RESPONSES OF *TYLENCHULUS SEMIPENETRANS* TO CRUDE EXTRACTS OF INDIGENOUS *CUCUMIS* FRUITS WITH AND WITHOUT EFFECTIVE MICRO-ORGANISMS IN CITRUS PRODUCTION

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200501684

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF AGRICULTURAL MANAGEMENT (HORTICULTURE) IN THE DEPARTMENT OF PLANT PRODUCTION, SOIL SCIENCE AND AGRICULTURAL ENGINEERING, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO, SOUTH AFRICA

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2013

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Agricultural Management (Horticulture) has not been submitted previously by me or anybody for a degree at this or any other University. Also, this is my work in design and in execution, while related materials contained herein had been duly acknowledged.

Candidate: Kgahliso Desmond Maile	Signature	Date
Supervisor: Professor P.W. Mashela	Signature	Date

DEDICATION

To my exquisite mother, Mrs Makhalata Gladys Lebyane, my late father, Mr Makuteni Lammick Lebyane, the parents that I have always wanted to be my parents and lastly to the bone of my bones, to the heart of my hearts, Miss Khutjo Moagi – for your unconditional love and support

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to the Limpopo Department of Agriculture, Fisheries and Forestry, for generously funding various aspects of my post-graduate (Master of Agriculture) study at the University of Limpopo. I also appreciate the Department of Labour and the National Research Foundation (NRF) of South Africa for financial support in the form of bursaries for my undergraduate and Honour's degrees, respectively. The Land Bank Chair - University of Limpopo funded various aspects of my Master's research and therefore, I am eternally grateful.

There are not enough words in my heart to express my profound and genuine gratitude towards Professor P.W. Mashela, my Honour's and Master's supervisor, for always being there for me and providing the much needed professional assistance and guidance on various aspects of my student life. Professor Mashela was instrumental in my being admitted into a degree programme in agriculture, while the prophets of doom had the notion that my disability disqualifies me to can successfully complete a degree in this field, let alone a post-graduate qualification. Heartfelt gratitude is also due to Dr T.P. Mafeo, Dr K.M. Pofu, Miss Rebogile Mphahlele, Miss Vivian Matabane, Miss Khutjo Moagi, Miss Deborah Mokgothu, Miss Angel Malele, Mr Edward Mathebula, Mr David Mamphiswana, Mr Taboga Mathiba, Mr Pontso Edmund Tseke and Mr Osvaldo Pelinganga for their indispensable technical assistance. I am proud to have been a member of the team in the life-changing VLIR Nematology Laboratory at the University of Limpopo. To the entire University community and the staff at Reakgona Centre for

Students with Disabilities, I am wordless for the opportunity and special treatment accorded me, and I will endlessly be grateful. To all Reakgona students, “prod on, victory is certain!”

I wholeheartedly thank my mother, Mrs Makhalata Gladys Lebyane, my late father, Mr Makuteni Lammick Lebyane, my elder brother, Mr Joel Andrew Lebyane, my younger brothers, Mr Sekwale Cavin Lebyane and Collen Lebyane, along with my beautiful younger sisters, Miss Moshimoleng Asnath Lebyane and Keletso Retty Lebyane, for all their unconditional love and much needed support. You have played an outstanding and indispensable role in my life. Hence, I will always remember my roots and unconditionally the good things you have done in my life. I will always appreciate your support from my heart. I also appreciate the family of the late Mr S.J. Serage on his behalf for the support they continued to provide even after his passing on. You have earned to be part of my family, forever. Apparently, it is not possible to acknowledge all the people who have contributed willingly and so much towards my achieving this lifetime goal: To all the underdogs, may God gloriously bless you with multiple blessings. Above all, I thank the Almighty God for wisdom, knowledge and understanding, graciously accorded me during all processes of this research. I return all the Glory in multiples of million to where it belongs: Your Throne... Finally, Reakgona!

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ABSTRACT

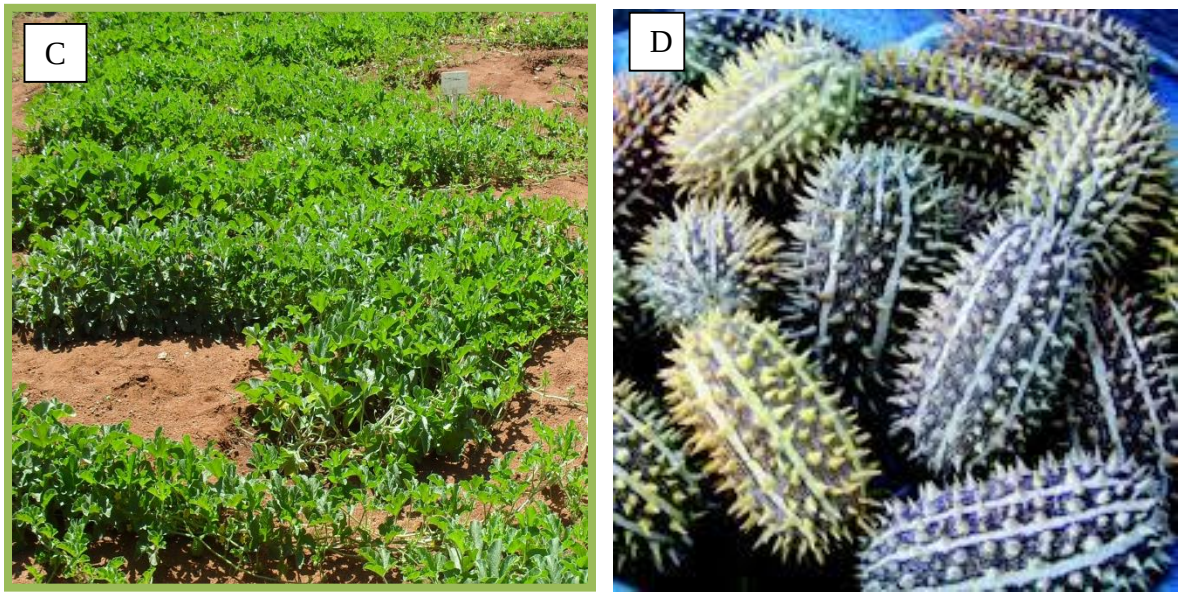
The ground leaching technology (GLT) system, using crude extracts of wild cucumber (*Cucumis myriocarpus*) and wild watermelon (*Cucumis africanus*) fruits, had been widely researched and developed in management of the root-knot (*Meloidogyne* species) nematodes in tomato (*Solanum lycopersicon*) production. In the GLT system, experiments were harvested at 56 days after inoculation with nematodes, which was approximately three generations of *Meloidogyne* species. Also, studies in GLT systems demonstrated that effective micro-organisms (EM) were not essential in the release of chemicals from crude extracts for nematode suppression, with suggestions that the system exclusively relied upon irrigation or rainwater for leaching out chemicals. However, the system had hardly been tested on other nematode species with longer life cycles and crops. The objective of this study was to investigate the influence of crude extracts of *C. myriocarpus* (cucurbitacin A-containing phytonematicide) and *C. africanus* (cucurbitacin B-containing phytonematicide) with and without EM on suppression of population densities of the citrus nematode (*Tylenchulus semipenetrans*) on rough lemon (*Citrus jambhiri*) over three generations of the nematode. Two studies, one on *C. myriocarpus* and the other on C. *africanus*, with and without EM, were conducted separately using the GLT system under greenhouse conditions with three generations of *T. semipenetrans* as the standard of application interval of the materials. Citrus seedlings were transplanted in 7-L plastic pots contain 6.5-L pasteurised river sand and Hygromix (3:1 v/v) and inoculated with approximately 25 000 second-stage juveniles (J2s) in 2 × 2 × 2 factorial experiment, where treatments were arranged in a randomised

complete block design (RCBD), with six replications. At 150 days after treatment, nematode and plant variables were collected and subjected to factorial analysis of variance. Under *C. myriocarpus* (Cm), EM × Cm interaction was not significant for nematodes (juveniles + eggs) in roots and juveniles in soil, while under *C. africanus* (Ca), EM × Ca interaction was highly significant for nematodes, but not for juveniles. Crude extracts of *C. myriocarpus* and *C. africanus* fruits contributed 21-36% and 38-59% to total treatment variation in nematodes, respectively. Relative to untreated control, crude extracts of *C. myriocarpus* fruit reduced nematodes by 22% in roots, but increased juveniles in soil by 93%. Similarly, *C. africanus* fruit reduced nematodes in roots by 80%, but increased juveniles in soil by 178%. The increase of juveniles in the soil was explained on the basis of opposing forces on nematode population densities under crude extracts of *Cucumis* and untreated control, along with the inherent nature of cyclic population growth in plant-parasitic nematodes. In plant variables, certain significant ($P \leq 0.05$) interactions consistently occurred under both *Cucumis* species. However, effects of the interactions were not consistent under the two *Cucumis* species. In most of the variables, the non-significant effects of EM × Cm interactions supported the view that the GLT systems were independent of microbial activities, while significant ($P \leq 0.05$) EM × Ca interactions suggested that the systems under *C. africanus* fruit could be viewed as being dependent upon microbial degradation activities. Growth of rough lemon rootstock was, to a certain extent, suppressed by application of crude extracts from *Cucumis* fruits, suggesting that the material were phytotoxic to this citrus rootstock. Under low nematode population densities, *T. semipenetrans* infection supported the view that nematode numbers below the damage threshold levels have

stimulatory effects on growth of plants as observed in plant height under conditions of this study. In conclusion, the approximately three nematode-generation-application interval of 150 days for crude extracts of fruits in *Cucumis* species was rather too long for the efficacies of the materials on suppression of the population densities of *T. semipenetrans* in rough lemon seedlings. Consequently, shorter application intervals, as demonstrated for *Meloidogyne* species would be appropriate, although caution has to be taken to ensure that phytotoxicity to the rootstock was avoided.

PICTURES OF INDIGENOUS *CUCUMIS* SPECIES FRUITS AND ROUGH LEMON SEEDLINGS

Legend A: Plants (A) and fruit (B) of wild cucumber (*Cucumis myriocarpus*).



Legend B: Plants (C) and fruit (D) of wild watermelon (*Cucumis africanus*).

Legend C: Rough lemon seedlings treated with *Cucumis myriocarpus* (E) and *Cucumis africanus* (F) fruits taken at harvesting, 150 days after treatment application.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction

The citrus nematode (*Tylenchulus semipenetrans*) is widely distributed in all citrus-producing regions of South Africa (De Villiers and Milne, 1976). Most commercially used citrus rootstocks are susceptible to *T. semipenetrans* (Mashela, 1992), resulting in a disease called slow decline of citrus (Hutchison *et al*., 1972; O'Bannon and Esser, 1985). Clinical symptoms of slow decline are similar to those associated with salinity injury stress and nutrient deficiency (Greenway and Munns, 1980; Levitt, 1980; Mashela, 1992). In citrus production, infection by *T. semipenetrans* results in accumulation of more salinity ions in leaves than in roots (Mashela and Nthangeni, 2002a), smaller fruits and leaves (Duncan, 2005), failure of citrus trees to respond to K fertilisation (Fouche *et al*., 1978), leaf chlorosis, sparse foliage and dieback of young twigs (Inserra *et al*., 1980; Mashela, 1992), with the result of overall poor yield of low quality (O'Bannon and Esser, 1985).

Following the withdrawal of fumigant nematicides from agrochemical markets, nematode-resistance had been the most preferred strategy for managing nematode population densities in citrus (Duncan, 2009). Various citrus rootstocks are available for managing *T. semipenetrans* (Duncan, 2009). However, resistance may be negated by virulent biotypes that are capable of overcoming genes that confer nematode-resistance (Inserra *et al*., 1980) and abiotic factors such as salinity stress (Mashela, 1992). Incidentally, nematode-resistant rootstocks in citrus are not salt-tolerant, *vice-versa*

(Mashela and Nthangeni, 2002a). Infection by *T. semipenetrans* breaks salt-tolerance in salt-tolerant rootstocks, while salinity breaks nematode-resistant in citrus rootstocks (Mashela, 1992). Phytonematicides are gaining international recognition in modern agriculture due to their environment-friendliness (Ballesteros *et al*., 1992). Considering the negative impacts of synthetic chemicals against non-target organisms, the need for new and environment-friendly phytonematicides are continuously rising (Arnold and McLachlan, 1996; Krol *et al*., 2000; Mitscher *et al*., 1987).

Synthetic nematicides had been increasingly suspended from agrochemical markets due to their environment-unfriendliness (Ledley *et al.*, 1999; UNEP, 2000; UNEP, 2005). Suspension of fumigant nematicides affect all crop-producing farmers (Mashela *et al*., 2011). In certain specialised crops, such as citrus, which also have specialised nematode pests, the suspension of synthetic nematicides has had profound effects for the South African land reform programme, where historically disadvantaged farmers were hoping to tap into the lucrative export markets of fresh fruits (Matabane, 2013).

Suspension of halogenated fumigant nematicides due to their negative environmental impact, particularly breakdown of ozone layer and high levels of toxicity, increased the evaluation of organic amendments on suppression of plant-parasitic nematodes (Bello, 1998). The Land Bank Chair of Agriculture - University of Limpopo, introduced, researched and developed the Indigenous Cucurbitaceae Technologies (ICT) in the management of plant-parasitic nematodes (Mashela *et al*., 2011). The ground leaching technology (GLT) within the ICT had been widely used in smallholder farming systems

(Mashela, 2002). In the GLT systems the application frequency of the products is 56 days, which is equivalent to three life cycles of the southern root-knot (*Meloidogyne incognita*) nematode and does not depend on microbial activities (Mashela *et al*., 2011). The ICT induces various technologies, which will be detailed later (Chapter 2). The ICT has since become an internationally-recognised research programme, with the possibility of producing commercial phytonematicide products (Mashela *et al*., 2011). However, the focus of ICT had been mainly on *Meloidogyne* species in tomato (*Solanum lycopersicon*) production.

1.2 Problem statement

Three life cycles are used as application interval standard of crude extracts of *C. africanus and C. myriocarpus* fruits in management of population densities of *M. incognita* under the GLT system. The researcher intends investigating whether three life cycles of *T. semipenetrans* would be suitable for use as application interval standard of crude extracts of the products under the GLT system.

1.3 Motivation

In order for commercial products to be realised and considered favourably for registration in terms of ICT, the products should cover a number of economically-important plant-parasitic nematodes within economically important industries. The successful suppression of population densities of *T. semipenetrans* in citrus industry would enhance the potential status of the two products since citrus constitute one of the

biggest crop industries both locally and internationally, while the citrus nematode is a cosmopolitan pest occurring in all citrus-producing areas in the world.

1.4 Aim and objectives

1.4.1 Aim

The aim of the study is to expand the use of ICT from *Meloidogyne* species in tomato production to the citrus nematode in citrus production using the GLT system.

1.4.2 Objectives

1 To determine the effect of crude extracts of *C. myriocarpus* fruit with and without effective micro-organisms (EM) on population densities of *T. semipenetrans* and growth of citrus when the application interval is equivalent to three life cycles of *T. semipenetrans*.

2 To determine the effect of crude extracts of *C. africanus* fruit with and without EM on population densities of *T. semipenetrans* and growth of citrus when the application interval is equivalent to three life cycles of *T. semipenetrans*.

1.5 Reliability, validity and objectivity

In this study, reliability of data was based on statistical analysis of data at the probability level of 5%, while validity was achieved through using factorial experiments (Leedy and

Ormrod, 2005). Objectivity was ensured by discussing results on the basis of empirical evidence (Leedy and Ormrod, 2005).

1.6 Bias

Bias was eliminated by using replications and randomisation of the treatments (Leedy and Ormrod, 2005).

1.7 Ethical considerations

The researcher would not act in such a way as to facilitate profit by third parties through the commercialisation or dissemination of the derived knowledge without the concurrence of the University of Limpopo. Where the use or dissemination of the acquired knowledge is envisioned, the researcher would ensure that the moral or legal rights of any potential claimants are respected, through consultation with them. The University policies, appropriate legal framework and ethical considerations as outlined here, would endure beyond the completion of this study.

1.8 Significance of the study

Findings of the research would indicate whether crude extracts of *C. africanus* and *C. myriocarpus* fruits with and without EM would be effective in suppression of nematode population densities of *T. semipenetrans* in citrus production. The findings would help in decision-making for expanding the use of the GLT systems from *Meloidogyne* species and tomato to other economically important nematodes and crop industries like the citrus nematode and citrus industry.

1.9 Format of dissertation

Following detailed outlining of the research problem (Chapter 1), the work done and not yet done on the research problem would be reviewed (Chapter 2). Then, each of the two subsequent chapters (Chapters 3 and 4) would address each of the two objectives in sequence. In the final chapter (Chapter 5), findings in all chapters would be summarised and integrated to provide the significance of the findings and recommendations with respect to future research, culminating in conclusions which would tie the entire study together.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

The crop losses induced by the citrus nematode (*Tylenchulus semipenetrans*) in heavily-infested Florida citrus orchards ranged from 10-20%, while in arid and high salinity-affected western states of the USA, yield losses in citrus were as high as 50% (Baines *et al*., 1962; Duncan, 2005). Cohn (1972) estimated annual global citrus yield losses due to the citrus nematode at 9-12%, while the South African estimate is not available.

After having been relied upon for over 50 years in management of population densities of plant-parasitic nematodes, the suspension of fumigant nematicides had noticeable consequences, particularly in citrus nurseries (Hague and Gowen, 1987). Research and development of alternative tactics to replace fumigant nematicides had since intensified (Bello, 1998; Mafeo and Mashela, 2009a,b; Mashela, 2007; 2002; Mashela *et al*., 2008; Pofu *et al*., 2009). Much work is being done to develop non-chemical and environment-friendly nematode management strategies such as the use of botanicals (Bello, 1998; Mashela, 2002, 2007; Mashela and Mpati, 2002; Mashela and Mphosi, 2002; Mashela and Nthangeni, 2002b; Mashela *et al.*, 2008; Rajendran and Saritha, 2005; Sukul *et al*., 2001) and organic soil amendments (Stirling, 1991). However, most of the phytonematicide strategies had been researched on annual crops, while research on perennial crops focused mainly on nematode resistant-rootstock strategies (Duncan, 2009; 2005).

Traditionally, in low-input agriculture farming systems, farmers use organic amendments to suppress plant-parasitic nematodes and to provide essential nutrients (Stirling, 1991). However, the use of conventional organic amendments to suppress plant-parasitic nematodes had numerous drawbacks (Mankau, 1968; Mankau and Minteer, 1962; McSorley and Gallaher, 1995a,b; Muller and Gooch, 1982; Rodriguez-Kabana, 1986; Stirling, 1991), including: (i) excessively large quantities (10-500 t/ha) of organic materials being required to achieve nematode suppression, (ii) high transport costs to haul the materials from the planting/collection site to the decomposition site and eventually to the field, (iii) long waiting periods for microbial decomposition, (iv) reduction in soil pH, and (v) inconsistent results in nematode suppression. Consequently, most marginal farming communities can hardly afford the use of conventional organic amendments in suppression of plant-parasitic nematodes.

Indigenous Cucurbitaceae Technologies (ICT) were researched and developed to address the management of plant-parasitic nematodes in low-input agricultural systems, particularly in marginalised farming communities in South Africa. The ICT comprises five technologies, *viz*. (1) ground leaching technology, (2) botinemagation technology, (3) nematode resistance technology, (4) agronomics technology and (5) chemical technology, which had since become a multi-disciplinary and internationally recognised research programme (Mashela *et al*., 2011). Due to increased interest among emerging farmers on citrus, the inclusion of ICT research and development in citrus would be appropriate in terms of the National Development Plan (NDP) framework in South Africa.

2.2 Indigenous *Cucumis* species

The review of indigenous *Cucumis* species in South Africa presented here is limited to (a) their pharmacological properties, (b) presence of potent chemicals, (c) attractiveness for use in nematode management, (d) their bioactivity effects and (e) minimum inhibition concentrations (MIC) against selected nematodes.

2.2.1 Pharmacological properties

The family Cucurbitaceae consists of 115 genera (Pitrat *et al*., 1999), most of which have been widely used for centuries in sub-saharae African traditional medicines (Rimington, 1938). According to Kristkova *et al*. (2003), South Africa is the centre of bio-diversity for wild watermelon (*Cucumis africanus*) and wild cucumber (*Cucumis myriocarpus*). Crude extracts of *C. myriocarpus* fruit and roots, along with the whole plant of *C. africanus,* contain pharmacological chemical properties used in the treatment of liver damage, weakening of the immune system, lumps, jaundice, acute and chronic viral hepatitis, hepatocirrhosis, persistent dyspepsia, epilepsy due to wind-phlegm, cancer, gonorrhea, boils and infections by intestinal roundworms (Mashela *et al*., 2011; Mphahlele *et al*., 2012).

2.2.2 Potent chemicals in *Cucumis* species

Potent chemicals in crude extracts of *Cucumis* fruit have been isolated and identified as cucurbitacins (Rimington, 1938). Plants in the family Cucurbitaceae contain a total of 12 cucurbitacins, with cucurbitacin A in *C. myriocarpus* fruit and roots being the only one that is soluble in water (Chen *et al*., 2005). Cucurbitacins are oxygenated tetracyclic

triterpenes with glycosides and originate from the mevalonic pathway (Inderjit and Malik, 2002). Cucurbitacins are used by plants *in vivo* for defence against nematodes, fungi and insects (Inderjit and Malik, 2002; Inderjit *et al*., 1995; Mashela, 2002). Generally, cucurbitacin A, which had been widely investigated, comprises two potent chemicals, *viz.* cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) (Jeffrey, 1978; Rimington, 1938), which have more or less similar molecular structures in size with aldicarb ($C_7H_{14}N_2O_2S$) and fenamiphos ($C_{13}H_{22}NO_3PS$), while cucurbitacin B ($C_{32}H_{48}O_8$) is also widely used in control of cancerous cells in medicine (Jayaprakasam *et al.*, 2003; Tannin-Spitz *et al*., 2007).

2.2.3 Origin in nematode management

Traditional practitioners orally-administered decoctions of crude extracts of *C. africanus* and *C. myriocarpus* fruits to control intestinal roundworms in humans *(Ascaris lumbricoides*), dogs (*Toxocara canis, Toxascaris leonine*), chickens (*Ascaridia galli*) and other domesticated animals (Mashela *et al*., 2011). Intestinal roundworms are zoo-parasitic nematodes (Mashela, 2007). Motivated by the efficacy of botanicals in control of internal parasites, Mashela and Mphosi (2002) opted to use crude extracts of *C. myriocarpus* fruit to suppress population densities of *Meloidogyne* species in pot trials, with results showing at least 90% suppression of the target pest. The material had both suppressive effects on nematode population densities and stimulative effects on growth of tomato *(Solanum lycopersicon)* plants (Mashela, 2002; Mashela and Nthangeni, 2002; Mashela *et al*., 2010), with the latter viewed as being curious since the stimulated

plant growth could not be attributed to essential nutrient elements which were accumulated in organs of the test plants (Mashela, 2002).

2.2.4 Bioactivity effects

Bioactivity effects of crude extracts of *C. myriocarpus* fruit on *M. incognita* race 2 *in vitro* resulted in nematode mortalities in the range 87-95%, while on *T. semipenetrans* mortalities ranged from 83-96% (Muedi *et al*., 2005).

2.2.5 Minimum inhibition concentrations

Pesticides have minimum inhibition concentration (MIC) for various pests. The MIC of crude extracts from *C. myriocarpus* fruit for both *M. incognita* race 2 and *T. semipenetrans* was 7 µg/ml (Muedi *et al*., 2005).

2.3 Indigenous Cucurbitaceae Technologies

The ICT comprises five technologies, which are briefly reviewed hereunder.

2.3.1 Ground leaching technology

In the ground leaching technology (GLT) systems, mature fruits of *C. africanus* or *C. myriocarpus* are cut into pieces, dried at 52°C (Makkar, 1999) for 72 h and ground in a Wiley mill to pass through a 1-mm-pore sieve (Mashela, 2002). The materials are applied at transplanting without first undergoing any microbial degradation (Mashela, 2002; Mashela and Mphosi, 2002). Crude extracts are generally spread in small quantities (2-5 g/plant) in a shallow hole around the base of the stem of the transplant,

which translate to 20-71 kg ground material/ha for 4 000 tomato plants (Mashela, 2002). The small quantities used in GLT systems precluded high transport costs to haul the materials to the fields (Mashela *et al*., 2011). When applied at transplanting, the waiting period for microbial decomposition was not an issue (Mashela, 2002), while the materials from the two plant species did not reduce soil pH (Mashela *et al*., 2011). Also, suppression of nematode numbers had been consistently achieved, regardless of the environment where the study was conducted (Mafeo, 2012; Mashela and Nthangeni, 2002b; Mofokeng *et al*., 2004; Mphosi, 2004; Shakwane *et al*., 2004).

Post-emergent application: Crude extracts of *C. myriocarpus* fruit suppressed plant-parasitic nematodes in greenhouse trials by over 90% (Mashela, 2002), in microplot trials by over 90% (Mofokeng *et al*., 2004; Shakwane *et al*., 2004) and in field trials by over 80% (Mashela *et al*., 2011). Also, crude extracts from the two materials increased soil electrical conductivity (EC) by 95-160%, but had no effect on soil pH (Mashela *et al*., 2011). Incidentally, the materials improved tomato fruit yield and plant growth (Mashela, 2007; 2002; Mashela *et al*., 2008; Mphosi, 2004). Efficacy of crude extracts from *C. myriocarpus* fruit, when compared with that of aldicarb and fenamiphos suggested that the three materials did not have significant differences in their nematode suppression and plant growth (Mashela *et al*., 2008).

In GLT systems, microbial decomposition had negligible role in the efficacy of crude extracts from *C. myriocarpus* fruit, since interactions between this material and *Bacillus* species were not significant ($P \geq 0.10$) (Mphosi *et al*., 2004). Mashela and Pofu (2012) demonstrated that the material promoted nodulation of *Bradyrhizobium japonicum* in

cowpea (*Vigna unguiculata*) production. Also, the independence of the GLT system from microbial activities was demonstrated through elimination of *Bacillus* species in predictive models when using crude extracts of castor (*Ricinus communis*) bean (Mashela and Nthangeni, 2002; Mofokeng *et al*., 2004) and fever tea (*Lippia javanica*) leaves (Mashela *et al*., 2010).

Pre-emergent application: Crop yield losses are, incidentally proportional to initial population densities (Pi) of nematodes (Seinhorst, 1967). Ideally, the use of a material in GLT systems should be as a pre-emergent bio-nematicide in order to keep Pi at the lowest level possible. *In vitro*, seed germination assays suggested that at 5 g crude extracts of *C. myriocarpus* fruit were highly phytotoxic to tomato, watermelon and butternut squash (Mafeo and Mashela, 2009a), along with maize, finger millet, sorghum and onion (Mafeo and Mashela, 2009b). In greenhouse trials, the material completely inhibited seedling emergence of all dicotyledonous crops tested (Mafeo and Mashela, 2010).

Cucumin, from crude extracts of *C. myriocarpus* fruit, was shown to have the capabilities of suppressing the division of cancer cells in animals (Van Wyk *et al*., 1997). However, the suppression occurred at higher concentrations which were toxic to healthy cells, while at reduced concentrations the material stimulated division of cancer cells. Quadratic relationships between cell divisions and concentrations of cucumin characterised responses of biological systems to increasing concentration of extrinsic factors (Salisbury and Ross, 1992). Using the observation of stimulatory effects on cells,

concentrations were reduced *in vitro* from 0 to 2.25 g/plant, with germination of tomato, watermelon and butternut squash resulting in significant ($P \leq 0.01$) quadratic relationships with increasing concentrations of the material (Mafeo and Mashela, 2009b). In the trials, increasing concentrations of crude extracts of *C. myriocarpus* fruit explained 91%, 97% and 91% of the total treatment variation in inhibition of seed germination in tomato, watermelon and butternut squash, respectively. Findings in that trial suggested that crude extracts of *C. myriocarpus* fruit had allelopathic effect on seed germination of test plants and therefore, the material was not suitable for use as a pre-emergent bio-nematicide. Confirmation studies were initiated using the Curve-fitting Allelochemical Response Dosage (CARD) computer model (Liu *et al*., 2003) to determine concentrations where crude extracts of *C. myriocarpus* fruit stimulated, had no effect and then inhibited germination of various crops in order to establish the pre-emergent quantities of crude extracts of *C. myriocarpus* fruit (Mafeo and Mashela, 2010; Mafeo *et al*., 2010).

In short, the CARD model provided the biological index - total sum of transformations (k), which expressed the sensitivities of the test plants to the bio-nematicide tested (Mafeo *et al*., 2010). Generally, k is inversely proportional to the degree of sensitivity of the test plant to the material (Liu *et al*., 2003). Initially, the studies involved 18 different plant species in the greenhouse trials, where each was subjected to 10 concentrations from 0 to 2.25 g. At harvest, 18 days after initiating the treatments, seedling height, radicle length, coleoptile length and coleoptile diameter were each subjected to the CARD model (Mafeo and Mashela, 2010; Mafeo *et al*., 2010). The CARD models

indicated that the 18 crops had various sensitivity (k) values to crude extracts of *C. myriocarpus* fruit, with clear stimulatory, neutral and inhibitory concentrations to emergence within the quadratic relation models (Mafeo and Mashela, 2010; Mafeo *et al*., 2010; Mafeo *et al*., 2011a,b). Pre-emergent quantities for applying crude extracts of *C. myriocarpus* fruit using GLT were computed and validated, where germination of crops was not significantly ($P \geq 0.10$) affected, but nematode numbers were significantly reduced (Mafeo, 2012).

2.3.2 Botinemagation technology

The GLT system is labour-intensive and could therefore, be costly for large-scale commercial farming systems (Pelinganga *et al*., 2012a). Development of a bio-nematicide from fermented crude extracts of *C. africanus* and *C. myriocarpus* fruits would therefore, enhance the application of the materials through irrigation water in commercial farming agriculture. However, since only cucurbitacin A in *C. myriocarpus* fruit is soluble in water (Chen *et al*., 2005), it was not certain whether crude extracts of *C. africanus* fruit could also serve as fermented crude extracts in suppression of nematodes since cucurbitacin B is insoluble in water (Chen *et al*., 2005). Thus, fermented crude extracts of *C. africanus* and *C. myriocarpus* fruits were tested and reduced population levels of *M. incognita* race 2 by 89% (range 80–100%) and 69% (range 52–79%), respectively, with the reproductive factor (Pf/Pi) values being below one (Pelinganga *et al*., 2012a). At low concentrations both materials had fertiliser effect on tomato plant growth, while at high dilutions the materials were phytotoxic. Results of the study (Pelinganga *et al*., 2012a) demonstrated that the two materials could serve as

potent bio-nematicides at low dilutions – in what had been termed “botinemagation” – the suppression of nematodes through application of botanicals through irrigation. Pelinganga *et al*. (2012b), after establishing the stimulatory concentrations, which were devised the concept of a 30-day month to determine the application intervals 16 and 17 days for *C. myriocarpus* and *C. africanus* fruits, respectively.

2.3.3 Nematode resistance technology

The two *Cucumis* species could be used as alternative crops in the production of traditional medicine and bio-nematicides. Also, preliminary studies had shown that *Cucumis* species were to a certain extent, compatible with watermelon (*Citrullus lanatus*) cultivars. Host-status and host-sensitivity of *C. africanus* and *C. myriocarpus* to *Meloidogyne* species were investigated in greenhouse, microplot and field trials (Pofu *et al.,* 2009; 2010a,b). Both *C. africanus* and *C. myriocarpus* were shown to be highly resistant to *M. incognita* races 2 and 4 and *M. javanica,* which are dominant in South Africa (Kleynhans *et al*., 1996). However, the two plant species were tolerant to the spiral nematode (*Helicotylenchus dihystera*) and the ring nematode (*Criconema mutabile*) under field conditions (Pofu *et al*., 2011a).

Mechanisms of resistance to *Meloidogyne* species in the two *Cucumis* species were established under greenhouse conditions (Pofu and Mashela, 2011). Generally, resistance in plant-parasitic nematodes had been broadly classified as pre-infectional and post-infectional with resistance manifesting prior and after infection, respectively (Kaplan and Keen, 1980). Establishment of resistance type in plant-parasitic nematodes

is essential, especially when germplasm could be introgressed into breeding lines since only post-infectional resistance is introgressible (Thurau *et al*., 2010). Resistance forms in *C. africanus* and *C. myriocarpus* were pre-infectional and post-infectional, respectively (Pofu *et al*., 2010a). Post-infectional resistance is introgressible (Kaplan and Keen, 1980). Consequently, resistant germplasm in *C. myriocarpus* could be useful for introgression in highly nematode-susceptible genera such as *Citrullus* cultivars (Pofu, 2012). In *Cucumis* studies (Pofu, 2012), most J2s of *Meloidogyne* species were converted into males, apparently due to their failure to establish feeding sites in *Cucumis* species. The observation of conversion of juveniles to males confirmed that of Fassuliotis (1970) in commercial *Cucumis* species with moderate resistance to *Meloidogyne* species. The biological importance of conversion of juveniles to males is that the latter do not feed since they are not required for reproduction (Ferraz and Brown, 2000).

Inter-generic grafting technology has incompatibility challenges due to different stem diameter sizes of scions and rootstocks in plant species from different genera (Tiederman, 1989). Grafts of watermelon, with relatively thick stem diameters when compared to *C. africanus* and *C. myriocarpus* with relatively thin stem diameters, had low survival rates of approximately 36% (Pofu and Mashela, 2011). Through research and development, procedures were developed to optimise the stem diameters of plants from the two genera, resulting in 100% survival of the grafts (Pofu and Mashela, 2011). In a subsequent greenhouse study (Pofu *et al*., 2011b), all inter-generic grafts survived, with *C. africanus* and *C. myriocarpus* seedling rootstocks retaining their capabilities to

reduce population densities of *M. incognita*. Under field conditions the procedure was also successful, with grafts flowering earlier and producing higher fruit yield than intact plants (Pofu *et al*., 2011a).

2.3.4 Agronomics technology

This technology, under the auspices of ICT, involves all aspects of the agronomy of indigenous plants in the Cucurbitaceae family. Originally, fruits used in GLT systems were collected from the wild. However, with the advent of the agronomics technology, fruits were eventually collected from locally-propagated plants. Mafeo (2005) developed sexual propagation protocols of *C. myriocarpus*. Incidentally seeds have autoallelopathy and the leaching of allelochemicals was necessary to improve germination (Mafeo and Mashela, 2009b). Nkgapele *et al*. (2011a,b) also investigated irrigation and fertilisation requirements of *C. africanus* and *C. myriocarpus* in pot trials, while Mafeo (2005) previously tested these requirements for *C. myriocarpus* under field conditions. In both trials, results suggested that moderate irrigation and fertilisation were required for achieving optimum yields in the two *Cucumis* species. Attempts are also being made to use *in vitro* propagation in order to eliminate autoallelopathic effects and the resultant poor emergence and therefore, non-uniform seedling rootstocks (Maila *et al.*, 2013).

2.3.5 Chemical technology

All aspects of the chemistry of the two Cucurbitaceae family with respect to identification, molecular structures, toxicities and residues in produce are investigated under this technology (Mashela *et al*., 2011). The molecular structures of the two

Cucumis species had been established in the late 1930s (Rimington, 1938). However, under the auspices of the ICT, not much had been done in this area except for minimum inhibition concentration (MIC) values for *Meloidogyne* and *Tylenchulus* species (Muedi *et al*., 2005) and LD_{50} values (Mashela, 2007).

2.4 Effective micro-organisms

Effective micro-organisms (EM) are used for various purposes in agriculture (Ncube, 2008), with the active ingredients being similar to those in a country where they are applied. In South Africa, EMROSA EM (Centurion, South Africa) is commercially available and comprised predominant populations of lactic acid bacteria, yeasts, smaller numbers of photosynthetic bacteria, actinomycetes and other types of organisms. In botinemagation, EMROSA EM is used to mine the chemicals out of crude extracts prior to application through irrigation system. Generally, in GLT, EM is used to test the hypothesis that the efficacy of this system on nematode suppression was independent of microbial degradation.

2.5 Gaps with respect to the research problem

The ICT had mainly focused on one nematode genus, namely, *Meloidogyne* species, in tomato production. In GLT system, application interval had been restricted to three life cycles of *Meloidogyne* species, which occur in approximately 56 days. Similar life cycles for *Tylenchulus* species would occur in approximately 150 days. The researcher intends using 150 days as application interval of *T. semipenetrans* to investigate how the population densities would behave under the two phytonematicides in the GLT system.

CHAPTER 3
EFFECTS OF CRUDE EXTRACTS OF *CUCUMIS MYRIOCARPUS* FRUIT ON *TYLENCHULUS SEMIPENETRANS* AND GROWTH OF ROUGH LEMON

3.1 Introduction

Cucurbitacin A is an active ingredient which is concentrated in fruits and roots of wild cucumber (*Cucumis myriocarpus*) (Rimington, 1983). This is the only cucurbitacin that is soluble in water (Chen *et al*., 2005) and consists of two constituents, namely, leptodermin ($C_{27}H_{38}O_8$) and cucumin ($C_{27}H_{40}O_9$) (Jeffrey, 1978; Rimington, 1938). Cucurbitacin A had been used in the management of plant-parasitic nematodes using the ground leaching technology (GLT) system, which was introduced, researched and developed as one of the post-planting alternative measures to manage the root-knot (*Meloidogyne* species) nematodes (Mashela, 2002). The use of ground *C. myriocarpus* fruit for suppression of plant-parasitic nematodes had been successful on *Meloidogyne* species in tomato (*Solanum lycopersicon*) production under diverse conditions (Mashela *et al*., 2011).

Active ingredients in GLT systems are released through irrigation and rainwater (Mashela *et al*., 2011), with the application interval of the material being equivalent to three life cycles of *M. incognita* (Mashela, 2002). However, the material had not been tested on the citrus nematode (*Tylenchulus semipenetrans*) in citrus plants. The objective of this study was to determine the effect of crude extracts of *C. myriocarpus* fruit with and without EM on population densities of *T. semipenetrans* and growth of citrus when the application interval was equivalent to three generations of *T. semipenetrans*.

3.2 Materials and methods

A greenhouse experiment was initiated during the last month of autumn (February-April) through winter (May-July) to the middle of spring (August-October) at the Plant Protection Skills Centre, University of Limpopo (23°53'10"S, 29°44'15"E). Twenty-cm-diameter (7 L) plastic pots were filled with 6.5 L at 3:1 (v/v) of pasteurised river sand and Hygromix (Hygrotech, Pretoria North, South Africa) and placed on greenhouse benches at 0.3 m inter-row and 0.2 m intra-row spacing. Uniform 5-month old citrus seedlings (*Citrus jambhiri*), purchased from Du Roi Nursery (Portion 21, Junction Farm, Letsitele) were hardened-off and each transplanted into pots containing the growing mixture. *Cucumis myriocarpus* fruit were locally collected from the field, dried for 72 h at 52°C in air-forced ovens in order to reduce volatilisation of the phytochemicals (Makkar, 1999) and then ground in a Wiley mill to pass through a 2-mm-pore sieve.

A factorial experiment, with eight treatments, namely. (1) untreated control, (2) nematode, (3) EM, (4) *C. myriocarpus*, (5) nematode + *C. myriocarpus*, (6) nematode + EM, (7) EM + *C. myriocarpus* and (8) nematode + EM + *C. myriocarpus*, was arranged in a randomised complete block design (RCBD), with 6 replicates. Nematode inocula were prepared by extracting eggs and second stage juveniles (J2s) of *T. semipenetrans* from roots collected at Zebediela Citrus Estate (24°11′S, 29°01′E) in 1% NaOCl solution (Hussey and Barker, 1973). A 30-ml-plastic syringe was used to place 25 000 eggs and J2s into 3-cm-deep holes on cardinal points of the stem, while plants without nematodes each received a 20 ml filtrate from the nematode aliquot in order to establish microbes associated with nematodes. All treatments were applied at transplanting in 3-cm deep

holes around the cardinal points of appropriate treatments. EM + *C. myriocarpus* fruit were applied at 50 ml and 2 g per pot, respectively, while for *C. myriocarpus* without EM solutions the same amount of water was added to settle the material before covering with the growing mixture.

Irrigation was scheduled using four Hadeco Moisture Meters (Hadeco Magic^{R}, New Delhi, India), with plants being irrigated with 500 ml when average moisture meter reading was less than 2 units. At 150 days after initiating the treatments, plant height was measured from the crown to the terminal end of the flag leaf, stems were cut at the crown and stem diameters measured at 3 cm above the severed ends using a digital vernier caliper. Shoots were oven-dried at 52°C for 72 h and weighed. Root systems were removed from pots, immersed in water to remove soil particles, blotted dry and weighed to facilitate the calculation of nematode density per total root system per plant. Nematodes were extracted from 5 g roots per plant through maceration and blending for 30 seconds in 1% NaOCl solution of water (Hussey and Barker, 1973) and passed through top-down nested 150 and 25-µm-pore sieves. Contents of the 25-µm-pore sieve were poured into 100 ml plastic containers for counting under a stereomicroscope. Soil per pot was thoroughly mixed and a 100 ml soil sample was collected for nematode extraction from a 250 ml soil subsample/pot using the modified sugar-floatation and centrifugation method (Jenkins, 1964). Nematode numbers from roots were converted to nematodes per total root system per plant, while those from soil were converted to 6 500 ml soil per pot to compute total nematode population densities (Pf)/plant.

Data were subjected to factorial analysis of variance (ANOVA) through the SAS software (SAS Institute, 2008). Prior to ANOVA, nematode numbers were transformed through $\log_{10}$ (x + 1) to homogenise the variances (Gomez and Gomez, 1984), but untransformed data were recorded. Second and first order interactions were further expressed using three-way and two-way tables, respectively, in order to allow for determination of the magnitude and direction of the main factors (Steyn *et al*., 2003).

3.3 Results

The first order (EM x Cm) interaction was not significant for nematodes nor was EM which had slight significant (P ≤ 0.10) effects while crude extracts of *C. myriocarpus* fruit had significant effect on this variable (Table 3.1). The *Cucumis* factor contributed 21%, 36% and 34% to the total treatment variation in nematode juveniles in root, soil and total (root + soil) nematodes, respectively. Relative to untreated control, crude extracts of *C. myriocarpus* fruit reduced nematode eggs and juveniles in roots by 22%, but increased juveniles in soil and total by 93% and 73%, respectively (Table 3.2).

The second order (EM x N x Cm) interaction was slightly significant (P ≤ 0.10) for dry root and shoot mass, contributing 4% and 5% to the total treatment variation of the two variables, respectively (Table 3.3). In contrast, this interaction was highly significant for root/shoot and stem diameter, contributing 11% and 47% to the total treatment variation, respectively. The first order (N x Cm) interaction was highly significant for dry root mass, dry shoot mass, plant height and stem diameter, but not for root/shoot ratio. In contrast the first order (EM x Cm) interaction had no effect on all plant variables,

while EM x N interaction had similar effects except that it contributed significantly by 15% to total treatment variation of root/shoot ratio. Both *Cucumis* and EM main factors had significant effects on all variables measured, except for plant height, while *T. semipenetrans* had no effect on all plant variables.

Table 3.1 Sum of squares for root nematode, soil nematode and final nematode population density (Pf) as influenced by effective micro-organisms (EM) and crude extracts of *Cucumis myriocarpus* (Cm) fruit at 150 days after treatment application (n = 48).

Source of variation	DF	Root nematode (J2s)		Soil nematode (J2s)		Pf	
		SS	%	SS	%	SS	%
Replication	5	0.61	19.06^{ns}	0.82	14.75^{ns}	0.61	12.98^{ns}
EM	1	0.35	10.94*	0.11	1.98^{ns}	0.00	0.00^{ns}
Cm	1	0.66	20.63**	1.98	35.61***	1.59	33.83***
EM × Cm	1	0.02	0.00^{ns}	0.00	0.00^{ns}	0.01	0.21^{ns}
Error	15	1.54	48.13	2.64	47.48	2.49	52.98
Total	23	3.20	100.0	5.56	100.0	4.70	100.0

ns = not significant at P ≤ 0.10, * = significant at P ≤ 0.10, ** = significant at P ≤ 0.05 and *** = significant at P ≤ 0.01.

Table 3.2 Effects of crude extracts of *Cucumis myriocarpus* (Cm) fruit on root nematode, soil nematode and final nematode population density (Pf) of *Tylenchulus semipenetrans* (N) at 150 days after treatment application (n = 48).

C. myriocarpus	Root nematode (J2s)	Soil nematode (J2s)	Pf
Cm_0	384	1867	2250
Cm_1	300	3593	3894
Impact (%)^y	-22**	93***	73***

Impact (%)^y = (treatment/control – 1) × 100.

In a three-way table the focus was on dry root mass, dry shoot mass, root/shoot ratio and stem diameter (Table 3.4), where the second order interaction had significant ($P \leq 0.05$) effects. Relative to $Em_0N_0Cm_0$, $Em_1N_1Cm_1$ interacted to increase dry root mass, dry shoot mass and root/shoot ratio by 9%, 11% and 14%, respectively, but reduced stem diameter by 6%. Relative to $Em_0N_0Cm_0$, nematodes alone ($Em_0N_1Cm_0$) reduced dry root mass, dry shoot mass, root/shoot ratio and stem diameter by 25%, 26%, 59% and 24%, respectively, while along with Cm ($Em_0N_1Cm_1$) they reduced root/shoot ratio by 9%. Relative to $Em_0N_0Cm_0$, *Cucumis* ($Em_0N_0Cm_1$) reduced dry root mass, dry shoot mass, root/shoot ratio and stem diameter by 16%, 18%, 25% and 20%, respectively.

Table 3.3 Sum of squares for dry root mass, dry shoot mass, plant height, root/shoot ratio and stem diameter as influenced by effective micro-organisms (EM), *Tylenchulus semipenetrans* (N) and crude extracts of *Cucumis myriocarpus* (Cm) fruit at 150 days after treatment application (n = 48).

Source of variation	DF	Dry root mass (g)		Dry shoot mass (g)		Plant height (cm)		Root/shoot ratio		Stem diameter (mm)	
		SS	%	SS	%	SS	%	SS	%	SS	%
Replication	5	56.76	8.49^{ns}	308.61	7.21^{ns}	676.34	15.39^{*}	26.60	4.57^{ns}	1.00	2.91^{ns}
EM	1	61.20	9.15^{***}	254.38	5.94^{***}	73.26	1.67^{ns}	54.61	9.38^{***}	1.86	5.42^{***}
N	1	1.27	0.19^{ns}	11.31	0.26^{ns}	51.88	1.18^{ns}	1.40	0.24^{ns}	0.04	0.12^{ns}
Cm	1	41.44	6.19^{**}	330.23	7.72^{**}	97.76	2.22^{ns}	69.12	11.88^{***}	1.08	3.15^{***}
EM × N	1	6.45	0.96^{ns}	140.43	3.28^{ns}	1.58	0.04^{ns}	84.80	14.57^{***}	0.38	1.11^{ns}
EM × Cm	1	5.07	0.76^{ns}	82.95	1.94^{ns}	136.35	3.10^{ns}	5.88	1.01^{ns}	0.79	2.30^{ns}
N × Cm	1	184.08	27.52^{***}	935.45	21.86^{***}	1351.50	30.75^{***}	103.84	17.84^{ns}	6.83	19.90^{***}
EM × N × Cm	1	27.30	4.08^{**}	198.86	4.65^{**}	0.35	0.01^{ns}	63.02	10.83^{***}	16.28	47.42^{***}
Error	35	285.36	42.66	2017.28	47.14	2006.24	45.65	172.71	29.68	6.07	17.68
Total	47	668.94	100.0	4279.49	100.0	4395.24	100.0	581.99	100.0	34.33	100.0

ns = not significant at P ≤ 0.10, * = significant at P ≤ 0.10, ** = significant at P ≤ 0.05 and *** = significant at P ≤ 0.01.

Table 3.4 A three-way table for dry root mass, dry shoot mass, root/shoot ratio and stem diameter as affected by second order interaction of effective micro-organisms (EM), *Tylenchulus semipenetrans* (N) and crude extracts of *Cucumis myriocarpus* (Cm) fruit at 150 days after treatment application (n = 48).

		Dry root mass (g)				Dry shoot mass (g)				Root/shoot ratio (g)				Stem diameter (mm)			
EM	N	Cm_0	$(\%)^y$	Cm_1	$(\%)^y$	Cm_0	$(\%)^y$	Cm_1	$(\%)^y$	Cm_0	$(\%)^y$	Cm_1	$(\%)^y$	Cm_0	$(\%)^y$	Cm_1	$(\%)^y$
EM_0	N_0	26.2	-	22.0	-16	58.1	-	47.8	-18	13.8	-	10.3	-25	8.8	-	7.0	-20
	N_1	19.7	-25	26.4	1	42.7	-26	58.3	0	5.6	-59	12.5	-9	6.7	-24	8.7	-1
EM_1	N_0	25.6	-2	25.7	-2	52.6	-9	55.7	-4	10.3	-25	12.7	-8	7.6	-14	8.6	-2
	N_1	23.6	-10	28.5	9	52.2	-10	64.8	11	11.9	-14	15.7	14	8.2	-7	8.3	-6

Impact $(\%)^y$ = (treatment/control – 1) × 100, where $EM_0N_0C_0$ = untreated control, $EM_0N_1C_0$ = nematode, $EM_0N_0C_1$ = *C. myriocarpus*, $EM_0N_1C_1$ = nematode + *C. myriocarpus*, $EM_1N_0C_0$ = EM, $EM_1N_1C_0$ = EM + nematode, $EM_1N_0C_1$ = EM + *C. myriocarpus*, $EM_1N_1C_1$ = EM + nematode + *C. myriocarpus*.

Relative to the untreated control (N_0Cm_0), *Cucumis* and nematode (N_1Cm_1) had no effect on plant height (Table 3.5). However, *Cucumis* (N_0Cm_1) alone and nematode alone reduced plant height by 11% and 9%, respectively.

Table 3.5 A two-way table for plant height as affected by first order interaction of *Tylenchulus semipenetrans* (N) and crude extracts of *Cucumis myriocarpus* (Cm) fruit at 150 days after treatment application (n = 48).

C. myriocarpus	N_0	Impact (%)[y]	N_1	Impact (%)[y]
Cm_0	103.23	-	94.17	-8.78
Cm_1	91.93	-10.95	104.43	1.16

Impact (%)[y] = (treatment/control – 1) × 100, where N_0C_0 = untreated control, N_1C_0 = nematode, N_0C_1 = *C. myriocarpus*, N_1C_1 = nematode + *C. myriocarpus*.

3.4 Discussion

Findings in this study involved a number of significant and non-significant interactions. Thus, the concept of interaction is briefly discussed. The fact that the first order interaction, EM × Cm, was not significant for nematode numbers, agrees with the original hypothesis which suggested that in GLT systems, microbial degradation of crude extracts of *C. myriocarpus* fruit is not essential since related active ingredients are released into soil solution through irrigation or rainwater (Mashela, 2002).

Reduction of nematode numbers in citrus roots was consistent with observation in other GLT studies, where the materials reduced *Meloidogyne* species in roots of tomato plants (Mashela and Mpati, 2002; Mashela *et al*., 2011). Increases of nematode numbers in soil and Pf under *Cucumis* treatment were unusual. However, since the growth of nematode population densities is cyclic (Pofu, 2008), interpretation of interactions of nematode results in this study requires great caution. One should not jump into conclusion that *Cucumis* increased nematode numbers.

Seinhorst (1965) advanced a mathematical explanation to describe the cyclic nature of growth in nematode population densities through the use of an equilibrium point (E) concept. In this concept, after nematode population densities pass E, due to subsequent competition for space (infection sites) and nutrient resources, nematode population densities decline with increasing time of infection. However, as the nematode population densities decrease, damaged roots generate new roots, which results in the upswing of nematode population densities (Pofu, 2008). In the current study, nematode population densities under nematode alone and *Cucumis* + nematodes behaved differently as graphically shown below (Figure 3.1). Due to crude extracts of *C. myriocarpus* fruit, upon initiation of *Cucumis* treatment, population densities in the inoculum started to decline, while those under control conditions increased. Should harvesting been done at T1, Pf under *Cucumis* could have been lower than under controls, as observed in other GLT studies (Mashela *et al.*, 2011). In this study, harvesting was done at T2, when population densities under control and *Cucumis* were in the downswing and upswings growth phases, respectively.

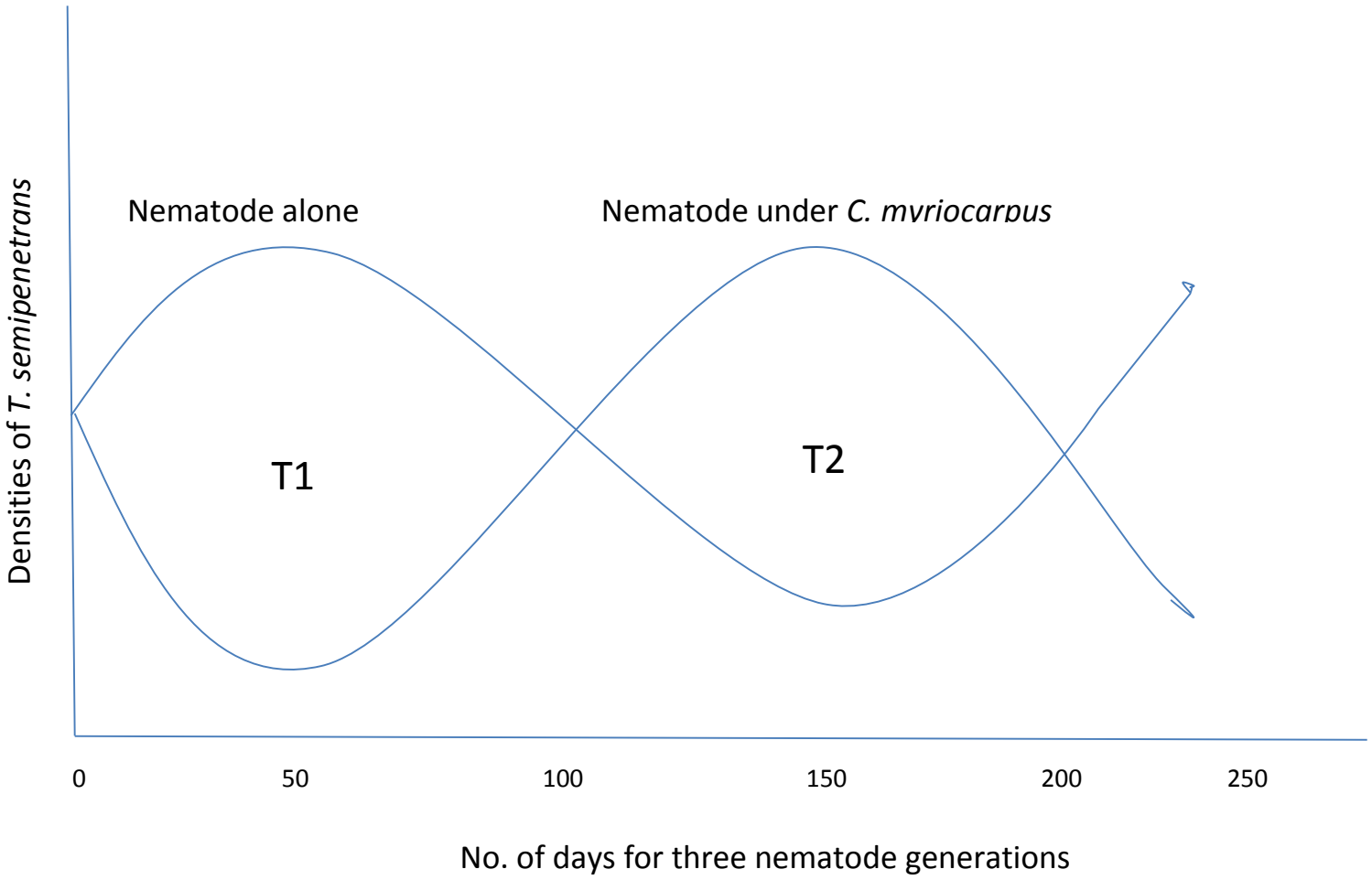


Figure 3.1 Relative cyclic population densities of *Tylenchulus semipenetrans* on rough lemon under untreated control and *Cucumis*-treated pots at 50 and 150 days after inoculation with 25 000 nematodes.

Mashela (2007) demonstrated that in the GLT system, crude extracts of *C. myriocarpus* fruit were active up to 56 days after application. In another 56-day study, crude extracts of fruit from this plant significantly reduced nematode population densities of *T. semipenetrans* (Mashela, 2007). In other short-term studies, the GLT systems at 56

days have had criticism that this short period does not allow sufficient time for crude extracts of *C. myriocarpus* to decompose (Mashela *et al*., 2011). In the current study, where the duration was almost three times the 56-day period, EM × *Cucumis* interaction for nematodes was not significant at 5% level of probability – which further supported the view that in GLT system, microbial decomposition is not required (Mashela, 2002).

The second order interaction (EM × N × Cm) was significant ($P \leq 0.01$) for most of the plant variables measured. At three-way table used to further analyse the data demonstrated that the interaction increased dry root mass, dry shoot mass and root/shoot ratio. This confirmed similar interactions in cowpea (*Vigna unguiculata*), where *Bradyrhizobium javanica* and nematodes improved plant growth (Mashela and Pofu, 2012). The EM × N × Cm interaction reduced stem diameter. Actually, this is the first observation where biological activities in citrus-nematode relations interacted to reduce stem diameter. Perhaps this could be ascribed to the fact that in abiotic × biotic × citrus interaction, this variable had hardly been measured (Mashela, 1992). Notwithstanding, reduced stem diameters in tomato plants infected by *Meloidogyne* species had been widely reported (Mashela *et al*., 2011). Various abiotic and biotic factors were shown to reduce root/shoot ratio, resulting in increased accumulation of non-structural carbohydrates, with the result that stem diameter declined (Mafeo, 2005; Mashela and Nthangeni, 2002a). Apparently, the reduction of stem diameter is a physical mechanisms used in reducing the concentrations of non-structural carbohydrates in order to avoid damaging osmotic potentials in root cells (Mashela and Nthangeni, 2002a).

In a three-way table, *T. semipenetrans* infection reduced dry root mass and root/shoot ratio, but had no effect on dry shoot mass and stem diameter. Generally, *T. semipenetrans* at low population densities is a non-aggressive nematode (O'Bannon and Esser, 1985), which is lost at high population densities (Mashela, 2007). Also, the effect of infection by this nematode is enhanced by abiotic factors such as salinity and/or any factor that reduces root/shoot ratio (Mashela, 1992; Mashela and Nthangeni, 2002a). Similarly, relative to untreated control, crude extracts of *C. myriocarpus* fruit alone drastically reduced all plant variables except plant height, which suggested that the product may be phytotoxic to citrus plants. Generally, crude extracts of *C. myriocarpus* fruit had been shown to be phytotoxic to various crops (Mafeo, 2012; Pelinganga, 2013). Actually, almost all commercial crops are highly sensitive to crude extracts of *C. myriopcarpus* fruit during germination (Mafeo, 2012). The CARD model demonstrated that three stages in response to increasing concentrations of allelochemicals could be identified, namely, stimulation, neutral and inhibition phases (Liu *et al.,* 2003). Mafeo (2012) adapted the CARD model to compute stimulatory concentrations of *C. myriocarpus* fruit in GLT systems for various crops. The model had since been successfully used to determine non-phytotoxic concentrations of fermented crude extracts of *C. africanus* and *C. myriocarpus* fruits in tomato production through what had since been termed botinemagation (Mashela *et al*., 2011; Pelinganga and Mashela, 2012; Pelinganga *et al*., 2012a,b). Botinemagation had been described as the use of botanicals for nematode suppression through irrigation systems, developed for large scale commercial systems, with the citrus industry being an ideal candidate (Mashela *et al.,* 2011).

Detailed analysis of the interactions demonstrated that in plant variables, EM alone and sometimes in interaction with other factors increased and/or reduced plant growth. Other reports (Ncube, 2008) suggested that EM could either increase or reduce plant growth. However, the mechanism involved is currently not clear.

3.5 Conclusions

The application interval of crude extracts of *C. myriocarpus* fruit in the GLT systems should be the normal period of 56 days since 150 days appeared to have been too long for the material to consistently suppress population densities of *T. semipenetrans.* Stimulatory concentrations of *C. myriocarpus* fruit should be computed, perhaps in the form of fermented crude extracts under botinemagation, in order to reduce the incidence of phytotoxicity. Detailed work under diverse environments is necessary in order to allow for the better understanding of the mechanisms involved in citrus-nematode-GLT systems.

CHAPTER 4
EFFECTS OF CRUDE EXTRACTS OF *CUCUMIS AFRICANUS* FRUIT ON *TYLENCHULUS SEMIPENETRANS* AND GROWTH OF ROUGH LEMON

4.1 Introduction

The potent chemical in wild watermelon (*Cucumis africanus*) fruit had been identified as cucurbitacin B ($C_{32}H_{48}O_8$), which is insoluble in water (Chen *et al*., 2005; Jeffrey, 1978). Potent chemicals from crude extracts of fruit of this plant had not been widely investigated on nematode suppression in the ground leaching technology (GLT) (Mashela *et al*., 2011). However, they consistently reduced nematodes in nematode-resistant *Cucumis* studies (Pofu *et al*., 2012) and botinemagation (Pelinganga, 2013; Pelinganga and Mashela, 2012; Pelinganga *et al*., 2012a,b).

Cucurbitacin B occurs in all organs of *C. africanus* plants (Jeffrey, 1978). Consequently, unlike *C. myriocarpus* plants where cucurbitacin A is confined to fruits and roots, *C. africanus* is not edible as a green vegetable. In GLT system, crude extracts of *C. africanus* fruit had not been documented in nematode suppression and growth of citrus plants. The objective of this study, therefore, was to determine the effect of crude extracts of *C. africanus* fruit with and without effective micro-organisms (EM) on population densities of *T. semipenetrans* growth of citrus when the application interval was equivalent to three generations of *T. semipenetrans*.

4.2 Materials and methods

The study was conducted parallel with that of *C. myriocarpus* under greenhouse conditions (Chapter 3). Thus, all aspects related to materials and methods were similar except that crude extracts of *C. africanus* fruit were used instead of *C. myriocarpus* fruit.

4.3 Results

The first order (EM × Ca) interaction was highly significant for eggs and juveniles in roots, but not for juveniles in soil and total eggs and juveniles (Pf) (Table 4.1). The EM treatment had no effect on population densities of *T. semipenetrans*, while crude extracts of *C. africanus* significantly affected the three variables, contributing 38%, 59% and 47% to total treatment variation in eggs and juveniles in roots, juveniles in soil and Pf, respectively. In a two-way table for root stages, relative to the untreated control, the EM_1Ca_1 reduced root stages by 70%, while EM and Ca separately reduced the variable by 56% and 80%, respectively (Table 4.2). Relative to untreated control, crude extracts of *C. africanus* fruit alone reduced eggs and juveniles in citrus roots by 80%, but increased juveniles in soil and Pf by 178% and 70%, respectively (Table 4.3).

Table 4.1 Sum of squares for root nematode, soil nematode and final nematode population density (Pf) as influenced by effective micro-organisms (EM) and crude extracts of *Cucumis africanus* (Ca) fruit at 150 days after treatment application (n = 48).

Source of variation	Root nematode (J2s)			Soil nematode (J2s)		Pf	
	DF	SS	%	SS	%	SS	%
Replication	5	1.02	7.93^{ns}	0.63	6.10^{ns}	0.25	4.66^{ns}
EM	1	0.59	4.58^{ns}	0.12	1.16^{ns}	0.11	0.03^{ns}
Ca	1	4.89	38.00***	6.04	58.53***	2.40	46.80***
EM × Ca	1	2.69	20.90***	0.03	0.29^{ns}	0.07	1.31^{ns}
Error	15	3.69	28.67	3.50	33.91	2.54	47.39
Total	23	12.87	100.0	10.32	100.0	5.36	100.0

ns = not significant at P ≤ 0.10, * = significant at P ≤ 0.10, ** = significant at P ≤ 0.05 and *** = significant at P ≤ 0.01.

Table 4.2 A two-way table for root nematode as affected by first order interaction of effective micro-organisms (EM) and crude extracts of *Cucumis africanus* (Ca) fruit at 150 days after treatment application (n = 48).

C. africanus	EM_0	$(\%)^y$	EM_1	$(\%)^y$
Ca_0	1252	-	539	-56
Ca_1	245	-80	374	-70

Impact $(\%)^y$ = (treatment/control – 1) × 100, where EM_0C_0 = untreated control, EM_0C_1 = *C. africanus*, EM_1C_0 = EM, EM_1C_1 = EM + *C. africanus*.

Table 4.3 Effects of crude extracts of *Cucumis africanus* fruit on root nematode, soil nematode and final nematode population density (Pf) of *Tylenchulus semipenetrans* at 150 days after treatment application (n = 48).

C. africanus	Root nematode (J2s)	Soil nematode(J2s)	Pf
Control	1252	1727	2978
C. *africanus*	245	4807	5052
Impact (%)[y]	-80***	178***	70***

Impact (%) = (treatment/control – 1) × 100.

The second order (EM × N × Ca) interaction did not have any effect on plant variables except for slightly significant (P ≤ 0.10) and highly significant (P ≤ 0.01) effects on dry root and dry shoot masses, respectively (Table 4.4). The interaction contributed approximately 4% and 12% to the total treatment variation in dry root mass and dry shoot mass, respectively. In contrast, the first order (N × Ca) interaction was highly significant (P ≤ 0.01) for plant height and stem diameter, contributing approximately 8% and 11% to the total treatment variation, respectively. The EM × Ca interaction did not have any effect on plant height and stem diameter, but had significant (P ≤ 0.05) effects on dry root mass, dry shoot mass and root/shoot ratio, contributing 17%, 8% and 9% to the total treatment variation of the three variables, respectively. The EM × N interaction had highly significant effect on dry root mass, plant height and root/shoot ratio,

contribution 13%, 13% and 14% to the total treatment variation of the variables, respectively.

Using a three-way table, relative to untreated control $Em_0N_0Ca_0$, $Em_1N_0Ca_1$, $Em_0N_1Ca_1$ and $Em_1N_1Ca_0$ reduced dry shoot mass by 8%, 12% and 13%, respectively (Table 4.5). *Cucumis africanus* alone reduced dry shoot mass by 4%, while nematode alone increased the variable by 13%. EM alone had no effect on dry shoot mass. EM × Nematode interaction had no effect on dry shoot mass and stem diameter (Table 4.6), but EM_1N_1 reduced dry root mass and plant height by 22% and 2%, respectively. The main factor, nematode alone increased dry root mass by 29%, but reduced plant height by 9%. Similarly, EM reduced plant height by 7%, but increased dry root mass by 16%.

Table 4.4 Sum of squares for dry root mass, dry shoot mass, plant height, root/shoot ratio and stem diameter as influenced by effective micro-organisms, *Tylenchulus semipenetrans* and crude extracts of *Cucumis africanus* fruit at 150 days after treatment application (n = 48).

Source of variation	DF	Dry root mass (g)		Dry shoot mass (g)		Plant height (cm)		Root/shoot ratio		Stem diameter (mm)	
		SS	%	SS	%	SS	%	SS	%	SS	%
Reps	5	46.98	5.71^{ns}	64.23	10.46^{ns}	583.91	33.12^{***}	0.197	17.00^{*}	5.71	18.75^{**}
Microbe (EM)	1	10.55	1.28^{ns}	9.72	1.58^{ns}	104.14	5.91^{**}	0.056	4.83^{*}	0.05	0.16^{ns}
Nematode (N)	1	10.93	1.33^{ns}	0.08	0.01^{ns}	1.80	0.10^{ns}	0.018	1.55^{ns}	1.15	3.78^{ns}
C. africanus (Ca)	1	39.06	4.75^{*}	20.54	1.03^{ns}	23.38	1.32^{ns}	0.025	2.16^{ns}	3.32	10.90^{***}
EM × N	1	108.30	13.16^{***}	3.85	0.63^{ns}	232.76	13.20^{***}	0.166	14.32^{***}	0.33	1.08^{ns}
EM × Ca	1	137.03	16.65^{***}	46.81	7.63^{***}	2.95	0.17^{ns}	0.110	9.49^{***}	0.75	2.46^{ns}
N × Ca	1	1.05	0.13^{ns}	0.19	0.03^{ns}	143.87	$8,16^{***}$	0.002	0.17^{ns}	3.21	10.54^{***}
EM × N × Ca	1	35.54	4.32^{*}	74.50	12.14^{***}	2.57	0.15^{ns}	0.002	0.17^{ns}	0.04	0.13^{ns}
Error	35	433.64	52.69	393.88	64.17	667.93	37.88	0.583	50.30	15.92	52.27
Total	47	823.06	100.0	613.81	100.0	1763.30	100.0	1.159	100.0	30.46	100.0

ns = not significant at P ≤ 0.10, * = significant at P ≤ 0.10, ** = significant at P ≤ 0.05 and *** = significant at P ≤ 0.01.

Table 4.5 A three-way table for dry shoot mass as affected by second order interaction of effective micro-organisms (EM), *Tylenchulus semipenetrans* (N) and crude extracts of *Cucumis africanus* (Ca) fruit at 150 days after treatment application (n = 48).

EM	N	Ca_0	$(\%)^y$	Ca_1	$(\%)^y$
EM_0	N_0	22.75	-	21.83	-4
	N_1	25.60	13	19.95	-12
EM_1	N_0	22.93	1	20.98	-8
	N_1	19.67	-13	22.95	1

Impact $(\%)^y$ = (treatment/control – 1) × 100, where $EM_0N_0C_0$ = untreated control, $EM_0N_1C_0$ = nematode, $EM_0N_0C_1$ = *C. africanus*, $EM_0N_1C_1$ = nematode + *C. africanus*, $EM_1N_0C_0$ = EM, $EM_1N_1C_0$ = EM + nematode, $EM_1N_0C_1$ = EM + *C. africanus*, $EM_1N_1C_1$ = EM + nematode + *C. africanus.*

Table 4.6 A two-way table for dry root mass, plant height and stem diameter as affected by first order interaction of effective micro-organisms (EM) and *Tylenchulus semipenetrans* (N) at 150 days after treatment application (n = 48).

	Dry root mass (g)				Plant height (cm)				Root/shoot ratio			
EM	N_0	$(\%)^y$	N_1	$(\%)^y$	N_0	$(\%)^y$	N_1	$(\%)^y$	N_0	$(\%)^y$	N_1	$(\%)^y$
EM_0	13.82	-	17.88	29	101.43	-	92.72	-9	0.61	-	0.71	16
EM_1	16.10	16	10.72	-22	94.12	-7	99.03	-2	0.71	16	0.55	-10

Impact $(\%)^y$ = (treatment/control – 1) × 100, where EM_0N_0 = untreated control, EM_0N_1 = nematode, EM_1N_0 = EM, EM_1N_1 = EM + nematode.

In EM × Ca interaction, there was no effect on plant height and stem diameter, but the interaction was highly significant ($P \leq 0.01$) for dry root mass, dry shoot mass and root/shoot ratio (Table 4.7). Relative to untreated control, EM_1Ca_1 increased dry root mass and root/shoot ratio by 18% and 25%, respectively, but reduced dry shoot mass by 8%. *Cucumis africanus* fruit alone reduced dry root mass, dry shoot mass and root/shoot ratio by 23%, 4% and 20%, respectively. In contrast, EM alone had no effects on dry root mass, dry shoot mass and stem diameter, but had slightly significant ($P \leq 0.10$) effect on root/shoot ratio. EM increased dry root mass and dry shoot mass by 16% and 23%, respectively.

Table 4.7 A two-way table for dry root mass, dry shoot mass and root/shoot ratio as affected by first order interaction of crude extracts of *Cucumis africanus* (Ca) fruit and effective micro-organisms (EM) at 150 days after treatment application (n = 48).

	Dry root mass (g)				Dry shoot mass (g)				Root/shoot ratio			
Ca	EM_0	$(\%)^y$	EM_1	$(\%)^y$	EM_0	$(\%)^y$	EM_1	$(\%)^y$	EM_0	$(\%)^y$	EM_1	$(\%)^y$
Ca_0	13.82	-	16.10	16	22.75	-	22.93	1	0.61	-	0.71	16
Ca_1	10.65	-23	16.25	18	21.83	-4	20.98	-8	0.49	-20	0.76	25

Impact $(\%)^y$ = (treatment/control – 1) × 100, where N_0C_0 = untreated control, EM_1C_0 = EM, EM_0C_1 = *C. africanus*, EM_1C_1 = EM + *C. africanus*.

In a two-way table, N_1Ca_1 had no effect on dry root mass, dry shoot mass and root/shoot ratio, but had highly significant ($P \leq 0.01$) effects on plant height and stem diameter (Table 4.8). Nematode alone reduced plant height and stem diameter by 9% and 12%, respectively, while *Cucumis* had negligible effects on the two variables, although the direction of the effect suggested that the material may reduce the variables.

Table 4.8 A two-way table for plant height and stem diameter as affected by first order interaction of crude extracts of *Cucumis africanus* (Ca) fruit and *Tylenchulus semipenetrans* (N) at 150 days after treatment application (n = 48).

	Plant height (cm)				Stem diameter (mm)			
C. africanus	N_0	(%)^y	N_1	(%)^y	N_0	(%)^y	N_1	(%)^y
Ca_0	101.43	-	92.72	-9	8.62	-	7.57	-12
Ca_1	99.40	-2	98.53	-3	8.33	-3	8.42	-2

Impact (%)^y = (treatment/control – 1) × 100, where N_0C_0 = untreated control, N_1C_0 = nematode, N_0C_1 = *C. africanus*, N_1C_1 = nematode + *C. africanus*.

4.4 Discussion

Findings in EM × *C. africanus* interaction with respect to nematode population densities contradicted those in EM × *C. myriocarpus* interactions (Chapter 3), where the interaction had no significant effect on any nematode variable. In this study the interaction was significant ($P \leq 0.05$) for eggs and juveniles in roots. This observation violates the long-standing belief that in GLT systems, when using crude extracts of *Cucumis* species, microbial degradation is not essential in the release of active ingredients. The observed interactions, however, should be interpreted with caution, more especially that crude extracts of this plant material increased juveniles in soil, which was similar to the observations under *C. myriocarpus* (Chapter 3). Thus, it is important to view the differences and similarities between the two materials on nematodes in relation to both the differences in their molecular structures and the duration from treatment to harvest, which was approximately three life cycles of *T. semipenetrans*.

Cucurbitacin A [cuccumin ($C_{27}H_{40}O_9$); leptodermin ($C_{27}H_{38}O_8$)] and cucurbitacin B ($C_{32}H_{48}O_8$) occur in C. *myriocarpus* and *C. africanus*, respectively (Jeffrey, 1978; Rimington, 1938). Different responses to the two *Cucumis* species had been consistently observed in resistance to *Meloidogyne* species (Pelinganga, 2013; Pofu, 2012). However, the duration is also quite important in the discussion and interpretation of observations as graphically shown previously (Chapter 3). The message that is emerging in this and the previous study (Chapter 3), is that the life cycle of a nematode species is not an appropriate yard stick in determining the application interval of these

two materials. In *M. incognita* the cycle is 19 days – depending on the prevailing temperature (Sikora and Fernadez, 2005) which translates to approximately three generations within a 56 day period which was recommended for *Meloidogyne* species (Mashela, 2002).

High population densities of the *T. semipenetrans* in soil with crude extracts of the two plant materials at 150 days also suggested that by this time, the materials might have negligible nematicidal or nematostatic residues in the soil, as graphically shown earlier (Chapter 3). In most cases, phytonematicides are applied at short intervals to eliminate this challenge. Pelinganga (2013) developed protocols for developing application intervals of phytonematicides from the two *Cucumis* species. In botinemagation, the application interval for crude extracts from *C. africanus* and *C. myriocarpus* are 17 and 16 days, respectively (Pelinganga, 2013). In the protocols, attention is taken to ensure that efficacy in nematode suppression is not achieved at the cost of phytotoxicity to the protected crops.

The reduction of nematode numbers under EM cultures had in the previous study (Chapter 3) or under botinemagation (Pelinganga, 2013) never been observed. Apparently, this could be the first of such reports in the current study. In EM cultures used in this study included actinomycetes bacteria, which release chitinases that have the capabilities to hydrolyse chitin (Ncube, 2008). The cuticle of plant-parasitic nematodes is, among other constituents, formed by chitin, along with the covering of the nematode bodies (cuticle), body cavities and stylets (Ferraz and Brown, 2000), which

may help to explain the current observations. Previously (Chapter 3), it was shown that phytonematicides have multi-site active ingredients, which enhances their capabilities in nematode suppression.

Significant ($P \leq 0.05$) interactions observed in the current study confirmed previous observations (Chapter 3). In the current study, subjection of interactions to three- or two-way tables revealed further interesting issues, which were not observed previously (Chapter 3). In a three-way table, relative to untreated control, nematode alone increased dry shoot mass, which was also observed in a two-way table for dry root mass. Generally, in plant-parasitic nematodes, at densities below the damage threshold point, nematode infection stimulates plant growth, resulting in the increase of variables similar to those in this trial (Wallace, 1973). Similar observations were reported in *Meloidogyne* species (Mashela *et al.*, 2011), while this appears to be the first report in *T. semipenetrans*. In this study at 150 days, population densities of *T. semipenetrans* in untreated controls were low in this and the previous trial (Chapter 3), which therefore, agrees with observations that at low densities nematode infection increased plant growth. Apparently, this is the first report of such findings for *T. semipenetrans* on rough lemon, although it is widely reported that *T. semipenetrans* is non-aggressive nematode (O'Bannon and Esser, 1985). *Cucumis* treatment, as explained previously (Chapter 3), at 150 days, the population density of *T. semipenetrans* had already increased to above the damage threshold levels, resulting in reduction of plant growth of citrus seedlings. In this and the previous study (Chapter 3), it is not clear why EM and Ca together

sometimes increased plant growth, while at other times the interaction reduced plant growth variables

4.5 Conclusions

In this study, as observed in the previous study (Chapter 3), the application interval of crude extracts of *C. africanus* fruit in the GLT systems should be the normal period of 56 days since 150 days appeared to have been too long for the material to consistently suppress population densities of *T. semipenetrans.* However, caution should be taken to ensure that phytotoxicity is avoided when the materials are used at shorter application interval to manage population densities of *T. semipenetrans*.

CHAPTER 5
SUMMARY, RECOMMENDATIONS AND CONCLUSION

5.1 Summary

The study investigated the responses of rough lemon (*Citrus jambhiri*) seedlings and population densities of the citrus nematode (*Tylenchulus semipenetrans*) to: (1) cucurbitacin A-containing phytonematicide from crude extracts of wild cucumber (*Cucumis myriocarpus*), with and without effective micro-organisms (EM) and (2) cucurbitacin B-containing phytonematicide from crude extracts of wild watermelon (*Cucumis africanus*), with and without EM. The ground leaching technology (GLT), which is being researched and developed as an alternative to synthetic nematicides was used. In GLT systems it was premised that (1) the plant materials do not require microbial degradation to release active ingredients for nematode suppression and (2) the application interval of the material is equivalent to three generations of the target nematode.

In this study, interactions between EM and crude extracts of *C. myriocarpus* fruit were not significant for all eggs and juveniles per unit, while those between EM and *C. africanus* fruit were significant at 5% level of probability for eggs and juveniles in roots. Also, in most of the plant variables, EM × *Cucumis* interactions were not significant at 5% level probability. The observation could be interpreted to imply that in GLT systems, microbial degradations were not essential for the efficacy of the materials in nematode suppression. The significant interaction between EM and *C. africanus* for nematode numbers would require additional work to justify a different explanation.

Generally, in *Cucumis*-treated plants, population densities of *T. semipenetrans* under both *C. myriocarpus* and C. *africanus* fruits were significantly higher than under untreated control. This apparent abnormality was described in terms of the cyclic nature of population densities of plant-parasitic nematodes, which was first described by Seinhorst (1965) using the concept of equilibrium point. Nematode densities under untreated control and *Cucumis* amendment started their growth towards an upswing and downswing directions, respectively. Due to the opposed cyclic nature, at 150 days, when plants were harvested, population densities under untreated control and *Cucumis* were having downswings and upswings, respectively. Thus, resulting in *Cucumis*-treated plants having high population densities, while untreated plants had low population densities.

Tylenchulus semipenetrans is perceived as a non-aggressive nematode (O'Bannon and Esser, 1985). The intensity of slow decline of citrus is dependent on the interaction of this nematode with abiotic factors such as salinity (Mashela, 1992). In this study infection by the nematode increased and/or decreased growth of rough lemon seedlings, depending on the prevailing population density. Generally, it had been shown under various studies that in population densities below the damage threshold, plant-parasitic nematodes increased plant growth (Mashela *et al*., 2010; Wallace, 1973), while above the damage threshold, parasitism reduced plant growth. This was the first report where *T. semipenetrans* infection increased growth of citrus.

5.2 Recommendations

Results in this study suggested the need to quantify non-phytotoxic quantities of the two *Cucumis* species in citrus for the management of population densities of *T. semipenetrans.* The Curve-fitting Allelochemical Response Dosage (CARD) model (Liu *et al*., 2003) could be used to determine biological indices of the materials on various citrus species in order to allow for computation of non-phytotoxic concentrations of the materials using protocols developed by Pelinganga (2013). Incidentally, the studies could be conducted using botinemagation (Pelinganga, 2013), since this technology is cost-effective than the GLT system (Mashela *et al*., 2011). Future studies should also include the influence of these materials on quality of citrus fruits.

5.3 Conclusions

Application of crude extracts of *C. myriocarpus* and *C. africanus* fruits consistently altered cyclic growth of population densities of *T. semipenetrans* in citrus, suggesting that the materials were highly effective in suppression of this nematode species. However, since population increases of *T. semipenetrans* are seasonal (Mashela, 1992), proper studies are required to determine when to apply the materials for nematode suppression. The adoption of botinemagation using any of the two materials by the citrus industry would enhance the commercialisation prospects of the two products.

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APPENDICES

APPENDIX 3.1 Analysis of variance table for *Tylenchulus semipenetrans* population densities in roots of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	0.61	0.12	1.19	0.36
Effective microbe (EM)	1	0.35	0.35	3.44	0.08
C. myriocarpus (Cm)	1	0.66	0.66	6.44	0.02
EM × Cm	1	0.02	0.02	0.20	0.66
Error	15	1.54	0.10		
Total	23	3.20			

APPENDIX 3.2 Analysis of variance table for *Tylenchulus semipenetrans* population densities in soil.

Source of variation	DF	SS	MS	F	P
Replication	5	0.82	0.16	0.93	0.49
Effective microbe (EM)	1	0.11	0.11	0.65	0.43
C. myriocarpus (Cm)	1	1.98	1.98	11.27	0.00
EM × Cm	1	0.00	0.00	0.01	0.93
Error	15	2.64	0.18		
Total	23	5.56			

APPENDIX 3.3 Analysis of variance table for *Tylenchulus semipenetrans* population densities in total (root + soil).

Source of variation	DF	SS	MS	F	P
Replication	5	0.61	0.12	0.73	0.61
Effective microbe (EM)	1	0.00	0.00	0.02	0.88
C. myriocarpus (Cm)	1	1.59	1.59	9.55	0.00
EM × Cm	1	0.01	0.01	0.08	0.79
Error	15	2.49	0.17		
Total	23	4.70			

APPENDIX 3.4 Analysis of variance for plant height of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	676.34	135.27	2.36	0.06
Effective microbe (EM)	1	73.26	73.26	1.28	0.27
Nematode (N)	1	51.88	51.88	0.90	0.35
C. myriocarpus (Cm)	1	97.76	97.76	1.71	0.20
EM × N	1	1.58	1.58	0.03	0.87
EM × Cm	1	136.35	136.35	2.38	0.13
N × Cm	1	1351.50	1351.50	23.58	0.00
EM × N × Cm	1	0.35	0.35	0.01	0.94
Error	35	2006.24	57.32		
Total	47	4395.24			

APPENDIX 3.5 Analysis of variance for stem diameter of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	1.00	0.20	1.16	0.35
Effective microbe (EM)	1	1.86	1.86	10.74	0.00
Nematode (N)	1	0.04	0.04	0.23	0.63
C. myriocarpus (Cm)	1	1.08	1.08	6.25	0.01
EM × N	1	0.38	0.38	2.19	0.15
EM × Cm	1	0.79	0.79	4.55	0.04
N × Cm	1	6.83	6.83	39.43	0.00
EM × N × Cm	1	16.28	1.28	93.92	0.00
Error	35	6.07	0.17		
Total	47	34.33			

APPENDIX 3.6 Analysis of variance for dry shoot mass of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	308.61	61.72	1.07	0.39
Effective microbe (EM)	1	254.38	254.38	4.41	0.04
Nematode (N)	1	11.31	11.31	0.20	0.66
C. myriocarpus (Cm)	1	330.23	330.23	5.73	0.02
EM × N	1	140.43	140.43	2.44	0.13
EM × Cm	1	82.95	82.95	1.44	0.24
N × Cm	1	935.45	935.45	16.23	0.00
EM × N × Cm	1	198.86	198.86	3.45	0.07
Error	35	2017.28	56.64		
Total	47	4279.49			

APPENDIX 3.7 Analysis of variance for dry root mass of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	56.76	11.35	1.39	0.25
Effective microbe (EM)	1	61.20	61.20	7.51	0.01
Nematode (N)	1	1.27	1.27	0.16	0.70
C. myriocarpus (Cm)	1	41.44	41.44	5.08	0.03
EM × N	1	6.45	6.45	0.79	0.38
EM × Cm	1	5.07	5.07	0.62	0.44
N × Cm	1	184.08	184.08	22.58	0.00
EM × N × Cm	1	27.30	27.30	3.35	0.08
Error	35	285.36	8.15		
Total	47	668.94			

APPENDIX 3.8 Analysis of variance for root/shoot ratio of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	26.60	5.32	1.08	0.39
Effective microbe (EM)	1	54.61	54.61	11.07	0.00
Nematode (N)	1	1.40	1.40	0.28	0.60
C. myriocarpus (Cm)	1	69.12	69.12	14.01	0.00
EM × N	1	84.80	84.80	17.18	0.00
EM × Cm	1	5.88	5.88	1.19	0.28
N × Cm	1	103.84	103.84	21.04	0.00
EM × N × Cm	1	63.02	63.02	12.77	0.00
Error	35	172.71	4.94		
Total	47	581.99			

APPENDIX 4.1 Analysis of variance table for *Tylenchulus semipenetrans* population densities in roots of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	1.02	0.20	0.83	0.55
Effective microbe (EM)	1	0.59	0.59	2.39	0.14
C. africanus (Ca)	1	4.89	4.89	19.90	0.00
EM × Ca	1	2.69	2.69	10.95	0.00
Error	15	3.69	0.25		
Total	23	12.87			

APPENDIX 4.2 Analysis of variance table for *Tylenchulus semipenetrans* population densities in soil.

Source of variation	DF	SS	MS	F	P
Replication	5	0.63	0.13	0.54	0.74
Effective microbe (EM)	1	0.12	0.12	0.53	0.48
C. africanus (Ca)	1	6.04	6.04	25.88	0.00
EM × Ca	1	0.03	0.03	0.14	0.71
Error	15	3.50	0.23		
Total	23	10.32			

APPENDIX 4.3 Analysis of variance table for *Tylenchulus semipenetrans* population densities in total (root + soil).

Source of variation	DF	SS	MS	F	P
Replication	5	0.25	0.05	0.29	0.91
Effective microbe (EM)	1	0.00	0.00	0.01	0.93
C. africanus (Ca)	1	2.51	2.51	14.81	0.00
EM × Ca	1	0.07	0.07	0.41	0.53
Error	15	2.54	0.17		
Total	23	5.36			

APPENDIX 4.4 Analysis of variance for plant height of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	583.91	116.78	6.12	0.00
Effective microbe (EM)	1	104.14	104.14	5.46	0.03
Nematode (N)	1	1.80	1.80	0.09	0.76
C. africanus (Ca)	1	23.38	23.38	1.23	0.28
EM × N	1	232.76	232.76	12.20	0.00
EM × Ca	1	2.95	2.95	0.15	0.70
N × Ca	1	143.87	143.87	7.54	0.00
EM × N × Ca	1	2.57	2.57	0.13	0.72
Error	35	667.93	667.93		
Total	47	1763.30	1763.30		

APPENDIX 4.5 Analysis of variance for stem diameter of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	5.71	1.14	2.51	0.05
Effective microbe (EM)	1	0.05	0.05	0.10	0.75
Nematode (N)	1	1.15	1.15	2.52	0.12
C. africanus (Ca)	1	3.32	3.32	7.29	0.01
EM × N	1	0.33	0.33	0.72	0.40
EM × Ca	1	0.74	0.74	1.63	0.21
N × Ca	1	3.21	3.21	7.07	0.01
EM × N × Ca	1	0.04	0.04	0.08	0.78
Error	35	15.92	0.45		
Total	47	30.45			

APPENDIX 4.6 Analysis of variance for dry shoot mass of rough lemon seedlings.

Source of variation	DF	SS	MS	F	P
Replication	5	64.23	12.85	1.14	0.36
Effective microbe (EM)	1	9.72	9.72	0.86	0.36
Nematode (N)	1	0.08	0.08	0.01	0.93
C. africanus (Ca)	1	20.54	20.54	1.83	0.19
EM × N	1	3.85	3.85	0.34	0.56
EM × Ca	1	46.81	46.81	4.16	0.05
N × Ca	1	0.19	0.19	0.02	0.88
EM × N × Ca	1	74.50	74.50	6.62	0.01
Error	35	393.88	11.25		
Total	47	613.81			

APPENDIX 4.7 Analysis of variance for dry root mass of rough lemon seedlings.

Replication	DF	SS	MS	F	P
Source	5	46.98	9.40	0.76	0.40
Effective microbe (EM)	1	10.55	10.55	0.85	0.24
Nematode (N)	1	10.93	10.93	0.88	0.36
C. africanus (Ca)	1	39.06	39.06	3.15	0.06
EM × N	1	108.30	108.30	8.74	0.01
EM × Ca	1	137.03	137.03	11.06	0.00
N × Ca	1	1.05	1.05	0.08	0.63
EM × N × Ca	1	35.54	35.54	2.87	0.08
Error	35	433.64	12.39		
Total	47	823.06			

APPENDIX 4.8 Analysis of variance for root/shoot ratio of rough lemon seedlings.

Replication	DF	SS	MS	F	P
Source	5	0.197	0.04	0.34	0.06
Effective microbe (EM)	1	0.056	0.06	0.10	0.09
Nematode (N)	1	0.018	0.02	0.03	0.25
C. africanus (Ca)	1	0.025	0.03	0.04	0.34
EM × N	1	0.166	0.17	0.28	0.00
EM × Ca	1	0.110	0.11	0.19	0.01
N × Ca	1	0.002	0.00	0.00	0.98
EM × N × Ca	1	0.002	0.00	0.00	0.98
Error	35	0.583	0.58		
Total	47	1.159	1.16		