even though the mechanism of the SBM is still unknown, these results indicate that secondary metabolites of the micro-organism present in SBM is, at least to some degree, most probably responsible for the decreased J2 oxygen consumption. This is supported since the F SBM products contained no micro-organisms, but had the same effect as the NF SBM products that contained microorganisms.

3.6 References

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Chapter 4: The effects of SoilBioMuti (SBM) on the motility and reproduction of *Meloidogyne incognita* and identification of bacterial groups in soil treated with the product

4.1 Introduction

The control of plant-parasitic nematodes in agricultural systems is becoming more challenging as the use of chemical nematicides are being reduced considerably due to their negative effect on animals, humans and the environment (Schneider *et al.*, 2003). In several soils micro-organisms, in particular nematophagous fungi and bacteria, occur that have natural anti-nematodal properties (Stirling, 1991; Cumagun and Moosavi, 2015). Such organisms have potential to replace chemical nematicides to a certain extent. Currently a number of bacteria are known to have negative effects on plant-parasitic nematode populations since it interferes with the reproduction of such pests. Various species of bacterial genera of *Bacillus*, *Pasteuria* and *Pseudomonas* have, for example, shown great potential for the biological control of plant-parasitic nematodes (Emmert and Handelsman, 1999; Siddiqui and Mahmood, 1999; Meyer, 2003).

Bacteria are the most abundant micro-organisms in soil (Grubinger, 2004), illustrating their potential to act against nematode pests in reducing their population densities. The SBM product evaluated during this study for its adverse effects against *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949 is a mixture that, amongst others, contains numerous soil bacteria, with some of them exhibiting anti-nematodal properties. Although the mechanism responsible for adversely affecting root-knot nematode biology and physiology is unknown, it has been reported that some of the bacteria, also those present in SBM, produce toxins/secondary metabolites that interfere with nematode–plant-host recognition, compete for nutrients also needed by nematodes, induce systemic resistance in root-knot nematode infected plants and ultimately promote plant health (Siddiqui and Mahmood, 1999; Tian *et al.*, 2006, Yang *et al.*, 2007). Such bacteria can contribute towards sustainable crop production in southern Africa in both commercial and subsistence farming systems (Baiphethi and Jacobs, 2009). The latter farming systems are characterised by practising of multiple cropping in relatively small fields and a need exists for natural, safe and cost-effective anti-nematodal agents that

could be used by such producers (Hussey, 1990). Conversely, the cost of applying nematicides in commercial crops is expensive and their efficacy inconsistent, mainly due to local environmental conditions (Riekert 1996a and b; Mc Donald *et al.*, 2017). The use of SBM as such an eco-friendly product can hence benefit local producers (commercial and subsistence), but except for the data reported in this study (see Chapter 3), no research has been done regarding its effect on root-knot nematodes. Therefore, the aims of this study were to determine i) the effects of SBM on the motility and ii) reproduction of *M. incognita* in laboratory (*in vitro*) and glasshouse and tunnel (*in vivo*) experiments, and ii) identify the bacterial groups present in SBM treated soil.

4.2 Materials and methods

4.2.1 *Meloidogyne incognita* population used

Meloidogyne incognita eggs and J2 were extracted from roots of infected tomato plants using Riekert's (1995) modified NaOCl-method as described in Chapter 3, paragraph 3.2.1. The same population of this species used for determining the effect of SBM on the J2 oxygen consumption (see Chapter 3; paragraph 3.2.1.), was used for the purpose of this study. To hatch J2, eggs that were extracted were placed on a 25- μ m-mesh sieve which in turn was submerged into a container filled with tap water (approximately 5 cm deep). This container with eggs was incubated in a temperature-regulated chamber at 26 °C for 48 h. The J2 that hatched during the first 24 h were discarded and only those that hatched during the next 24 h were used to ensure optimal viability of J2.

4.2.2 *In vitro* laboratory experiment

Treatments for J2 motility assays consisted of SBM solution (100 %) being diluted to represent four different concentrations, viz. 2.5, 3, 4 and 5 % using sterile tap water. A sterile tap-water control was also included. These treatments were non-filtered and are further referred to as NF SBM. However, 50 ml of each concentration was removed and filtered through a 0.20 μ m syringe filter (GVS Altea Technology single use syringe) to produce a second set of treatments and are referred to as F SBM. The F SBM product concentrations were hence expected to contain no micro-organisms (including bacteria), but did contain the secondary metabolites of the micro-organisms that were removed by the filtering proses.

Subsequently the hatched J2 were collected in sterile tap water in a glass flask, placed on a magnetic stirrer and kept in suspension to determine the population density. Eight aliquots of 10 µl of the J2 suspension were collected with a glass pipette and placed in a De Grisse counting dish (De Grisse, 1963). The number of J2 was determined for each of the six aliquots using a stereo microscope (100× magnification). The mean number of J2/10 µl water was calculated and the number of J2 extrapolated to finally obtain 150 J2/20 µl water. Subsequently 20 µl of the J2 suspension was pipetted into each well of a plastic container to obtain 150 J2/well. The next step entailed that 3 ml of each of the SBM treatments and the tap-water control was added to the 150 J2 in each well. Each treatment had six replicates that were arranged in a randomised complete block design (RCBD). This same treatment setup was duplicated for the F SBM product concentrations and its tap-water control. The well plates were covered with cling plastic and incubated at 26 °C and J2 motility data recorded 3, 6 and 24 h after onset of the experiment. Six replicates were included for each treatment the data subjected to Repeated Measures ANOVA using Statistica for Windows, Version 12 (<http://www.statsoft.com>).

4.2.3 *In vivo* glasshouse experiment

After the completion of the *in vitro* experiment (see Paragraph 4.2.2.), the experiment was extended to an *in vivo* glasshouse experiment. The *M. incognita* J2 suspended in the wells for the *in vitro* experiment were removed from the various product treatments (both F SBM and NF SBM) and the tap-water controls they were suspended in for the 24 h period and inoculated directly onto the roots of susceptible tomato seedlings (cv. Floradade). These seedlings were planted in soil treated with Telone® II @ 150 l/ha (active substance 1,3 dichloropropene, 1.110 g/l). After 30 days the root system of each tomato plant was removed, washed free of soil using tap water and weighed. The number of root-knot nematode egg masses per root system was subsequently counted by staining the root systems with a 0.1% Phloxin B solution for 20 min. The pink-stained egg masses were then counted using a commercial magnifying glass and a light source. The number of egg masses represents the number of egg-laying females (E.L.F.) per root system. The egg-laying female indice (E.L.F.) were rated on a scale from 0-5, where 0=no egg masses, 1=1 to 2 egg masses, 2=3 to 10 egg masses, 3=11 to 30 egg masses, 4=31 to 100 egg masses and 5=more than 100 egg masses per root systems (Hussey and

Boerma, 1981). After counting of the egg masses, eggs and J2 were extracted from each root system, using the adapted NaOCl-method of Riekert (1995). Eggs and J2 were counted and Oostenbrink's reproduction factors (Rf) calculated using the following equation by Windham and Williams (1988): $Rf = \text{final population density (Pf)} / \text{initial population density (Pi)}$. Nematode data were subjected to a Factorial ANOVA (Statistica for Windows, Version 12), with treatments as the main factor. Means were separated by the Tukey (HSD) Test at $P \leq 0.05$.

4.2.4 *In vivo* tunnel experiments

Two experiments were conducted during the 2013/14 and 2014/15 seasons, respectively, to evaluate the effect of a SBM under natural environmental conditions in a tunnel (Fig. 4.1.). Garden soil (6 % clay, 4 % silt, 90 % sand 0.3 % organic material and pH (H₂O) of 6.55) was purchased from a local supplier (Sandman) and fumigated with Telone II as described in Paragraph 4.2.3.

4.2.4.1 First experiment

Maize stover/hay (stalks and leaves; obtained from a local farmer Mr Hendrik Riekert) was pasteurised for 72 h at 60 °C. Soil and hay were mixed at a 1:1 v/v rate and added to pots (20-l capacity) (Fig. 4.1.) to which SBM was added to the designated treatments (see below). This was done using a wheelbarrow and spade, which were rinsed with a 4 % NaOCl solution after every treatment substrates were mixed, followed with tap water. This was done to prevent cross contamination between treatments with and without SBM.

The SBM and hay treatments were applied ca. three months before planting of maize. A freshly prepared stock SBM product sample was obtained of which a 2.5 % concentration was prepared using sterile tap water. The trial consisted of six treatments in a RBCD of which each treatment was replicated eight times.

The different treatments were:

- i) 2.5 % SBM + pasteurised hay
- ii) Pasteurised hay only (without SBM)
- iii) 2.5 % SBM + unpasteurised hay
- iv) Unpasteurised hay only (without SBM)
- v) 2.5 % SBM only (withouth hay)
- vi) Untreated control (no SBM and no hay).



Fig. 4.1. Pots containing garden soil mixed with hay as well as soil only (control) to which SoilBioMutl was added to evaluate its efficacy towards *Meloidogyne incognita* reproduction in a tunnel experiment at Potchefstroom during the 2013/14 growing season.

Approximately 10 000 eggs and J2, from the same *M. incognita* population used in the OCR (see Chapter 3) and J2 motility experiments (see Paragraph 4.2.2) were inoculated to the top 15 cm soil layer of each pot after SBM and hay treatments were applied. This procedure allowed settling of the nematodes in the soil substrate and soil was kept moist to allow optimal conditions for survival of the nematodes. The pots were covered with a black plastic sheet for the three months ‘incubation period’ of the SBM and hay as advised by Mr Snyman. Two seeds each of a ‘Farmer-bred’ maize variety (undisclosed identity) and a popular commercial cultivar (DKC 80-10RR) were then planted in each pot. No fertiliser, synthetically-derived or organic-based, was added to the soil in the pots. All pots, treated and non-treated were watered weekly by filling the trays with tap water.

The experiment was terminated 74 days after planting, during which the aerial parts of the maize plants were excised from the roots and weighed. After removing the roots of these plants from each pot, the roots were rinsed with tap water to remove excess soil and debris and weighed. Root samples were then subjected to the adapted NaOCl extraction protocol for extraction of root-knot nematodes (Riekert, 1995). Concurrently, rhizosphere soil samples were taken from each pot and subjected to the decanting and sieving-, followed by the sugar-flotation methods (Hooper *et al.*, 2005). The temperature in the tunnel was measured for the duration of the experiments since the planting of the two genotypes, using an iButton® DataLogger (Anonymous, 2016a) and ranged between 17.7 ± 1.54 and 35.8 ± 7.8 °C.

4.2.4.2 Second experiment (2014/15 season)

The same procedures were generally followed as described for the 2013/14 experiment, except for the following adaptations that were made:

- i) a 5 % SBM was used instead of a 2.5 % dosage rate and treatments changed as indicated below,
- ii) hay was provided by the producer of the product Mr Snyman[†] from Rustenburg, North-West Province and was not pasteurised. Mixing of the soil and hay was done by hand using a shovel and wheelbarrow adding the same volume of fumigated soil to the same volume of hay (1:1 v/v rate),
- iii) only one maize genotype, cv. DKC 80-10RR, was used,
- iv) a 'counterpart' product' Wonder™, commercially available by owner company Efekto Care (Pty) Ltd (Registration No. 2009/006357/07) was included to enable comparison with SBM,
- v) 100 g of dried chicken manure was added to all pots (as advised by Mr Snyman[†]),
- vi) soil samples were obtained for DNA and NGS analyses to determine the bacteria present in the soil. Three subsamples per treatment were prepared from the six replicates due to the high cost of NGS analyses and
- vii) the experiment was terminated 60 days after planting.

The 'counterpart' product Wonder™ is a natural microbial-based product that 'enriches soil naturally, creates nutrient-rich humus, is non-toxic, biodegradable' and act as a compost activator to enhance the natural decomposition of garden waste.

The microbes present in this product are, however, not available on the label (Anonymous, 2016b).

Treatments for the second experiment were as follows:

- i) Untreated control
- ii) 5 % SBM (without hay)
- iii) 5 % SBM + hay
- iv) Wonder™ (without hay)
- v) Wonder™ + hay

Mixing of hay and soil was done as described for the first experiment (see Paragraph 4.2.4.1). After the pots were filled with the different soil and/or soil:hay substrates, the pots were left in the tunnel for a month. This was done to allow the organisms contained in the SBM to multiply and break down the organic soil amendment that was represented by the hay and chicken manure. During that month the pots were watered to field capacity once a week using an over head sprinkler irrigation system. This kept the soil and/or soil:hay substrates moist, but not saturated. After this incubation period, 2 500 eggs and J2 of *M. incognita* were inoculated in two clearly marked 4-cm deep holes that were made in the soil at opposite's sides of each pot. Three days after nematode inoculation, two seeds of the DKC 80-10 maize genotype were planted in the same 4-cm deep holes where nematodes were inoculated. Ultimately, each pot received 5 000 eggs and J2. After planting and inoculation, the pots were watered twice weekly for the duration of the trial period.

The temperature in the tunnel was measured since planting of the maize cultivar, using an iButton® DataLogger (Anonymous, 2016a) and ranged between 15.1 and 35.7 °C. Trial layout for the experiment was a RCBD, with six replicates included for each treatment. Nematode and plant data were subjected to Factorial ANOVAs (Statistica for Windows, Version 12), with treatments as the main factor. Means were separated by the Tukey (HSD) Test at $P \leq 0.05$.

4.2.5 Molecular identification of bacterial species present in soil samples

The same methods were used to determine which bacterial genera were present in the soil of each treatment as described for DNA isolation for Next Generation Sequencing (NGS) in Chapter 2.

4.3 Results

4.3.1 *In vitro* laboratory experiments

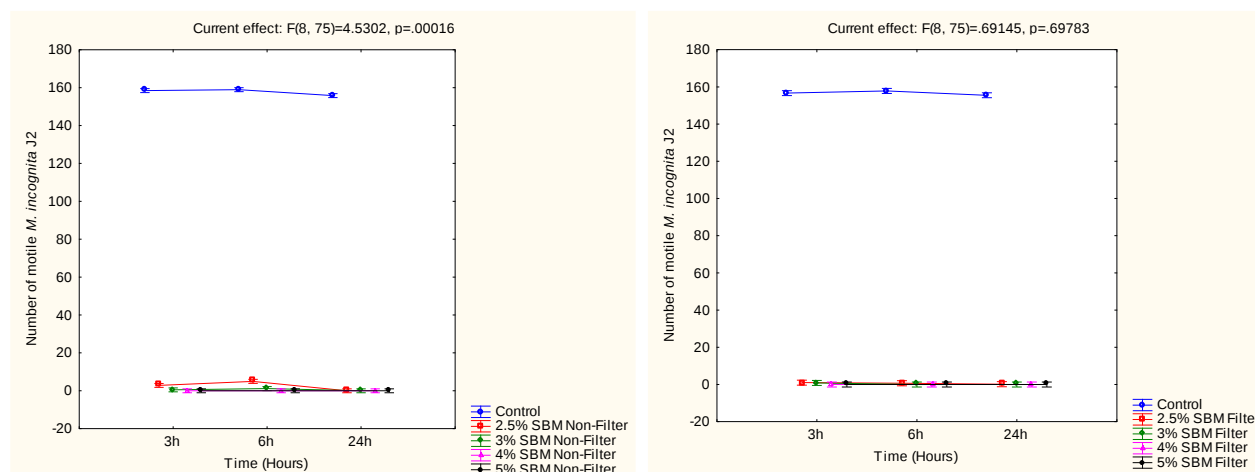


Fig. 4.2. Data of *in vitro* experiments that were done to determine the effect of different concentrations of non-filtered SBM (NF SBM) (A) and filtered (F) SoilBioMuti (SBM) (B) on the motility of *Meloidogyne incognita* second-stage juveniles (J2) over a 24 hour period.

For both experiments a significant difference ($P \leq 0.05$) was recorded between the SBM treatments and the sterile tap-water controls (Fig. 4.2.A and 4.2.B). Nearly 100 % of the J2 suspended in the tap-water controls of both experiments remained motile throughout the 24 h experimental period. No significant differences existed among the various F SBM treatment concentrations (3, 6 and 24 h) of which less than 10 % were motile. However, for the 2.5 % NF SBM treatment a significantly ($P \leq 0.05$) higher (but still below 10 %) number of J2 was motile at 6 h after onset of the experiment compared to the other NF SBM treatments (Fig. 4.2.A).

4.3.2 *In vivo* glasshouse experiment

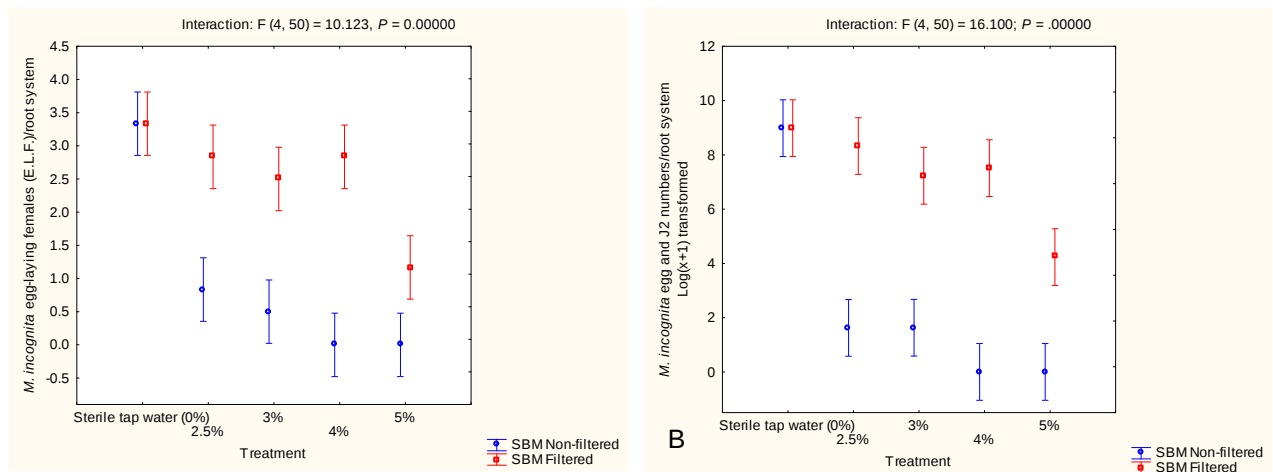


Fig. 4.3. Egg-laying female (E.L.F.) data (A) and the number of egg and second-stage juveniles (J2) of *Meloidogyne incognita* per root system of tomato plants of the susceptible cultivar Rodade 30 days after inoculation in an *in vivo*, glasshouse experiments.

Significant interactions existed for *M. incognita* concerning E.L.F. indice data ($P \leq 0.001$; F-ratio = 10.1) (Fig. 4.3.A) and final population (Pf) densities per root system ($P \leq 0.004$; F-ratio = 1.65) (Fig. 4.3.B) as obtained for the different SBM concentrations at termination of the glasshouse experiments. This indicates that the F SBM concentrations reacted differently from the NF ones for this root-knot nematode species for both parameters.

The sterile tap water control (0 % SBM) resulted in the highest E.L.F. index values (Fig. 4.3.A) and eggs and J2/root system (Fig. 4.3.B), being significantly ($P \leq 0.005$) higher than that of all the NF SBM treatments. However, for the F SBM treatments, the sterile tap-water control did differ significantly from the 5 % concentration for both E.L.F. and egg and J2 numbers/root system. Although the 5 % SBM concentration (F and NF) had the lowest E.L.F. index values and egg and J2 numbers/root system it did not differ significantly from the other SBM concentrations.

4.3.3 *In vivo* tunnel experiments

4.3.3.1 First experiment

4.3.3.1.1 Nematode data

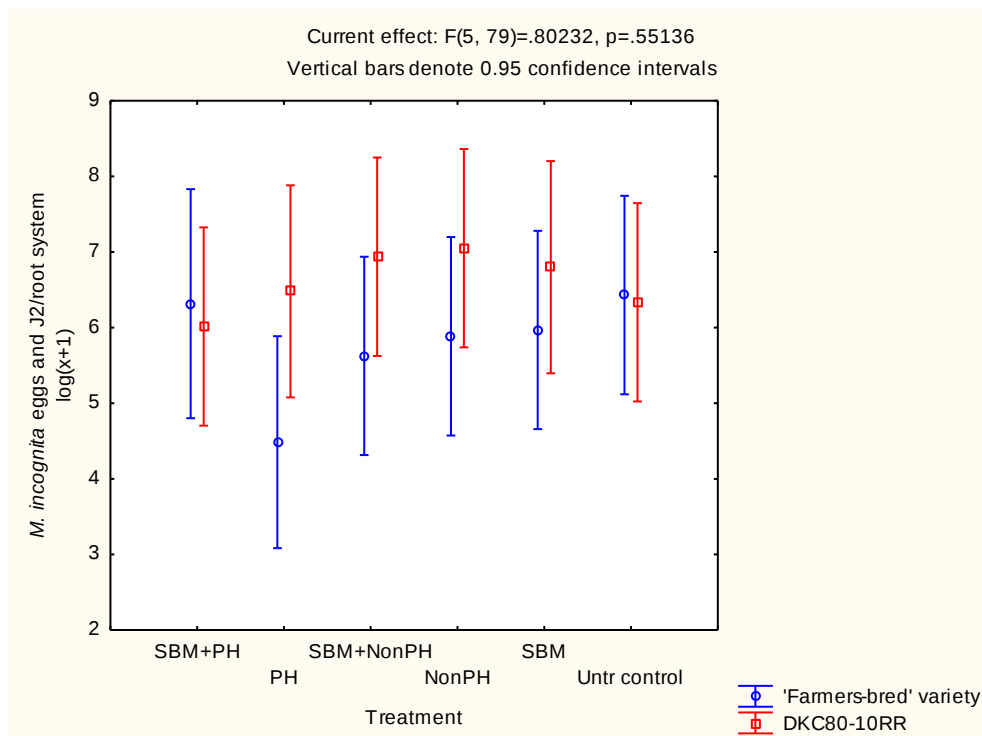


Fig. 4.4. Log transformed data of final population densities of *Meloidogyne incognita* in maize roots 74 days after planting of two maize genotypes in soil to which SoilBioMuti and other treatments were applied in a tunnel experiment during the 2013/14 season (PH: Pasteurised hay; SBM: Soil Bio-Muti; NPH: Non-pasteurised hay; Untr control = Untreated control).

No interactions existed between treatments and maize genotypes for *M. incognita*, indicating that both genotypes reacted similarly to the treatments. Also, no significant differences existed among the treatments for the Pf per root system for either genotype (Fig. 4.4.).

The pasteurised hay (PH) and non-pasteurised hay (NPH) treatments did not differ in terms of the nematode-parameter data recorded.

4.3.3.1.2 Plant data

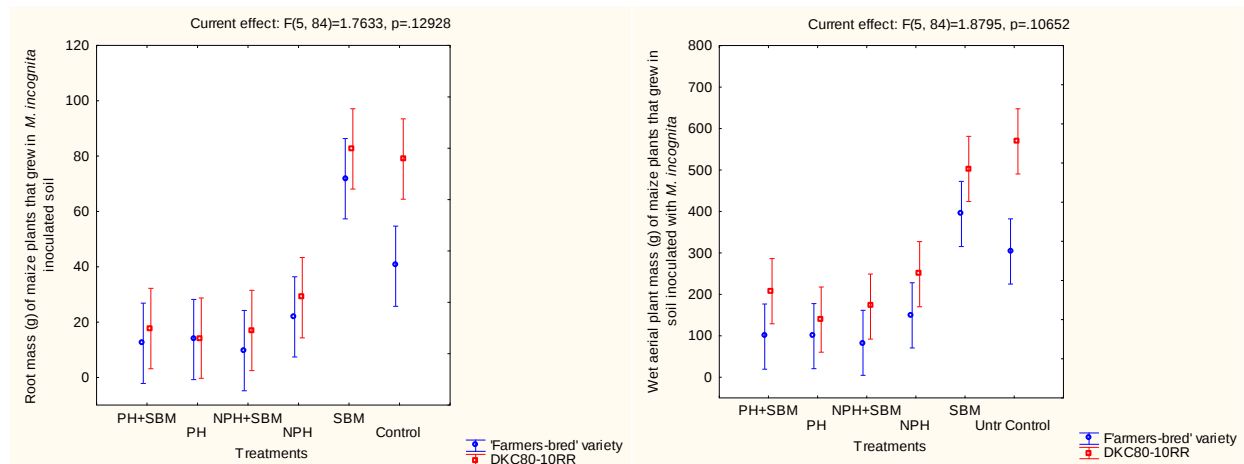


Fig. 4.5. Wet root (A) and aerial (B) plant mass (g) of maize plants of two maize genotypes 60 days after planting in soil inoculated with *Meloidogyne incognita* to which SoilBioMuti and other treatments were applied in a tunnel experiment during the 2013/14 growing season (PH: Pasteurised hay; SBM: Soil Bio-Muti; NPH: Non-pasteurised hay).

No significant interactions existed with regard to root and aerial plant mass for treatments x genotype for *M. incognita* (Fig. 4.5.A and 4.5.B). This implies that the genotypes generally reacted similarly to the treatments, although it was different (but not significantly different) for the untreated controls. Significant ($P \leq 0.05$) differences existed among the treatments for the root mass of maize plants of cv. DKC 80-10RR as well as those for the 'Farmers-bred' variety that were grown in pots inoculated with *M. incognita* (Fig. 4.5.A). For both genotypes, root systems of plants that grew in soil to which only SBM was added, had significantly ($P \leq 0.05$) higher root masses than those to which hay (P and NP) were added. Interestingly, the mass of root systems of all maize plants that grew in soils to which hay were added were significantly ($P \leq 0.05$) lower than those without hay (including the untreated control). Although the root systems of both genotypes were generally similar in mass for both the SBM and hay combined treatments, the root mass of cv. DKC 80-10RR plants were significant ($P \leq 0.05$) higher for the untreated control compared to that of the 'Farmers-bred' variety.

Significant ($P \leq 0.05$) differences existed among the treatments with regard to the wet aerial mass of maize plants of the two genotypes that were inoculated with *M.*

incognita (Figs. 4.5.A and 4.5.B). Plant masses of plants from both genotypes that grew in soil to which only SBM was added, were significantly ($P \leq 0.05$) higher than those to which hay were added, including the untreated control.

The pasteurised hay (PH) and non-pasteurised hay (NPH) treatments did not differ in terms of the plant-parameter data recorded.

4.3.3.2 Second experiment

4.3.3.2.1 Nematode data

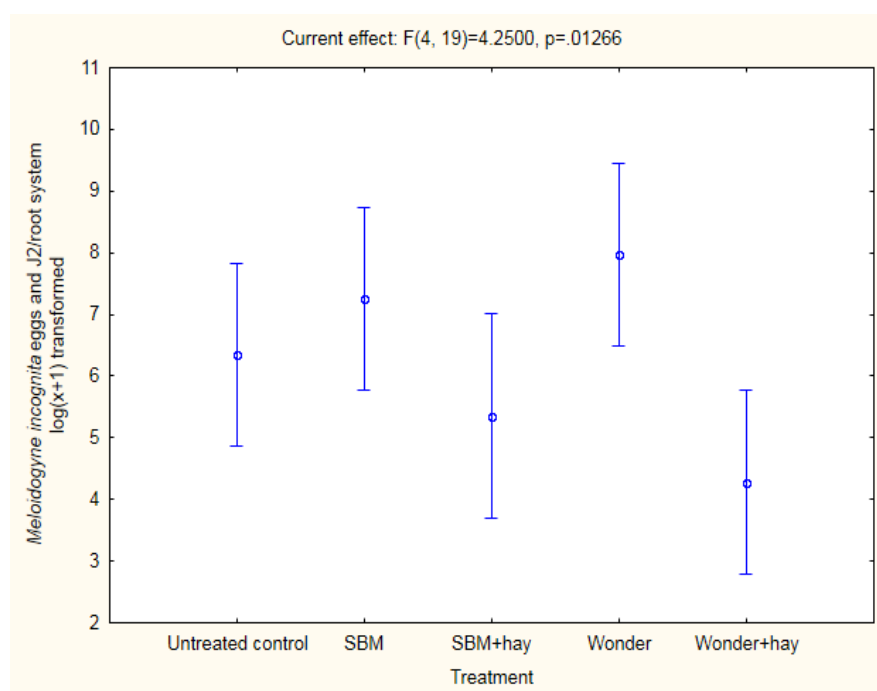


Fig. 4.6. Log transformed data of final population densities (Pf) of *Meloidogyne incognita* in roots 60 days after planting of the 'Farmers-bred' maize variety in soil to which SoilBioMuti (SBM) and other treatments were applied in a tunnel experiment during the 2014/15 season.

No significant differences were evident among the different SBM treatments evaluated against *M. incognita* (Fig. 4.6.). Substantial variation, however, existed in the data of the six replicates of each treatment. The SBM and Wonder™ treatments to which hay were added had the lowest egg and J2 numbers per root system. These treatments, for example, resulted in 83 (Wonder™ + hay) and 77 % (SBM + hay) reductions in egg and J2 population densities of *M. incognita* compared to the untreated control. Both the Wonder™ and SBM treatments maintained the highest egg and J2 population densities/root system.

4.3.3.2.2 Plant data

Table 4.1. Plant data for the maize genotype ‘Farmer-bred’ variety 60 days after planting in soil inoculated with *Meloidogyne incognita* and to which SoilBioMuti and other treatments were applied in a tunnel experiment during the 2014/15 growing season.

Treatment	Aerial plant mass (g)	Root mass (g)	Aerial plant length (cm)
1. Untreated control	245 ¹	16 ¹	171 ¹
2. SBM	249	17	159
3. SBM + hay	293	17	179
4. Wonder™	258	22	169
5. Wonder™ + hay	291	16	165

¹Means in each column did not differ significantly from each other according to the Tukey HSD Test ($P \leq 0.05$).

No significant differences were evident among the treatments for the three plant parameters (aerial plant mass, root mass and plant length) determined for plants (Table 4.1.).

4.3.4 Molecular identification of bacterial species present in soil samples

Numerous microbial groups have been identified from soil samples of treatments from the second tunnel experiment as is displayed in Fig. 4.7. The bars indicate the diversity of microbes identified from soils at termination of the experiment (60 days after *M. incognita* inoculation). However, due to the vast number of microbe groups identified, interpretation of the graph at a glance is not only challenging but also overwhelming. Hovering with the mouse of a lap- or desktop on each specific coloured bar displayed for each treatment in the graph, however, results in the identity of the microbe group represented by it being displayed.

The extensive body of data that have been generated for this specific activity is hence rather represented as a summary of the most abundant microbial groups that dominated in terms of the number of hits recorded during the NGS data analyses on the MG-RAST server for the soil samples from each treatment. Focus was therefore placed on the larger groups rather than individual species and the most abundant

microbial groups indicated in Fig. 4.7. hence summarised in Table 4.2. From this table it is apparent that the microbial community profile of SBM-treated soils appeared similar to that of the untreated control, which is also apparent when looking at these two treatments presented in Fig. 4.7. Although some minor changes occurred in microbial group constitution of SBM-treated compared to untreated control soils, additional work will be required for confirmation. Interestingly, the addition of hay to the soil has led to the introduction of more β -proteobacteria and γ -proteobacteria in both the SBM and WonderTM treatments. Also, the abundance of microbial groups in the latter treatments was substantially higher compared to their counterpart treatments without hay. The WonderTM treatment, however, reduced the incidence of α -proteobacteria and the Bacilli group.

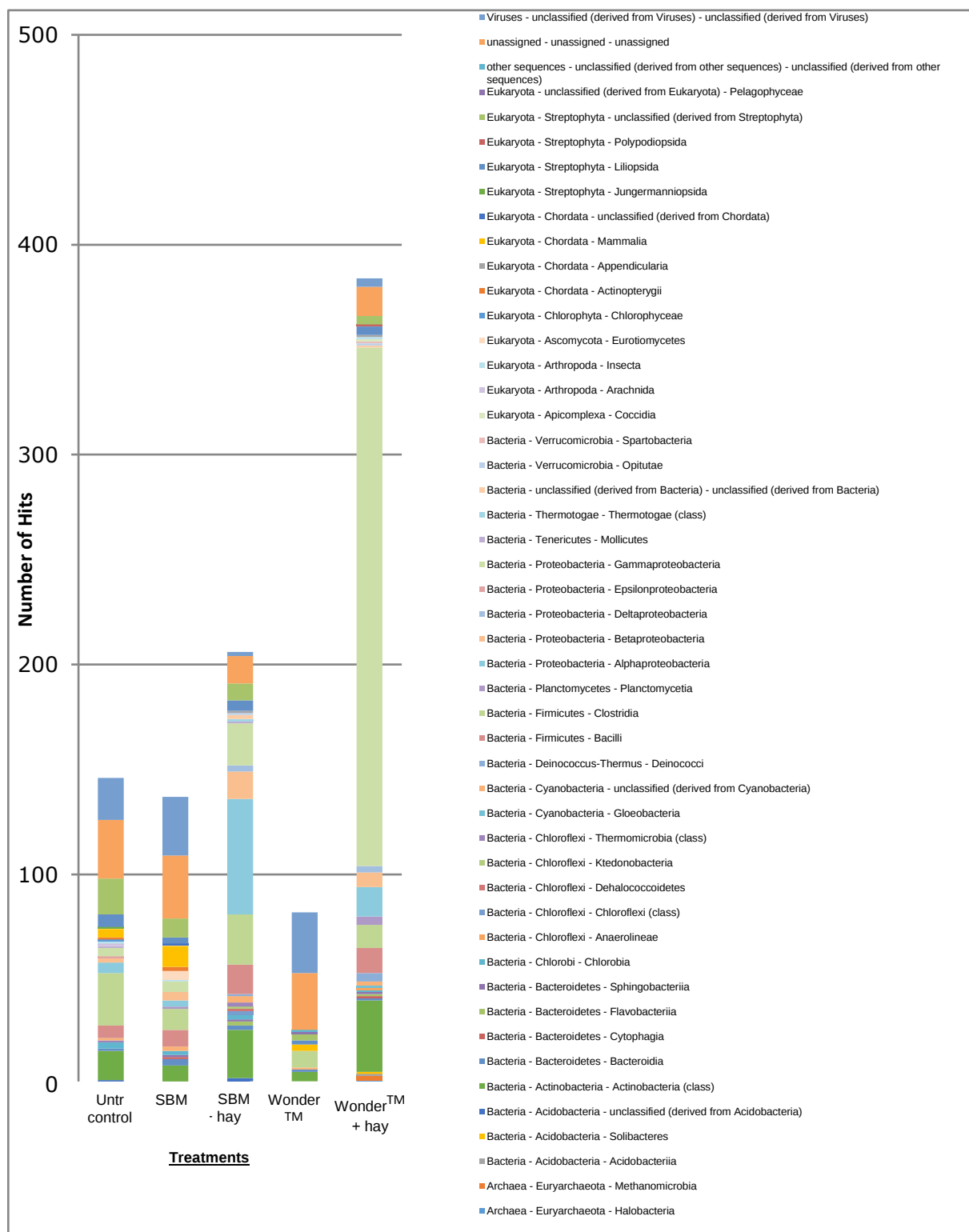


Fig. 4.7. The presence of microbes (bacteria and viruses) in soil 60 days after inoculation with *Meloidogyne incognita* to which SoilBioMuti (SBM) and WonderTM were applied for its evaluation against this nematode pest in a tunnel experiment during the 2014/15 growing season.

Table 4.2. A concise summary of abundant microbes present (indicated with a ■) in soil 60 days after inoculation with *Meloidogyne incognita* and to which SoilBioMuti (SBM), Wonder™ and hay were added to study their effects on the nematode pest in a tunnel experiment during the 2014/15 growing season.

Treatment	Microbes						
	Unassigned	Bacteria					
	Bacteria & virusses	Firmicutus			Proteobacteria		
		Actinobacteria	Bacilli	Clostridia	Alphaproteobacteria	Betaproteobacteria	Gammaproteobacteria
Untreated control	■ (dominated)	■	■	■	■		
SBM	■ (dominated)	■	■	■	■		
SBM + hay		■		■	■ (dominated)	■	■
Wonder™	■ (dominated)	■		■			
Wonder™ + hay		■	■		■	■	■ (dominated)

4.4 Discussion

The anti-nematodal characteristics of SBM determined during *in vitro* laboratory studies showed superior efficacy in reducing the motility of infective J2 of *M. incognita*. Various studies have demonstrated paralyse of J2 of this and other *M. incognita* after suspension in products containing bacteria (Nordmeyer and Dickson, 1989; Mendoza *et al.*, 2008; Meyer *et al.*, 2002; Terefe *et al.*, 2009; Jansen-Girgan *et al.*, 2016). This specific assay is hence an accepted and valuable tool to determine whether products with anti-nematodal characteristics (biologically or synthetically-derived) can disrupt the behavior of infective J2 stages of *Meloidogyne* spp. by means of paralyzing or killing them.

Although the F and NF product concentrations of SBM significantly reduced J2 motility in this study, it is suggested that the mode of action of microbes against root-knot nematodes are the toxins/antibiotics present in the secondary metabolites excreted by such organisms and not direct parasitism of the J2 by the micro-organism itself (Lamovšek *et al.*, 2013). Furthermore, reproduction of *M. incognita* in roots of the susceptible tomato (cv. Floradade) following suspension of J2 in SBM showed that the active substances in the product do not kill or permanently paralyse the infective stages of this root-knot nematode species. Meyer *et al.* (2002) reported a reduction in J2 hatching and mobility when *Burkholderia cepacia* was for example tested for its anti-nematodal effect.

Another positive outcome of this study was the substantial decrease in the reproduction potential of *M. incognita* as a result of submerging the infective J2 in SBM for 24 h, before inoculating them on roots of tomato cv. Floradade in a glasshouse study. Results of this study varied regarding the success of SBM to reduce *M. incognita* levels. Such inconsistencies in results were also found by Medeiros *et al.* (2009) who used endophytic-bacteria for controlling *M. incognita* on melons. This may probably be due to inherent genetic variability among J2 used, with some of them exhibiting enough energy to penetrate roots of the host plants and others not.

Data generated from tunnel experiments under prevailing environmental conditions were challenging in terms of conclusively demonstrating the anti-nematodal effects

exhibited by SBM. This was mainly due to the substantial variation of data recorded within replicates of the respective treatments and can be an indication of the limited knowledge available on interactions between SBM and other microbes, soil physical and chemical parameters (and others which are not known at this stage). Results from the first tunnel experiment with the 2.5 % SBM, showing stunting and yellowing of maize leaves, can however be ascribed to the lack of an organic fertiliser (in this case chicken manure that is recommended for use during application of SBM). Although results from the second tunnel experiment, with 5 % SBM, yielded substantial reductions in root-knot nematode population densities, considerable variation within the replicates of each respective treatment existed. For example, a 77 % reduction in *M. incognita* population densities was obtained for the SBM + hay treatment compared to the untreated control. Although this indicates superior efficacy of SBM in reducing *M. incognita* J2 in the soil substrate under natural occurring conditions, it needs to be verified in field experiments too. However, the substantial reduction in *M. incognita* reproduction is in agreement with results by Seo *et al.* (2012) who reported 52.1 and 38.7% reductions in *M. incognita* reproduction in roots of Oriental melons (*Cucumis melo* L. cv. Geumssaragi-euncheon,) due to drenching soil in pots to which seedlings were planted with 2x and 1x dosage mixtures of *Lactobacillus farraginis*, *Bacillus cereus*, and *Bacillus thuringiensis* strains. An interesting outcome of this tunnel experiment is, however, that the 'counterpart' product Wonder™ + hay reduced *M. incognita* population densities to an even higher extent (83 %) than the SBM + hay and suggested that the use of Wonder™ can contribute towards reducing *M. incognita* population densities although it is not registered for this purpose. Also, the owner company and producers are most probably not aware of this phenomenon.

Furthermore, results from the second greenhouse experiment suggest that the addition of hay contributed towards the reduction in population densities of *M. incognita*, which was in contrast for the SBM treatments without hay. In fact, the latter treatments resulted in similar or higher population densities of *M. incognita* compared to the untreated control. It is known that the addition of organic matter in the form of plant material, which in this study was represented by hay, generally has a detrimental effect on *Meloidogyne* spp. population densities where maize is grown (Odeyemi *et al.*, 2011; Olabiyi, 2013). Interesting also was that both SBM and

Wonder™ treatments to which hay were added also showed the highest abundance of microbial communities according to molecular, NGS analyses. Whether the dominating alphaproteobacteria (SBM + hay) and gammaproteobacteria (Wonder™ + hay) contributed towards reducing root-knot nematode species population densities in the second tunnel experiment can, however, not be confirmed at this stage. Identification of bacterial species in soil samples to which SBM were added at the beginning of the tunnel experiment hence did not yield conclusive results that beneficial bacteria were able to establish themselves and proliferate in such soils. Although the microbes present in Wonder™ is not specified on the label, its general attributes as specified generally related to that of SBM, being also biodegradable, non-toxic and hence environmentally friendly, cost-effective and enriches the quality of soils (Anonymous, 2016b). Therefore, the product was used in this study in an attempt to compare the effect of SBM to a 'counterpart' product.

Only the Bacilli group, initially also identified from the fresh stock SBM samples, was identified from such soil samples. It is a prerequisite that biological agents such as bacteria/fungi used for their adverse effects on pest organisms are able to establish themselves in soils and proliferate to optimise their anti-pest activity (Kumar and Gopal, 2015). Interesting, however, was that the microbial complex in the untreated soil and SBM-treated soil were quite similar. It is important to bear in mind, such as in this case, that fumigation of soil with synthetical fumigants (in this case Telone II®) does not kill all microbes (James, 1989). The presence of microbes in fumigated soil in this study can thus partially explain this phenomenon. Also, the addition of hay to SBM and Wonder™ treatments to the soil in the second tunnel experiment resulted in higher numbers and variety of microbial groups identified compared to the untreated control and SBM treatments. No explanation can, however, be given for this phenomenon at this stage. Due to the fact that the hay was not sterilised before use, it could be an addition of organisms that were present on hay before hand.

4.5 Conclusions

Data generated during this study not only represent important information about the anti-nematodal effect of SBM against *M. incognita*, but also revealed that the community structures of microbes in SBM- and Wonder™-treated soil did not really differ. Information obtained during this study should be complemented by further

investigations in on-farm field sites to obtain more conclusive results regarding the use of SBM as an eco-friendly, potential alternative with anti-nematodal characteristics at commercial level.

4.6. References

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Chapter 5: Conclusions and the way forward

The dominance of the root-knot nematode species *Meloidogyne incognita* (Kofoed & White, 1919) Chitwood, 1949 in local maize production areas result in considerable damage to such crops, especially in sandy soils (Riekert, 1996; Riekert and Henshaw, 1998). Due to the progressive withdrawal of various synthetic nematicides from markets (Nordmeyer and Dickson, 1989; Ferraz and de Freitas 2004; Meyer *et al.*, 2002; Mendoza *et al.*, 2008; Terefe *et al.*, 2009; Jansen-Girgan *et al.*, 2016; Haydock *et al.*, 2013), exploitation of biological products with anti-nematodal characteristics became crucial. Crude extracts of a novel, soil-derived biological product SoilBioMuti (SBM), containing a variety of micro-organisms have been evaluated for its potential to alleviate *M. incognita* damage experienced by local maize producers.

Identification of the bacterial genera contained in SoilBioMuti using traditional plating (with two agar media; nutrient and MRS) as well as advanced molecular (Next Sequence Generation; NGS) techniques rendered interesting and novel information. Significantly higher bacterial counts ($\pm 99\%$) in the fresh SBM compared to those in the 'cooled' SBM found during this study have also been reported in literature (Dongyai *et al.*, 2010). Furthermore, it is known that the use of MRS agar promotes the cultivation and proliferation of lactic-acid producing bacteria (Anonymous, 2016) as has been found in freshly-prepared SBM samples. NGS results revealed the presence of 45 bacterial genera contained within the three freshly prepared stock SBM samples tested. These constituted non-pathogenic/beneficial ($\pm 50\%$), plant-pathogenic, human pathogens and miscellaneous bacterial genera for which limited/dubious knowledge is available. Beneficial bacterial genera represented several genera that are known for their anti-nematodal effects and included *Acetobacter*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Bradyrhizobium*, *Burkholderia*, *Frankia*, *Glutacetobacter*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Microbispora*, *Micromonospora*, *Paenibacillus*, *Pediococcus*, *Phyllobacterium*, *Rhodococcus* (can also act as animal pathogens), *Streptosporangium*, *Streptomyces* and *Weissella*. However, the presence of animal-, human- and plant-pathogenic genera is concerning. Despite novel information being generated on the constitution of SBM, the active substances that can be responsible

for adverse effects on *M. incognita* biology (see paragraphs that follow) has not been concluded on and needs to be elucidated.

Novel knowledge on the adverse effect of 2.5 % SBM treatments regarding the oxygen consumption of *M. incognita* J2 has also been generated. According to data obtained, 100 J2 were optimal to use for determining the oxygen consumption rates (OCR) of these pests when suspended in SBM. The OCR recorded for non-filtered NF SBM containing J2 of 42% compared to its counterpart treatment without J2 is also an insight to this field of Nematology. However, the OCR of J2 suspended in sterile tap water did not differ significantly from that of the NF SBM containing J2, which was expected since the latter product contained living, respiring microbes. Interesting was that the two F SBM treatments had the lowest OCR and differed significantly from that of the NF SBM treatments and the tap-water control containing J2. According to the author of this dissertation no information about the effect of micro-organisms on the OCR of *Meloidogyne* or other nematode pest species is available, except for that by Nordmeyer and Dickson, 1989) who demonstrated a significant OCR reduction for *M. incognita* J2 when exposed to avermectin B2A with the active substance being secondary metabolites of the soil bacterium *Streptomyces avermitilis* (Coyne *et al.*, 2009) to be 61.3 %. This was significantly higher than OCR reductions of this species' J2 compared to synthetically-derived nematicides with aldicarb and carbofuran as active substances, but not ethoprop, fenamiphos and oxamyl. Results from the SBM study hence showed that secondary metabolites of micro-organisms isolated from the isolated NF product were most probably responsible for suppressing J2 OCR. This is in agreement with results of various studies that advocate the mode of action of bacteria and other microbes against *Meloidogyne* spp. being expected to be the toxins present in secondary metabolites of such micro-organisms and not their direct parasitism of these nematode pests (Padgham and Sikora, 2007; Mendoza *et al.*, 2008; Lamovšek *et al.*, 2013). Ultimately, the NF SBM stock solution, unofficially-used for its anti-nematodal characteristics (amongst others) proved to be effective in substantially reducing the OCR of *M. incognita* J2 during this study, and indicates the potential of this product to adversely impact on the physiology of this root-knot nematode species.

In vitro evaluations on the effect of different SBM product concentrations (2.5, 3, 4 and 5 %) on J2 motility and *in vivo* testing of the reproduction abilities of *M. incognita* also produced novel information. For motility assays, all SBM concentrations for all SBM treatments (F and NF) significantly reduced J2 motility (below 10 %) compared to the tap-water controls in which nearly 100 % of the J2 remained motile throughout the 24 h experimental period. This was also reported by various authors for *M. incognita* and other *Meloidogyne* spp. when microbes or microbe-containing products were evaluated for their anti-nematodal effects (Meyer *et al.*, 2002; Seo *et al.*, 2012). For reproduction assays, greenhouse data showed that all NF SBM product concentrations (used for the motility experiment) reduced the egg-laying female indices significantly compared to that recorded for the tap-water control. This represent valuable information of the ability of J2 to recover to a certain extent after being suspended in the SBM product concentrations for 24 h. Such data have also been recorded in other studies for *Meloidogyne* spp. (Nordmeyer and Dickson, 1989; Meyer *et al.*, 2002). Data from the first tunnel experiment yielded no conclusive data that 2.5 % SBM treatments (with and without hay) inhibited *M. incognita* reproduction, while that generated during a follow-up experiment revealed 83 and 77 % reductions in nematode reproduction in soils treated with WonderTM + hay, and 5 % SBM + hay, respectively. However, substantial variation occurred among the six replicates of these experiments, rendering the data obtained to be verified particularly under field conditions. SBM, however, had no significant effect on various plant-growth variables measured for both tunnel experiments, except for those to which hay were added that were significantly lower than their counterparts without hay. Interesting, however, is that the addition of WonderTM and hay could be exploited further for its adverse effects on *M. incognita* as was found during this study where it was used as a 'counterpart' product for SBM. Use of this product can hence benefit producers since it fits the criteria for non-toxicity and not harming the environment or animals and/or humans. However, it has not been evaluated for its adverse effects on nematodes except for during this study according to the knowledge of the author of this study. Hence, producers and most possibly the owner company do not know about its positive effect in terms of reducing *M. incognita* population densities.

In summary, although results from this study is to a large extent convincing about the abilities of SBM as an anti-nematodal alternative to reduce *M. incognita* (and most probably other *Meloidogyne* spp.) further research in this regard is questionable.

This is due to the following challenges that are foreseen to be experienced:

- i) the presence of bacterial genera that are harmful to animals and humans and that could lead to diseases when animals and humans get into contact with SBM treated soils,
- ii) the presence of plant-pathogenic bacterial genera of which certain species is known to cause crop damage when present in soils,
- iii) the substantial presence of 'unassigned/unidentifiable' microbes present in SBM due to the absence of numerous of these bacteria genera from the databases used for characterisation purposes,
- iv) proper quality control during the production process of SBM to ensure that the contents (in terms of microbial abundance and diversity) is the same for each batch,
- v) complete identification of other microbes present in SBM that may also have anti-nematodal effects, e.g. bacteria, fungi and others to elucidate the causal micro-organisms should the product be considered for registration to be used by producers.

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ANNEX 1: SoilBioMuti Material Safety Data Sheet

P.O. Box 14544,
(0)82 804 3130
Bredell,
(0)86 242 3366
1623
South Africa.
fred1@wizz.co.za

Tel : +27

Fax : +27

E-mail :

MATERIAL SAFETY DATA SHEET

TRADE NAME: Soil Bio-Muti { Grond Bio-Muti }

PRODUCT BAR CODE(S): None

1) COMPANY IDENTIFICATION

Uniglide Lubricants S.A. cc.
P. O. Box 14544
Bredell
1623
South Africa

ROUTINE INQUIRIES: (0)82 804 3130 OR 083 483 7770
FACSIMILE No.: (0)86 242 3366

EMERGENCY TELEPHONE NUMBERS: ; +27(0)82 804 3130

2) CHEMICAL DESCRIPTION: Organic Soil Micro-Organisms in
suspension in water used for rapid rehabilitation of
soils depleted of plant nutrients.

PRODUCT COMPONENTS

COMPONENTS % WEIGHT	CAS No.
Water	57
Micro-Organisms	3
Dissolved Molasses	30

3) PHYSICAL & CHEMICAL PROPERTIES

FLASH POINT (DEGREES C)	Water Based – does not oxidise
APPEARANCE	Dark Murky Brown Liquid
PH OF UNDILUTED PRODUCT	7
EVAPORATION	Evaporates releasing only water vapour

4) HAZARDS IDENTIFICATION

Non Hazardous.

5) HEALTH HAZARD DATA

EYES	NOT EXPECTED TO CAUSE EYE IRRITATION.
ORAL	IF SEVERAL MOUTHFULS ARE SWALLOWED, NOMINAL DISCOMFORT, NAUSEA AND DIARRHEA MAY OCCUR.
INHALATION	BREATHING THE VAPOUR MAY BE UNPLEASANT BUT NO ILL EFFECTS WILL BE EXPERIENCED.
SKIN	WILL NOT CAUSE ANY HARM OR IRRITATION.
LONG TERM TOXIC EFFECTS	NONE.

6) EMERGENCY FIRST-AID PROCEDURES

EYES	IRRIGATE WITH EYEWASH SOLUTION OR CLEAN WATER, HOLDING THE EYELIDS APART FOR AT LEAST 5 MINUTES.
SKIN	NO ILL EFFECTS
INGESTION	DO NOT INDUCE VOMMITING. IF A LARGE VOLUME HAS BEEN INGESTED DIARRHEA MAY OCCUR WHICH CAN BE EASILY TREATED WITH OVER-THE-COUNTER MEDICATION SUCH AS IMMODIUM.
INHALATION	THIS MATERIAL DOES NOT EMIT FUMES OR VAPORS AND ONLY HAS AN EARTHY SMELL WHICH MAY BE OFFENSIVE TO SOME.
ADVICE TO DOCTOR	TREAT THE SAME AS INGESTION OF SOIL.

7) EXPOSURE CONTROL / PERSONAL PROTECTION

EYES NONE.

SKIN NONE.

INHALATION. NONE.

VENTILATION NONE.

EXPOSURE LIMITS NONE.

8) FIRE AND EXPLOSION HAZARD DATA

IGNITION TEMPERATURE EXTIGUISHANT DOES NOT BURN
(DEGREES C)

FLAMMAIBLE LIMITS Not Applicable
(% BY VOLUME)

FLASH POINT Not Applicable
(DEGREES C)

FIRE EXTINGUISHING NONE NECESSARY
AGENTS

EXPLOSION HAZARDS NONE

9) ACCIDENTAL SPILL OR LEAK PROCEDURES

LEAKAGE CAN BE WASHED AWAY WITH WATER OR ANY MILD SOAP OR
DETERGENT.
PROCEDURES BIO-DEGRADABLE THREFORE NO DISPOSAL PROCEDURES
REQUIRED.

10) STABILITY AND REACTIVITY DATA

HAZARDOUS
POLYMERIZATIONS NONE

PRODUCTS OF
COMBUSTION NONE.

STABILITY AND REACTIVITY STABLE AND NON-REACTIVE.

11) HANDLING & STORAGE

TRANSPORT & STORAGE	AVOID CONTACT OF LIQUID WITH DIRECT SUNLIGHT. PACK IN OPAQUE OR DARKENED CONTAINERS AND STORE OUT OF DIRECT SUNLIGHT, EXCESSIVE HEAT (OVER 30 °C) OR EXCESSIVE COLD (UNDER 10 °C).
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12) TRANSPORT INFORMATION

TRANSPORTATION TRANSPORT BY AIR, SEA, MEASURES	NOT CLASSIFIED AS HAZARDOUS FOR OR LAND.
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13) TOXOLOGICAL INFORMATION

ADDITIONAL COMMENTS	NOT HAZARDOUS BY INHALATION, SKIN CONTACT OR INGESTION. ECOLOGICALLY, A LIQUID WITH NO VOLATILITY, BENEFICIAL TO SOIL.
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High-Pressure Equipment Information: Accidental high-velocity injection under the skin of materials of this type may result in serious injury. Seek medical attention at once should an accident like this occur
Take this information with you if you seek medical treatment.

ENVIRONMENTAL	BENEFICIAL TO SOIL NON-TOXIC AND FULLY BIO-DEGRADABLE.
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14) ECOLOGICAL INFORMATION

BIODEGRADABILITY	-	FULLY BIODEGRADABLE.
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15) REGULATORY INFORMATION

THIS PRODUCT IS NON-FLAMMABLE NON-TOXIC AND THEREFORE NON-HAZARDOUS.

16) DISPOSAL INFORMATION

WASTE DISPOSAL

MAY BE WASHED AWAY OR POURED INTO
WATER COURSES STORM WATER DRAINS OR
SEWAGE SYSTEMS WITHOUT ANY NEGATIVE
EFFECTS.

17) OTHER INFORMATION

The above information is based on data available to us and is believed to be correct. However, NO WARRANTY is expressed or is to be implied regarding the accuracy of this information, the results to be obtained for the use thereof, or the hazards connected with the use of material. Since the information contained herein may be applied under conditions beyond our control and with which we may be unfamiliar, we do not assume any responsibility for the results of its use. This information is furnished upon the condition that the persons receiving it shall make their own determinations of the effects, properties, and protection procedures which pertain to their particular conditions.

Some background to Soil Bio-Muti by the late Mr Nico Snyman†

The name Soil Bio Muti is derived from a word Muti, which means medicine in most of the African languages; the Bio, pertains to the multitude of different bio-organisms present in the solution; Soil comes from the virgin soil plus natural ingredients of which the solution has been made to effect total Soilution (recovery of depleted soil) in a short time. The Soil Bio-Muti Soilution has been augmented in his own Bio-Bank by the co-worker (N T Snyman) of applicant, North West University,.

Soil Bio-Muti Soilution

Soil is not just soil where we see nothing. Soil is alive with populations of microbes that vary by the way that you treat and feed them, work with them and farm with them. Then you discover that food production is easy, plants healthy and yields high. Pests that previously caused problems (created by ourselves) disappeared when the soil was treated right. In the end farming (or Maize-production) is very easy and uncomplicated if you treat the soil right with Soil Bio-Muti.

Our motto is: From the Soil into the Soil.

A comprehensive and detailed explanation of each project:

THE SOIL BIO- MUTI SOILUTION PROJECT was Initially started by Nicolaas Tjaart Snyman (B.Sc.Agric.. (Agron) 1963) per chance on their (him and his two sons's) farm in Zambia when they had a total crop failure after about 6 years.in the tropical regions.

NATURAL Ecology was disturbed

He discovered the reason was accumulation of stump root eelworm that ate all the hair roots of the plants and maize preventing absorption of moisture (in that high rainfall area) resulting in plant deterioration. He reasoned that the natural ecology and microbe population that should control the eelworm was disrupted on account of burning, cultivation and chemicals. They then started making compost from leaves and virgin topsoil (where no burning, no fertilization, and no cultivation have taken place) from the bush and saw that it helped restoring the soil. But it was a slow,

tedious process and not viable taking away the healthy organic material and destroying the bush.

At that stage he started looking for an existing bio-bank that could provide the whole population of natural organisms for restoration of soil. There were none. He however came upon several individuals and commercial entities that only sold isolated single bio-organisms or a few saprophytic organisms eg. EM which mostly helps with the breakdown of compost. However he met the late Dr P.W Cloete who had a bio-bank built on the Bio-Dynamic principles by catching organisms for a year on the oxborn principles of Rudolph Steiner (1925) and then make a compost tea from it.. Dr Cloete who had serious health problems and was in a wheelchair. did good work on orchids, cattle and deep organisms, that merit an acclaim, but had no luck in the Maize fields of Bothaville which he handed over to the care of the applicant before his death.

OWN BIOBANK

The co-worker of the North West University, Mr N T Snyman, started his own Bio-Bank on total different methods which cut the tediousness from a year to one month and included all the organisms in virgin soil. He continued with practical trials on the maize, which he was doing for the past seven years already. On own initiative with own funding on his farm in Rustenburg District he persevered up to the stage where the results became very pronounced and yields extraordinary high. He discovered:

1. The first step is to create a Bio-Bank which is based on Rudolph Steiners Methods (of 1924) but which I have adapted to be less time consuming, much easier and commercially viable.
2. b) In our Bio-Muti Bio-bank a virgin soil sample is taken and all the microbial contents of the soil is grown collectively to have it available in a solution (which we call Soil Bio-Muti) which is easy to apply to the soil, (more than 875,000 bacteria per millilitre) or in the drinking water of animals.
c) This solution contains all the necessary bacteria and fungi that are essential to restore the rumen flora in livestock and for a healthy soil.:
3. MICRO-ORGANISMS PRESENT:
(The fungi and yeasts, the forest floor bacteria that make compost;
the rhizobium bacteria that binds Nitrogen on legume roots;

the humus making bacteria that feeds the higher plants and binds and aggregates;

the michorrhiza that feeds the plant and binds water etc.as well as a lot of the unknown.)

- These were confirmed by costly DNA analysis.
- Photos taken by Electron Scan Microscope section of The University of North West has proofed the results and a X20,000 enlargement will be added for your perusal.