AGGRESSIVENESS AND IDENTIFICATION OF *TYLENCHULUS SEMIPENETRANS* BIOTYPE IN SOUTH AFRICA

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[redacted]

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree Master of Agricultural Management (Plant Protection) 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, and related materials contained herein had been duly acknowledged.

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DEDICATION

To my beloved husband who I have always wanted to be my husband.

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semipenetrans isolates from Limpopo and Mpumalanga citrus growing area on two citrus seedling rootstocks.

ABSTRACT

Studies were initiated to investigate (1) the aggressiveness of the citrus nematode (*Tylenchulus semipenetrans* Cobb) isolates from two provinces in South Africa (2 experiements) and (2) the biotype of *T. semipenetrans* in South Africa. In the aggressive study, isolates from Limpopo and Mpumalanga Provinces were used on Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) and rough lemon (*Citrus jambhiri*) seedling rootstocks under greenhouse conditions (18 experiements). Each seedling was inoculated with 0, 10 000, 20 000, 30 000 and 40 000 J2s of *T. semipenetrans* isolates, which were arranged in a randomised complete block design, with six replications. At 120 days, the reproductive factor of *T. semipenetrans* isolate from Mpumalanga Province was significantly higher than that from Limpopo Province. Similarly, due to its higher relative impact on the reproductive factor values, the Mpumalanga isolate reduced plant growth variables more than the Limpopo isolate. Consequently, the Mpumalanga isolate was viewed as being more aggressive than the Limpopo isolate, suggesting that there might be genetic variability and/or adaptation in populations from the two locations. A national study, comprising *T. semipenetrans* isolates from 18 citrus-producing district municipalities in South Africa was then initiated under greenhouse conditions using isolates from each district – for a total of 18 separate experiments. Three differential hosts, *viz*. rough lemon, *P. trifoliata* and olive (*Olea europaea*), served as treatments, arranged in a randomised complete block design, with 15 replications. Initially, an

orchard was randomly selected in each of the six citrus-producing provinces, *viz*. Eastern Cape, KwaZulu Natal, Limpopo, Mpumalanga, North West and Western Cape. Three-month old differential host seedlings were inoculated with approximately 10 000 J2s of *T. semipenetrans* and allowed to establish and grow under greenhouse conditions. At 120 days, penetration indices and standardised reproductive potentials/g roots demonstrated that *T. semipenetrans* failed to reproduce and develop on olive, but reproduced and developed on the other two hosts. Using *T. semipenetrans* biotype classification system, findings suggested that the biotype in citrus-producing district municipalities was Poncirus biotype. This biotype reproduces on *P. trifoliata* and hybrid rootstocks, which therefore, suggested that trifoliate orange and its hybrid rootstocks were not suitable for use in managing population nematode densities of *T. semipenetrans* in South Africa. In conclusion, results of this study demonstrated that the South African *T. semipenetrans* biotype was Poncirus, which suggested different management decisions and strategies for the citrus industry with regard to the management of this nematode.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

The South African Citrus industry, after Spain, leads export markets in the production of fresh fruits (Rabe and Von Broemben, 1991). The industry has a total of approximately 2 million trees, which are predominantly situated in six provinces, namely, Eastern Cape, KwaZulu Natal, Limpopo, Mpumalanga, North West and Western Cape. Climatically, the provinces differ from tropical, subtropical to Mediterranean, while edaphically they differ from low to high potential Agricultural soils.

1.1.1 Description of the problem

The citrus nematode (*Tylenchulus semipenetrans* Cobb 1913) causes immense damage in citrus trees (Milne, 1982), with affected trees exhibiting reduced vigour, leaf chlorosis, dieback of young twigs, leaf abscission and reduced fruit yield and quality (O'Bannon and Esser, 1985) – a disease collectively referred to as slow decline of citrus (Cobb, 1913; Thomas, 1913). Management of *T. semipenetrans* had since the withdrawal of synthetic nematicides depended on the use of resistant rootstocks (Duncan, 2009). However, due to widespread occurrence of nematode biotypes, it is increasingly difficult to rely on nematode-resistant genotypes without first confirming the aggressiveness and/or identity of the local biotype using empirical tests. Biotypes are nematodes within the same species which are morphologically similar, but have differential host preferences (O'Bannon and Esser, 1985). Biotypes of the citrus nematode had been reported to overcome nematode resistance in citrus rootstocks (Inserra *et al*., 1980). Thus, regular tests on dynamics of biotypes within a

citrus-producing region are important to ensure sustainable use reliant of nematode-resistant rootstocks.

Generally, the citrus nematode is non-aggressive and does not kill trees (O'Bannon and Esser, 1985), but the overall health of infected trees gradually decline overtime (Van Gundy and Kirkpatrick, 1964). In various citrus-producing regions of South Africa, the extent of slow decline may not depict the state of nematode numbers (Kwaye *et al*., 2008), In other words, healthy looking trees may have high nematode numbers, while trees with extensive decline symptoms may have few nematodes. Slow decline of citrus results in reduced fruit yield (O'Bannon and Esser, 1985), which have been associated with reduced fruit size due to K deficiency (Mashela, 1992). Incidentally, the citrus-producing regions have wide differences in quality water for irrigation, which differs from high to poor quality irrigation water (Matekere, 2012).

Cohn (1976) conducted survey of the citrus nematode in South Africa and established that there were areas with quite high population densities of this nematode. Previously, the population densities of this nematode were managed using synthetic nematicides. However, when legislative restrictions were placed in 2005, most highly effective products were withdrawn from the agrochemical markets. Incidentally, reduction in fruit size results in economic losses since fruits are graded by size, with large fruits retaining premium prices (Rabe and Von Broemben, 1991). In some regions, nematode-infected trees suffer from alternate fruit bearing, with on-years characterised by numerous small fruits, while off-years have uneconomically few, but excessively large fruits.

Although *T. semipenetrans* may be less aggressive (O'Bannon and Esser, 1985), its effects are environment dependent (Harding, 1954). Various studies have since shown that this nematode interacts with salinity in a manner which results in cyclic nematode populations (Machmer, 1958; Mashela *et al*., 1992a). Additionally, salinity might interfere with nematode resistance (Mashela *et al*., 1992b), while infection by *T. semipenetrans* interferes with salt-tolerance in salt-tolerant citrus rootstocks (Castle *et al*., 1993; Mashela and Nthangeni, 2002). Incidentally, all commercially available *T. semipenetrans*-resistant rootstocks are highly susceptible to salinity, while all salt-tolerant rootstocks are susceptible to infection by this nematode (Mashela and Nthangeni, 2002). Consequently, although resistant rootstocks are considered the most effective method to manage plant-parasitic nematodes, resurgence of new virulent isolates of *T. semipenetrans* that are capable of overcoming resistance in resistant genotypes may negate the perceived economic importance of these rootstocks (Inserra *et al*., 1980).

Worldwide, economic competition for high quality water among households, agriculture and mining increasingly persuades irrigation growers to resort to the use of marginal water (De Clercq *et al*., 2009; Ould Ahmed *et al*., 2010). Intensive urbanisation of high potential agricultural soil with much reliance on chlorinated water and industrial detergents that contain high concentrations of Ca^{2+} and Na^{+} exacerbate salinity challenges in agriculture (Keremane and McKay, 2006). Upon disposal, much municipality and industrial wastewater intrude into natural resources, increasing contamination of surface and underground water (Ghassemi *et al*., 1995). Globally, in the early 1990s salinity was estimated to have affected 19.5% and 2.1% of irrigated and dry-land, respectively (Ayers and Westcot, 1994), with the current status being probably much higher. Estimated global crop losses due to salinity were

in the 1990s at US$11 billion per annum (Ghassemi *et al*., 1995). Probably, the current status has worsened. Slow decline symptoms of citrus are severe in areas with salinity problems (Duncan *et al*., 1995; O'Bannon and Esser, 1985). Interaction of the citrus nematode and salinity is of practical concern because various salinity ions in irrigation water have been increasing in most citrus-producing regions in the world (Nabors, 1984; Syvertsen *et al*., 1989).

1.1.2 Impact of research problem

Following the suggestion that the citrus nematode biotype in South Africa was the Mediterranean (Cohn, 1976), most citrus plantings were done on trifoliate orange (*Poncirus trifoliata* L.) rootstocks (Rabe and Von Broemben, 1991). By the 1980, 21% of the citrus plantings in South Africa were under *P. trifoliata* rootstocks. However, by 1994, most growers have felled citrus trees on *P. trifoliata* due to its host-status to *T. semipenetrans* biotype that occurs in South Africa, and its accumulation of salinity ions (Rabe and Von Broemben, 1991).

1.1.3 Possible causes of the research problem

The citrus nematode biotype in South Africa has not been empirically identified. *Poncirus trifoliata* rootstock is a non-host to *Mediterranean* biotype (Inserra *et al*., 2009). Since, the South African citrus nematode feeds and reproduces on *P. trifoliata*, possibilities exist that the biotype was misidentified. Also, due to differences from region to region, possibilities exist that aggressiveness of the nematodes differs regionally.

1.1.4 Proposed solutions

The successful management of nematodes using nematode resistant calls for the accurate identification of the nematode biotypes. Aggressiveness and differential host tests would resolve the issues raised under possible causes of the research problem. Consequently, isolates should be collected from various citrus regions and tests performed under greenhouse conditions to determine the aggressiveness and differential host-status of the citrus nematode in South Africa.

1.2 Problem statement

The citrus industry is important in job creation and the overall economy of South Africa. However, the contribution of this industry in job creation and the overall economy is threatened by the high incidence of slow decline of citrus, which is a function of the environment, the rootstock and the citrus nematode. In South Africa, for various unexplained reasons, empirical studies on the aggressiveness and the identity of the citrus nematode biotype have not been undertaken. The researcher participated in the National Citrus Nematode Biotype (NCNB) study to determine the aggressiveness and to identify the citrus nematode biotype in South Africa.

1.3 Motivation

Currently, although South Africa is, after Spain, number two, in export of citrus fruits, certain aspects of the biology of *T. semipenetrans* have not been quantified. Empirically-supported information on aggressiveness and the citrus nematode biotype would enhance management decisions, nematode-resistant rootstock breeding programmes and legislative regulatory practices in South Africa.

1.4 Aim

The aim of this study was to characterize the aggressiveness and to identity the citrus nematode biotype in citrus-producing regions of South Africa.

1.5 Objectives

1. To determine the aggressiveness of *T. semipenetrans* isolates from Limpopo and Mpumalanga Provinces.
2. To investigate whether *T. semipenetrans* biotype in citrus-producing district municipalities of South Africa could was the Mediterranean biotype.

1.6 Hypotheses

1. The aggressiveness of *T. semipenetrans* isolates from Limpopo and Mpumalanga Provinces is not different.
2. *Tylenchulus semipenetrans* biotype in citrus-producing district municipalities of South Africa was not the Mediterranean biotype.

1.7 Structure of the dissertation

The dissertation was designed using the Senate-approved technical format of the University of Limpopo. Findings were summarised in the abstract, followed by detailed background to the research problem (Chapter 1), which was in turn followed by a review of relevant literature on the research problem (Chapter 2). Empirical studies comprised those for achieving Objective 1 (Chapter 3) and Objective 2 (Chapter 4). Finally, findings were summarised, with related recommendations being provided regarding the empirically-based identified *T. semipenetrans* biotype (Chapter 5).

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

The identified problem statement encompasses the fact that empirical studies on aggressiveness and identity of the citrus nematode biotype in South Africa had not been undertaken for various unexplained reasons. Review of literature, therefore, was limited to (1) work done on the research problem statement, (2) work not yet done and (3) how the identified gaps in the problem could be addressed in this study.

2.2 Work done on the problem statement

The focus was primarily on the aggressiveness and biotypes of the citrus nematode with reference to the citrus industry-nationally and internationally.

2.2.1 Aggressiveness of *Tylenchulus semipenetrans*

Aggressiveness is a quantitative measure of the general reproductive potential and damage potential of a given nematode without regard to the resistant genes (Shaner *et al*., 1992). Thus, comparisons on aggressiveness of nematode isolates should be done on genotypes with a variety of resistant genes. In plant-parasitic nematology, aggressiveness is described using the same concepts as those in nematode resistance, namely (i) host-status and (ii) host-sensitivity (Seinhorst, 1967), which describe the reproductive and damage potential of nematodes, respectively, on a given host. In most cases, aggressiveness tests, just like the biotype tests are undertaken in rootstocks with different degrees of host-status (Inserra *et al*., 2009), except that in biotype tests host-sensitivity is not performed. Also, aggressiveness is different from competition since in the latter the two nematode isolate are placed in

the same pot, while aggressiveness they are tested simultaneously but in separate pots. In this review, evidence of aggressiveness studies using the citrus nematode isolate could not be found.

2.2.2 Biotypes of *Tylenchulus semipenetrans*

Biotypes of the citrus nematode had been identified in various countries using differential host tests (Baines *et al*., 1974; Baines *et al*., 1969; Gottlieb *et al*., 1986; Inserra *et al*., 2009, 1980, Lamberti *et al*., 1976; O'Bannon *et al*., 1977; Stokes *et al*., 1969; Verdejo-Lucas, 1992). Worldwide, since the withdrawal of most nematicides from agrochemical markets, population densities of the citrus nematode are being increasingly managed through nematode-resistant rootstocks (Duncan, 2009; Inserra *et al*., 2009). However, in nematode management reliance on any exotic nematode-resistant genotype is complicated by inter- and intra-continental existence of biotypes, which are morphologically identical, but could be separated using differential host tests and/or molecular markers (Devran and Sogut, 2011).

In the citrus nematode, six biotypes had since been identified in various citrus-producing countries (Baines *et al*., 1974, 1969; Gottlieb *et al*., 1986; Inserra *et al*., 2009, 1980; O'Bannon *et al*., 1977; Stokes, 1969; Verdejo-Lucas, 1992). Using the classification of *T. semipenetrans* biotypes (Inserra *et al*., 1980), the most commonly distributed biotypes are the Citrus, Mediterranean and Poncirus biotypes. The three biotypes infect and reproduce equally on *Citrus* species, but differentially on Carrizo citrange (*Citrus sinensis* L. × *Poncirus trifoliata L*.), grape (*Vitis vinifera* L.), olive (*Olea europaea* L.), persimmon (*Diospyrus kaki* L.) and *P. trifoliata*. Originally, Baines *et al*. (1974) demonstrated that four *T. semipentrans* biotypes existed in California and designated them as C1, C2, C3 and C4 biotypes. According to Baines

et al. (1969), the California C3 biotype infects grape (*Vitis vinifera*) but not olive (*Olea europaea*), while C1 and C2 biotypes reproduce on grape and C1, while C2 and C4 reproduce on persimmon and olive, but poorly on trifoliate orange. O'Bannon *et al*. (1977) studied the host range of C1 and C3 using isolates from Arizona, Texas and Florida on *Citrus*, trifoliate and Carrizo citrange. In that study, C3 biotype was separated and called the 'Poncirus biotype' because it reproduced on *P. trifoliata.* Lamberti *et al*. (1976) reported another citrus nematode biotype from Italy that did not reproduce on olive and trifoliate but infected grape and persimmon which was code-named the Mediterranean biotype. In total, six citrus nematode biotypes had been identified worldwide, five of these biotypes reproducing on *Citrus*, *Poncirus* and their hybrids, while the sixth citrus nematode biotype from Florida was reported to reproduce only on *Andropagon rhizomatus* grass (Stokes, 1969). On the basis of additional host-range studies with the citrus nematode in several countries, it was possible to demonstrate that the five biotypes that are distributed worldwide can actually be reduced to four biotypes (O'Bannon and Ford, 1977):

- Poncirus biotype reproduces actively on *Citrus species, P. trifoliata,* their hybrids and grape, but not on olive. This biotype, which probably originated in Japan, appeared to be widespread in California, India and possibly elsewhere.
- Citrus biotype which reproduces poorly on *P. trifoliata* but infects *Citrus* species, Carrizo citrange, Troyer citrange, olive, grape and persimmon. California C2 and C4 were included in the Citrus biotype because they did not consistently reproduce on all *P. trifoliata* selections (Baines *et al*., 1969).

- Mediterranean biotype is very close to the Citrus biotype, with more or less the same host range, except that it does not reproduce on olive. This biotype occurs in all citrus-producing countries of the Mediterranean Basin region.
- Grass biotype reproduces only on *A. rhizomatus* and has only been reported in Florida.

Probably, other unidentified *T. semipenetrans* biotypes are present in other areas of the world. Using *T. semipenetrans* biotype classification as reviewed by O'Bannon and Ford (1977); Inserra *et al.* (1980) simplified the citrus nematode biotype classification in order to enhance empirical studies (Table 2.1).

Table 2.1 Simplified classification of *Tylenchulus semipenetrans* biotypes (Inserra *et al.*, 1980).

Biotype	Differential hosts					
	Citrus	Grape	Persimmon	Carrizo	Trifoliate	Olive
Citrus	+	+	+	+	-	+
Mediterranean	+	+	+	+	-	-
Poncirus	+	+	+	+	+	-

Symbol (+) indicates nematode reproduction; symbol (–) indicates poor infection.

2.2.2.1 Distribution of *Tylenchulus semipenetrans* biotypes

The host range for the citrus nematode is confined to citrus, olives, persimmons, grapes and several ornamental crops (O'Bannon and Ford, 1977). The nematode has a worldwide geographical distribution (O'Bannon and Ford, 1977) and occurs in 95% of citrus in California, while it is also common in eastside San Joaquin Valley on grapes (Baines *et al*., 1974, 1969), in Florida (Inserra *et al*., 2009, 1980; O'Bannon and Ford, 1977; O'Bannon *et al*., 1977), in Italy (Lamberti *et al*., 1976), in Spain (Verdejo-Lucas, 1992), in South Africa (Mashela *et al*., 2012; Kwaye *et al*., 2008) and possibly in other citrus-producing countries of the world (Inserra *et al*., 2009), The distribution of a particular nematode species can be the result of factors that include the host plant, environment and the nematode as depicted in the disease triangle (Agrios, 2005). Distribution may reflect a lengthy association with natural vegetation or a relatively recent introduction to a new region (McKenry and Roberts, 1985). Generally, climate or temperature preference, soil textural preference, host crop preference, area of initial introduction and movement of contaminated plant or

agricultural equipment, each can significantly influence nematode distribution, depending on subsequent environmental adaptation and parasitic habitat of a particular nematode species (McKenry and Roberts, 1985).

The distribution of the citrus nematode biotypes is not well defined (Inserra *et al*., 1980). From results of host preference studies, it appears that the citrus biotype occurs predominantly in California and Italy (Baines *et al*., 1974; Inserra *et al*., 1980). The Mediterranean biotype had been reported in the Mediterranean Basin (Inserra *et al*., 1980). Poncirus biotype is also present in California, Israel and Japan (Baines *et al*., 1974; Gottlieb *et al*., 1986). The citrus nematode populations from Florida, Arizona and Texas may either be the Citrus or Mediterranean biotypes, because they infect and reproduce poorly on *P. trifoliata*, while their infection potential on olive trees is unknown (O'Bannon *et al*., 1977).

Citrus and Mediterranean biotypes are probably more widely distributed than the Poncirus biotype, because rootstocks of the genus Citrus such as limes (*Citrus aurantifolia* Swingle), rough lemon (*C. jambhiri*) and sour orange (*Citrus aurantium* Swingle) have been more commonly used worldwide than *P. trifoliata* (Inserra *et al*., 1980). The adoption of *P. trifoliata* as a major rootstock in Japan and other parts of Asia to produce dwarf trees and the successive introduction of this rootstock and its nematode-resistant hybrids in other parts of the world has possibly induced the selection and dissemination of the Poncirus biotype (Inserra *et al*., 1980).

2.2.2.2 Origin of citrus in South Africa

Cohn (1976) traced the original route of citrus trees to South Africa and on the basis of that route, concluded that *T. semipenetrans* biotype was Mediterranean. In order to enhance the understanding of the logic which was for many years used *in lieu* of empirical evidence as fact, a brief review of the origin of citrus in South Africa is necessary. Citrus is native to the subtropical and tropical regions of Asia and the Malay Archipelago, from where it has since spread to other tropical and subtropical regions of the world (Castle *et al.,* 1993; Duncan, 2009). Due to inter-continental trades in the 1600s, by the end of the 15th century different citrus species had reached almost all the tropical and subtropical sections of the Eastern Hemisphere, except for the Southern Africa countries (Webber, 1943).

In South Africa, citrus trees were first introduced by Jan van Riebeeck from the island of St. Helena on 11 June 1654 and planted in his private garden (Webber, 1943). The first fruits were ripe by 25 July 1661. Meanwhile, other citrus trees were increasingly received from India since by July 1661 there were a total of 1162 young orange, lemon and pummelo trees growing in various parts of the Cape (Webber, 1943). Incidentally, St. Helena was commonly used by the merchandisers from Europe to India, *vice versa*, as a station for fresh supplies and water – just like Cape of Good Hope (Webber, 1943). Apparently, fruit trees were taken from India and/or Europe and planted at St. Helena and Cape of Good Hope for their provision of vitamin C.

During the early years of spreading citrus, citrus nurseries were established in the soil and uprooted with a soil ball for planting elsewhere (Webber, 1943). The

technique was a conduit for the distribution of root pests – particularly *T. semipenetrans*, which was then unknown. Although the origin of citrus in South Africa is not really known, most evidence, as reviewed here, suggested that citrus in South Africa had its origin from East Asia, which is indigenous to *Poncirus* biotype. The historical distribution of citrus to South Africa is important because it gives another view that *T. semipenetrans* biotype in South Africa could as well had originated in Asia, which is the centre of biodiversity for citrus.

2.3 Work not yet done on the problem statement

2.3.1 Aggressiveness of *Tylenchulus semipenetrans* isolates

Aggressiveness variables are similar to those for determining host-status and host-sensitivity. Generally, both aggressiveness and host-status depend on inoculation studies under controlled conditions such as greenhouses. Incidentally, the result depends much on the initial population density of nematodes. Although the protocols for aggressiveness studies are similar to those of nematode resistance, this has not been tested for *T. semipenetrans* isolates in South Africa.

2.3.2 Biotypes of *Tylenchulus semipenetrans*

Detailed knowledge of the biotype(s) in a given citrus-producing region(s) is important in (i) the selection and development of appropriate rootstock breeding programmes, (ii) developing regulatory legislation. Failure to have empirically-based knowledge of the exact biotype may result in unintentional recommendations of highly susceptible rootstock for the existing biotype in a particular country or region. This could be quite risky since a citrus orchard constitutes a long-term investment. In most major citrus-producing countries like Spain, Italy, Israel, USA and Japan, *T. semipenetrans* biotypes had been empirically-identified (Baines *et al*., 1974; Gottlieb

et al., 1986; Inserra *et al*., 1980; O'Bannon and Esser, 1985; O'Bannon *et al*., 1977; Verdejo-Lucas, 1992). In contrast, in Argentina, Australia, Brazil and Venezuela the exact *T. semipenetrans* biotypes were, in the early 1980s, not yet established since they had not been subjected to the distinguishing olive differential host plant test (Inserra *et al*., 1980; O'Bannon and Ford, 1977). Similarly, to date, the citrus biotype in South African had not been empirically identified.

2.3.3 The danger of unverified claims

After tracing the historical route of the first citrus seedlings into South Africa from the Mediterranean Basin countries through St. Helena Island, Cohn (1976) meticulously proposed that the citrus nematode biotype in South Africa was the Mediterranean. Four years later, Inserra *et al*. (1980: citing Cohn, 1976), stated that the Mediterranean biotype was in all citrus-producing countries of the Mediterranean Basin, South Africa and India. Subsequently authors stated as fact that the South African biotype was the Mediterranean, without empirically-based evidence (Inserra *et al*., 2009; O'Bannon and Esser, 1985).

The assertion that the South African citrus nematode biotype was the Mediterranean resulted in increased use of *P. trifoliata* and the related hybrid rootstocks in various citrus-producing regions – since this biotype does not infect trifoliate orange. However, citrus growers observed that *P. trifoliata* rootstock was potentially not suitable for South African conditions, resulting in its use declining from 21% to 5% by early 1990s (Rabe and Von Broemben, 1991). Originally, it was thought that the rootstock was failing due to its high sensitivity to Na ions, which was further exacerbated by observations in Florida, USA, that cyclic salinity increased

populations of *T. semipenetrans* (Mashela *et al*., 1992a), while salinity reduces resistance to this nematode in nematode-resistant rootstocks (Mashela *et al*., 1992b).

2.4 Addressing the identified gaps

Preliminary studies on differential host studies for determining the citrus biotype in South Africa were not successful due to excessive inoculations (Kwaye *et al*., 2008). Differential host plants were each inoculated with 40 000 J2s, resulting in reproductive factors less than unity. During almost the same time, Mashela (2007) demonstrated that the equilibrium point (E) of *T. Semipenetrans* was at 10 000 nematodes. According to Seinhorst (1965) inoculation using population levels above E, invariably results in reproductive factors below unity. In a subsequent study, where Pi = 10 000 J2s, after 120 days the reproductive factors were properly segregated by the differential host plants used in the trial (Mashela *et al*., 2012). However, using reproductive factors in host status for identifying the citrus nematode biotype was technically wrong for two reasons: (1) The rootstocks sizes are genetically different and (2) using total nematode numbers (soil plus root stages) is incorrect since in most non-host plants nematodes hardly enter the root system (Pofu and Mashela, 2011). Consequently, the current researcher used the concept of penetration index and standardisation of nematode numbers per gram roots (Pofu and Mashela, 2011) to enhance the assessment of the citrus nematode biotype in South Africa.

CHAPTER 3
AGGRESSIVENESS OF *TYLENCHULUS* ISOLATES

3.1Introduction

Slow decline of citrus, induced by the citrus nematode (*Tylenchulus semipenetrans* Cobb, 1913), is increasingly becoming a limiting economic factor (Duncan, 2009). Differences in the degree of symptoms in slow decline of citrus trees had been explained in reference to abiotic factors such as soil type (Harding, 1954), soil nutrition (Fouche *et al*., 1978) and salinity (Levy and Syvertsen, 2004). Findings in Texas and Florida, USA, demonstrated that salinity increased population densities of *T. semipenetrans* (Mashela *et al*., 1992a, b), while *T. semipenetrans* infection reduced salt tolerance (Mashela and Nthangeni, 2002). Generally, various factors are at play to induce slow decline symptoms (Levi and Syvertsen, 2004). In most cases, with the exception of implicating high nematode population densities and/or the existence of physiological races or biotypes, differences in the degree of slow decline symptoms of citrus trees had been confined to the narrow view that *T. semipenetrans* is generally a non-aggressive nematode (O'Bannon and Esser, 1985).

Aggressiveness is a quantitative measure of the general reproductive potential and damage potential of a given pest without regard to the resistant genes (Shaner *et al*., 1992). Generally, comparisons on aggressiveness of nematode isolates is done on genotypes with different degrees of resistant genes, using the concepts of reproductive potential and damage potential, which are analogous to those used in nematode-plant compatibility (Seinhorst, 1967). Reproductive potential (host-status) is determined using one of three ways, namely, (1) reproductive factor (RF), a

proportion of final nematode population density (Pf) to initial nematode population density (Pi), (2) infectivity potential, expressed as adult nematodes per unit of roots, and (3) reproductive potential, which is expressed as eggs + second-stage juveniles (J2s) per unit of roots, with variations and/or adaptations.

The major challenge in determining reproductive potential is its dependency on Pi. Generally, when Pi is greater than the Seinhorst (1967) equilibrium point (E), RF is less than one, due to nematode competition for resources such as infection site and nutrition (Seinhorst, 1967; Taylor and Sasser, 1978). Accordingly, E for a nematode on a particular plant species should be empirically established in order to ensure that the inoculation level is below E. In their differential host studies, Kwaye *et al*. (2008) used Pi = 40000 eggs + J2s of *T. semipenetrans*, with the result that in all differential hosts RF was less than one, making it difficult to interpret the results. Almost during the same time, Mashela (2007) empirically established that E for *T. semipenetrans* on rough lemon (*Citrus jambhiri*) was approximately 15000 eggs + J2s and then recommended approximately 10000 eggs + J2s as the appropriate Pi level for host-status experiments that would run for three months after inoculation. The recommended Pi agreed with that used in *T. semipenetrans* biotype studies in Spain (Verdejo-Lucas *et al*., 1997), which was previously validated in Morocco (Mokrini and Abbad-Andaloussi, 2011) and in South Africa (Mashela *et al*., 2012).

The fact that soil conditions play an indispensable role in the degree of slow decline of citrus (Harding, this may also suggest that population nematode densities should accordingly adapt themselves to different soil conditions. Nematodes have the rare ability of entering cryptobiosis when adverse condition approaches gradually. In

event that adverse conditions such as soil texture are permanent, introduced nematodes have to adapt or die. Due to the scarcity of land in South Africa, citrus had been expanding to areas with marginal soils. One such as Musina in Limpopo Province with heavy soils, while Champagne in Mpumalanga Province has light soil. Preliminary surveys demonstrated that each of the two sites was heavily infested with *T. semipenetrans* (Kwaye *et al*., 2008). The objective of this study was to compare the aggressiveness of *T. semipenetrans* isolates collected from Musina and Champagne after a 30-year adaptation period in their respective environments.

3.2 Materials and methods

Aggressiveness study was conducted under greenhouse conditions at the Plant Protection Skills Centre, University of Limpopo (23°53"10'S, 29°44"15'E). Ambient day/night temperatures averaged 28/21^{o}C, with maximum temperatures controlled using thermostatically-activated fans. The 30-cm-diameter plastic pots were filled with 10 L growing mixture, comprising 3:1 (v/v) steam-pasteurised sand (300^{o}C, 3 h) and Hygromix (Hygrotech, Pretoria North, South Africa). Pots were placed on greenhouse benches at inter- and intra-row spacing of 0.3 m. Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) and rough lemon were selected for use since they are moderately resistant and highly susceptible to *T. semipenetrans,* respectively (Castle *et al*., 1993). Uniform 5-month-old nematode-free Carrizo citrange and rough lemon seedlings were transplanted a day after irrigating the growing mixture to field capacity.

3.2.1 Location of isolates and experimental site

Citrus trees in Musina Citrus Estate (MCE) and Champagne Citrus Estate (CCE) had different degrees of slow decline symptoms (Kwaye *et al*., 2008). Soil at CCE (24°11'S, 29°54') was of Wasbank form, with orthic A layer over E horizon overlaying hard plinthic B soil (MacVicar, 1969). Clay content of E horizon was 31%, with 96% fine sand, which could be classified as Hoopstad soil series (MacVicar, 1969). The Estate is in the Lowveld region of South Africa, with sub-arid climate and annual average rainfall of less than 250 mm. In contrast, soil at MCE (22°16'S, 29°54'E) was of the Westleigh form, with orthic A over soft plinthic B. Clay content of soft plinthic B was 53%, with fine 30% sand, which was classified as Sibasa soil series MacVicar, 1969). The Estate is on the escarpment, within the tropical region and has sub-humid climate with annual mean rainfall being in excess of 700 mm. Each site had 30-year-old Delta Valencia trees on rough lemon (*Citrus jambhiri* Lush) rootstocks.

3.2.2 Collection and preparation of isolates

Isolates from CCE and MCE were code-named CCE and MCE isolates, respectively. On each site, nematode population densities had never been managed since the establishment of the orchards in the early 1980s.Nematode isolates for inoculation were obtained from citrus root samples collected from 25 randomly selected healthy looking trees per hectare. Approximately 500 g roots/tree was collected at approximately 0.5 m from the trunk. Roots were composited, transported in cooler boxes to the University of Limpopo, cut into small pieces, placed into 500 ml bottles filled with 1% NaOCl solutions and then mechanically shaken for 10 minutes. Contents from bottles were passed through top-down nested 150-, 45- and 25-µm-opening sieves, with contents of 25-µm- sieve washed into 250 ml beakers.

Baermann trays were used for egress in growth chamber at ±25^{o}C in order to obtain J2s for inoculation (Rodriguez-Kabana and Pope, 1981).

3.2.3 Experimental design, inoculation and cultural practices

The study, comprised a 2 × 5× 2 factor experiment, arranged in a randomised complete block design, with six replications. The first, second and third factors comprised nematode isolates (CCE, MCE), nematode (0, 5 000, 10 000, 15 000 and 20 000 J2s) and rootstock type respectively. Trees were inoculated three days after transplanting by dispensing approximate numbers of J2s in soil by placing into 5-cm-deep holes on cardinal points of stems using a 20-ml plastic syringe. Filtrates through the 25-µm sieve from two locations were mixed (1:1 v/v); with untreated control plants receiving 20 ml filtrate to establish any microbes associated with the isolates. Pests were managed as described previously (Kwaye *et al*., 2008).

Roots and tops of 12-month old seedlings were trimmed three months before transplanting. Two days after transplanting, each plant was fertilised using 3 g 2:3:2 (22) per pot to provide 186 N, 126 N and 2 g 2:1:2 (43) which provided 0.47 N, 0.43 K, 0.43 P, 1.21 Mg, 1 Fe, 0.10 Cu, 0.47 Zn, 1.34 B, 4.02 Mn and 0.09 Mo mg/ml tapwater. Four sets of Hadeco Moisture Meter (Hadeco Magic, New Delhi, India) were inserted to 20-cm-depths in randomly selected pots to monitor soil moisture tension. Plants were irrigated with 1 000 ml tapwater as soon as the moisture meters had average readings below 2 units.

3.2.4 Data collection

At 120 days after inoculation, plant height was measured from the soil surface to the tip of the flag leaf, with shoots severed at soil level. Root systems were removed from pots, immersed in tapwater to remove soil particles, blotted dry and weighed. Nematodes were extracted from 5 g roots/plant through maceration and blending for 30 seconds in 1% NaOCl (Hussey and Barker, 1973). The materials were passed through top-down nested 150-, 45- and 25-µm opening sieves. Contents of the 25-µm opening sieve were poured into 100-ml-plastic containers, bought to 100-ml mark and tightly closed for counting under a stereomicroscope. Shoots and the remaining roots were dried at 70°C for 72 h. Dry roots were adjusted for 5 g fresh roots from which nematodes were extracted. Soil per pot was thoroughly mixed and a 250-ml soil sample collected, with nematodes extracted using the modified sugar-floatation and centrifugation method (Jenkins, 1964). Nematode numbers/5 g roots were converted to nematodes per total root system, while nematode/250 ml soil were converted to total soil/pot in order to allow for the determination of (Pf)/plant for computing RF values.

3.2.5 Data analysis

Kwaye *et al*. (2008) previously used CCE isolate as one of the test nematode in differential host tests. Thus, since host-status data for CCE isolate on Carrizo citrange and rough lemon were available, the CCE isolate was used as a standard. RF and plant yield data were subjected to SAS software (SAS Institute, Cary, NC) to determine the contribution of various sources of variation to the total treatment variation in variables measured.

3.3 Results

Data were subjected to factorial analysis of variance. Due to the presence of significant interactions (Appendices 3.1-3.5), mean treatment values could not be compared and therefore, t-tests were performed on variables at each level of nematode infection.

3.3.1 Reproductive potential

The second order interaction, nematode isolate × nematode level × rootstock type, along with the individual main factors were significant at 1% level of probability for the reproductive factor (Table 3.1). Nematode isolate, nematode level, rootstock type and the second order interaction contributed 38%, 16%, 11% and 11% to the total treatment variation of the reproductive factors, respectively. Due to significant differences of the second order interaction, main factors could not be compared, allowing comparisons of the nematode isolates within each level of nematode inoculation within each seedling rootstock. Relative to CCE nematode isolate, the MCE nematode isolate had reduced reproductive potential in all levels of inoculation in both seedling rootstocks, with the magnitudes increased by 48-82% and 34-95% in Carrizo citrange and rough lemon, respectively (Table 3.2).

3.3.2 Damage potential

Selected first order interactions, nematode isolate and rootstock type had significant effect on damage potential of the two differential host plants. Nematode isolate x rootstock type contributed 3% and 1% to total treatment variation on dry root mass and plant height, respectively (Table 1). Nematode level × rootstock type interaction contributed 2% and 3% to total treatment variation in dry shoot mass and dry root

mass, respectively. Nematode isolate contributed 74%, 52% and 76% total treatment variation in dry shoot mass, dry root mass and plant height, respectively. In contrast, rootstock type contributed 5%, 18% and 12% total treatment variation to the three variables, respectively.

At individual inoculation level, relative to CCE nematode isolate, the MCE nematode isolate increased dry shoot mass and dry shoot mass on Carrizo citrange at 500 and 20 000 levels of inoculation by 14% and 12%, respectively (Table 3). At other levels the relative effects were not significant except at 10000-20000 inoculation levels where dry shoot mass was reduced by 10-17% and plant height by 12% at 15000 inoculation level. In contrast, on rough lemon the MCE nematode consistently reduced dry shoot mass and dry root mass by 11-21% and 16-51%, respectively, while this effect was not consistent in plant height.

Table 3.1 Contribution of source of variation to reproductive potential of *Tylenchulus semipenetrans* isolates from two citrus-producing regions in South Africa and damage potential of Carrizo citrange and rough lemon seedling rootstocks at 120 days after inoculation (n = 120).

Source Of Variation	DF	Reproductive potential of nematode isolates		Damage potential					
				Dry shoot mass		Dry root mass		Plant height	
		SS	%	SS	%	SS	%	SS	%
Replication	5	1.370	3^{ns}	206.58	1^{ns}	974.88	2^{*}	3278.65	2^{**}
Nematode isolate (a)	1	16.008	38^{***}	14749.30	74***	24314.10	52^{***}	166242.00	76^{***}
Nematode level (b)	4	6.723	16^{***}	75.38	0^{ns}	526.14	1^{ns}	810.45	0^{ns}
Rootstock type (c)	1	4.376	11^{***}	925.52	5^{***}	8448.31	18^{***}	27211.60	12^{***}
a × b	4	1.411	4^{*}	42.45	0^{ns}	334.85	1^{ns}	1242.01	1^{ns}
a × c	4	0.330	1^{ns}	282.94	1^{*}	1254.10	3^{***}	2144.82	1^{**}
b × c	1	1.294	3^{ns}	395.53	2^{***}	1247.27	3^{***}	285.33	0^{ns}
a × b × c	4	4.742	11^{***}	221.64	1^{ns}	665.55	1^{ns}	1435.41	1*
Error	95	5.429	13	3147.41	16	8921.88	19	16024.70	7
Total	119	41.683	100	20046.70	100	46687.00	100	218675.00	100

^{ns} implied that the contribution of the source of variation to total treatment variation was not significant at 10% level of probability, while for *, ** and ***implied that the contribution was significant at 10%, 5% and 1% level of probability, respectively.

Table 3.2 Reproductive factor (RF) of *Tylenchulus semipenetrans* isolates from Musina Citrus Estate (MCE) relative to RF of *T. semipenetrans* isolates from Champagne Citrus Estate (CCE) in Carrizo citrange and rough lemon at 120 days after inoculation (n = 120).

Nematode	Carrizo citrange			Rough lemon		
Level	CCE	MCE	RI (%)[z]	CCE	MCE	RI (%)
5 000	1.95	1.00	−48**	3.44	1.55	−55**
10 000	0.71	0.33	−53**	2.33	1.53	−34**
15 000	0.69	0.36	−48**	0.92	0.18	−80**
20 000	0.51	0.09	−82**	1.32	0.06	−95**

[z]Relative impact (%) = (MCE/CCE − 1) × 100 within the rows, with** denoting significant differences in row means within a rootstock at P ≤ 0.05

Table 3.3 Relative impact (RI) of *Tylenchulus semipenetrans* isolates from Musina Citrus Estate (MCE) when compared to those from Champagne Citrus Estate (CCE) on dry shoot mass, dry root mass and plant height of differential seedling rootstocks Carrizo citrange and rough lemon at 120 days after inoculation (n = 120).

	Dry shoot mass (g)						Dry root mass (g)						Plant height (cm)					
	Carrizo citrange			Rough lemon			Carrizo citrange			Rough lemon			Carrizo citrange			Rough lemon		
Nematode Level	CCE	MCE	RI (%)	CCE	MCE	RI (%)	CCE	MCE	RI (%)	CCE	MCE	RI (%)	CCE	MCE	RI (%)	CCE	MCE	RI (%)
0	26.8	26.8	0^{ns}	44.3	44.3	0^{ns}	9.8	9.8	0^{ns}	27.7	27.7	0^{ns}	88.7	89.0	0^{ns}	162	161	-1^{ns}
5000	24.3	27.9	14^{**}	42.8	36.6	-15^{**}	9.2	9.5	4^{ns}	32.5	23.4	-28^{**}	82.7	79.7	-4^{ns}	159	165	4^{ns}
10000	29.5	27.4	-7^{ns}	49.1	38.9	-21^{**}	10.7	9.6	-10^{**}	30.4	25.7	-16^{**}	87.7	84.1	-4^{ns}	164	153	-7^{ns}
15000	26.2	32.4	1^{ns}	38.8	31.2	-20^{**}	9.8	8.2	-17^{**}	33.8	16.7	-51^{**}	86.7	76.4	-12^{**}	182	137	-25^{**}
20000	25.5	24.6	1^{ns}	43.6	38.6	-11^{**}	8.5	9.5	12^{**}	27.8	26.2	-6^{ns}	80.9	85.5	6^{ns}	185	168	-9^{*}

RI (%) = (MCE/CCE) ─ 1) × 100 compares data within the nematode level.

^{ns}, * and ** denote that RI (%) within the nematode level was non-significant at 10% and significant at 10% and 5% level of probability, respectively.

3.4 Discussion

Consistently less than one (RF) values on the two seedling rootstocks at higher inoculation levels confirmed observation on olive (*Olea europaea* L.), trifoliate (*Poncirus trifoliate* L.*)* and Carrizo citrange when inoculated with 40 000 J2s of this test nematode (Kwaye *et a*l., 2008). Generally, RF values in this study were consistent in demonstrating that Carrizo citrange and rough lemon are genetically different. In Carrizo citrange, RF values for both isolates were equal or above unity at 5 000 J2s, while in rough lemon RF values were above one up to 10 000 J2s for both isolates. The latter is consistent with E at 10 000 J2s of *T. semipenetrans* on rough lemon (Mashela, 2007). Generally, the less than unity RF values suggested that the nematode did not have the potential to feed since feeding is a pre-requisite for nematode development and reproduction (Ferraz and Brown, 2002). The low Pf values in roots of both rootstocks cannot be explained exclusively in terms of nematode resistance since rough lemon is highly susceptible to *T. semipenetrans*. At high inoculation levels, the population nematode density of *T. semipenetrans* attained E soon (Seinhorst, 1967), especially in Carrizo citrange, which confirmed other nematode studies infecting nematode-resistant plants (Pofu *et al*., 2010a). Generally, it makes sense that E in nematode-resistant plant is lower than that in susceptible plants.

The interesting pattern was that of the relative impacts of RF values in nematodes. In either rootstock, regardless of inoculating level, RF values of CCE isolate were much higher than those of MCE isolates. However, RF values alone,

in nematode-resistant trials, cannot provide a water-tight conclusion that the two isolates had different degrees of aggressiveness (Pofu *et al*., 2010a). The result simply indicated that relative to the CCE isolate, the reduced relative impact of the MCE isolate on the reproductive factors suggested that the CCE isolate had significantly higher reproductive potentials within the same rootstock at all levels of inoculation under similar growing conditions. Thus, both RF and plant growth results are necessary.

The impact of the MCE isolate on various yield components in Carrizo citrange was consistently on dry root mass at all J2s levels, while the same was not true for dry root shoot mass and plant height. This could possibly be attributed to the fact that Carrizo citrange is moderately resistant to *T. semipenetrans*. However, as seen in the significant relative impact of the MCE isolate to the CCE isolate in roots, over the long-term the MCE isolate would reduce growth components. Taken together with the observed high RF and potential reduction in growth components of Carrizo citrange and its RF values in rough lemon and relative reductive effect on growth components, one could, therefore, suggest that, the MCE isolate was more aggressive than the CCE isolate. Generally, in most cases, in plant-parasitic nematodes inter-specific aggressiveness is well-documented (Eddaoudi *et al*., 1997; Ornat *et al*., 2001). The extent of nematode damage on plants is highly variable and is influenced by cultivar, climatic factors, edaphic factors and the relative aggressiveness of nematode populations (Kaplan and Gottwald, 1992).

Seinhorst (1965) reported that nematode infection might lead to growth stimulation or growth reduction, depending upon nematode density. The difference in plant growth might be a result of interactions between inhibitory and stimulatory processes (Wallace, 1973). The work presented in the current study, to ascertain degree, confirms Seinhorst (1965), who was later also confirmed on other plant species (Mashela, 2012; Wallace, 1971). However, information on intra-specific aggressiveness using isolates from different areas is scant.

Differences in the degree of slow decline of citrus had been noticed as early as the 1910s (Cobb, 1913). Later, variations in the degree of slow decline were associated with soil type (Harding, 1954). However, the notion that *T. semipenetrans* was a non-aggressive nematode (O'Bannon and Esser, 1985) and the observed significant interaction between this nematode species and salinity (Mashela *et al.*, 1992a,b), might have largely contributed towards the exclusive focusing on environmental factors at the expense of biological differences expressed through aggressiveness of the isolates. Genetically-determined actions can be conditioned by the environment - as outlined in the field of epigenetics (Salisbury and Ross, 1992). Probably, this is the first report to suggest that aggressiveness of *T. semipenetrans* from citrus-producing regions with different edaphic factors may differ. Consequently, the two isolates should also be subjected to molecular markers to establish if indeed the isolates from

the two provinces were genetically dis-similar or whether the observed differences were a question of epigenetics.

CHAPTER 4

SOUTH AFRICA *TYLENCHULUS* BIOTYPE

4.1Introduction

Worldwide, population densities of the citrus nematode (*Tylenchulus semipenetrans*) are increasingly being managed using nematode-resistant rootstocks (Duncan, 2009). However, in managing *T. semipenetrans*, reliance on a particular nematode-resistant genotype is complicated by the existence of nematode biotypes (Inserra *et al*., 1980). Biotypes are morphologically identical nematode species which could be identified using differential host plants and/or molecular markers (Devran and Sogut, 2011). Globally, *T. semipenetrans* biotypes had been identified in citrus-producing countries (Baines *et al*., 1974; Lamberti *et al*., 1976; Stokes, 1969). The most common biotypes include the Citrus, Mediterranean and Poncirus biotypes, which infect and reproduce equally on citrus species, but differentially on Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*), grape (*Vitis vinifera*), olive (*Olea europaea*), persimmon (*Diospyrus lotus*) and trifoliate orange (*P. trifoliata)*.

Detailed knowledge of the biotype in a given citrus-producing region is important in the selection and development of appropriate rootstocks. Failure to have empirically-based facts on the exact biotype may result in unintentional recommendations of a highly susceptible rootstock for the existing biotype in a particular country or region, which could be risky since a citrus orchard constitutes a long-term investment. In most major citrus-producing countries like

Spain, Italy, Israel, USA, Japan and Mediterranean basin countries, *T. semipenetrans* biotypes had been empirically-characterised (Baines *et al*., 1974; Gottlieb *et al*., 1986; Inserra *et al*., 1980; O'Bannon and Esser, 1985; O'Bannon *et al*., 1977; Verdejo-Lucas, 1992). In contrast, in Argentina, Australia, Brazil, India and Venezuela the exact *T. semipenetrans* biotypes were not yet established in the early 1980s since they had not been subjected to the distinguishing differential host plant – the olive (*Inserra et al*., 1980; O'Bannon and Ford, 1977).

After tracing the historical route of the first citrus seedlings into South Africa from the Mediterranean basin countries through St. Helena Island, Cohn (1976) proposed that the citrus nematode biotype in South Africa was the Mediterranean. Four years later, Inserra *et al*. (1980: citing Cohn, 1976), stated that the Mediterranean biotype was in all citrus-producing countries of the Mediterranean basin countries, South Africa and India. Later others continued to) state as fact that the South African biotype was the Mediterranean (Inserra *et al*., 2009; O'Bannon and Esser, 1985). Cohn's (1976) assertion resulted in increased use of *P. trifoliata* and hybrid rootstocks in citrus-producing regions – since this biotype does not infect trifoliate orange. However, citrus growers later observed that *P. trifoliata* was potentially not suitable for South African conditions, resulting in its use declining from 21% to 5% by the early 1990s (Rabe and Von Broemben, 1991). Originally, it was thought that the rootstock was under performing due to its high sensitivity to Na ions (Cooper, 1961). The observations

in Florida, USA, that cyclic salinity increased populations of *T. semipenetrans* (Mashela *et al*., 1992a), while salinity reduced resistance to this nematode in rootstocks (Mashela *et al*., 1992b), further exacerbated the review of using *P. trifoliata* in South Africa.

A baseline study was initiated in 2008 to empirically-confirm the South African *T. semipenetrans* biotype. Preliminary results using isolates from citrus orchards in Mpumalanga and Limpopo Provinces were contradictory (Kwaye *et al*., 2008). Isolates from Mpumalanga Province had reproductive factor (RF) values less than one on citrus, grape, Carrizo citrange, trifoliate orange and olive (Kwaye *et al*., 2008). However, those from Limpopo Province had RF values greater than one in all tested differential hosts, except for olive, where the RF value was less than unity. The disparity in RF values was traced to the size of the inoculum levels, where 40000 and 10000 second stage juveniles (J2s)/plant were used for isolates from Mpumalanga and Limpopo Provinces, respectively (Kwaye *et al*., 2008). Earlier Mashela (2007) had used a series of inoculum levels to determine the Seinhorst (1967) equilibrium point (E) for *T. semipenetrans* on rough lemon, with the results suggesting that the E point was approximately 15000 J2s/plant. Beyond E, RF values are less than one, suggesting that 10 000 J2s/plant were appropriate for use as inoculum level in investigating the South African *T. semipenetrans* biotype through differential host tests. The latter confirmed previous observations for isolates collected from Zebediela Citrus Estate, Limpopo Province (Kwaye *et al*., 2008).

A subsequent trial was conducted using 10 000 J2s/plant with isolates from six citrus-producing provinces on three differential hosts (olive, trifoliate orange, rough lemon) to validate the biotype. In all differential hosts, regardless of the province, RF values were greater than one, except for olive, where the variable was less than one (Mashela *et al*., 2012). Using the citrus nematode biotype classification (Inserra *et al*., 1980), *T. semipenetrans* biotype in South Africa was provisionally classified as Poncirus biotype (Mashela *et al*., 2012). Rootstock sizes of the three differential hosts are different and therefore, it is technically inappropriate to use RF in assessing the citrus nematode biotype, as done elsewhere (Kwaye *et al*., 2008; Mashela *et al*., 2012). Pofu and Mashela (2011) introduced the concept of relative penetration index (RPI) which compares the number of nematode juveniles inside the root system to those in the soil. Previously (Kwaye *et al*., 2008; Mashela *et al*., 2012), nematode numbers were not standardised per gram of root. Using RPI and standardisation a better view could emerge regarding the validation of the citrus nematode biotype in South Africa. The objective of this study, therefore, was to validate the citrus nematode biotype in South Africa using the RPI and standardisation concepts.

4.2 Materials and methods

South Africa comprises 46 district municipalities and 6 metropolises. Eighteen district municipalities with at least 100 000 fruit-bearing citrus trees were each selected to participate in the National Citrus Nematode Biotype (NCNB) study, under the auspices of the UL. A list of commercial citrus growers with at least 1000 citrus trees within the participating districts was compiled from the database of the Citrus Research International (Pty) Ltd (2 Barker Street, 1200, Nelspruit, Mpumalanga). CRI officers graciously assisted the research team with introduction to randomly selected participating growers. Participating districts constituted five in Limpopo, four in KwaZulu-Natal, three in Western Cape, two each in Eastern Cape and Mpumalanga and one each in Northern Cape and North-West Provinces.

4.2.1 Collection and preparation of isolates

Nematode isolates were obtained from citrus roots collected exclusively from Delta Valencia on rough lemon rootstocks. On each participating farm, root samples were collected from 2 ha within the drip area at approximately 0.5 m distance from the trunk of 25 randomly selected healthy-looking trees/ha. Soil particles were removed on-farm by gently rinsing roots in a bucket of water, composited, placed in a plastic bag, mixed with 1% NaOCl solution and then shaken vigorously. Aliquots were passed through a 1-mm-opening sieve into four 2-L bottles/farm and transported to the laboratory in cooler boxes. Contents from the bottles were passed through top-down nested 150-, 45- and 25-µm-opening

sieves, with contents from the 25-µm-opening sieve being washed into 250 ml beakers. Baermann trays were used for egress in a growth chamber at ±25^{o}C in order to obtain second stage juveniles (J2s) for inoculation (Rodriguez-Kabana and Pope, 1981).

4.2.2 Differential host tests and cultural practices

Differential host studies were conducted under greenhouse conditions, at the Plant Protection Centre, University of Limpopo (23°53”10’S, 29°44”15’E). Two-year-old differential host plants, namely, rough lemon, trifoliate orange and olive were selected for use on the basis of baseline results (Kwaye *et al*., 2008; Mashela *et al*., 2012). Plant roots and tops were trimmed and transplanted into 30-cm-diameter plastic pots, filled with 3:1 (v/v) 10 L steam-pasteurised (300^{o}C at 2 h) sandy soil and Hygromix (Hygrotech, Pretoria West). Fertilisation, irrigation and pest management practices were as described previously (Chapter 3).

4.2.3 Experimental design and inoculation

Pots were placed on the greenhouse bench at 30-cm inter-row and 60-cm intra-row spacing, with ambient day temperatures mechanically maintained at 25/17^{o}C maximum/minimum. Differential host plants were arranged in a randomised complete block design, with 15 replications. Second stage juveniles were applied 2 months after transplanting, with each plant receiving 10000 J2s, which were

dispensed into the growing medium by placing into 5-cm deep holes on cardinal points of stems using a 20-ml plastic syringe.

4.2.4 Data collection

At harvest, 120 days after inoculation with nematodes, data were collected and processed as described previously (chapter 3), except that nematodes from roots were extracted from 10 g roots/plant and converted to nematodes per total root system, while nematode/250 ml soil were converted to total soil/pot in order to allow for computation of RPI (Pofu and Mashela, 2011). Nematode numbers were standardised per gram roots.

4.2.5 Data analysis

Interactions of locations where isolates were collected and seasonal effects within the greenhouse were each significant at 5% level of probability (Gomez and Gomez, 1984), resulting in each district municipality and greenhouse experiment being viewed as a separate experiment. Relative penetration index was computed using the relationship RPI = $[(Pf_{root}/Pf_{soil}) - 1]$, while standardised nematode numbers were transformed using $\log_{10}(x + 1)$ to homogenise the variance (Gomez and Gomez, 1984) prior to analysis of variance with SAS software (SAS Institute, Cary, NC). However, untransformed nematode data were reported. Treatment means for each variable were separated using Tukey test. Unless otherwise stated, treatment means discussed were significant at 5%

level of probability. Data interpretation was achieved using the *T. semipenetrans* biotype classification system (Table 4.2).

4.3 Results

4.3.1 Relative penetration index

Tylenchulus semipenetrans isolates from all districts had negative RPI values on olive roots, which were adjusted to –1 per experiment (Table 4.3). In contrast, on trifoliate orange and rough lemon RPI values were positive, except for trifoliate orange in three districts, namely, Amatole in Eastern Cape, Zululand in KwaZulu-Natal and West Coast in Western Province, where RPI values were negative.

4.4.2 Reproductive potential

Eggs + J2s per g roots in olive were equivalent to zero (Table 4.4). In contrast, in trifoliate orange and rough lemon the numbers were significantly higher than those in olive. However, the differences in nematode numbers in trifoliate orange and rough lemon were inconsistent, probably due to the seasonal effect under greenhouse conditions.

4.4.3 Reproductive factor

Trifoliate orange and rough lemon rootstocks had RF values greater than one in isolates from all districts, while those on olives were less than one (Table 4.1). Also, in isolates from almost all districts, except for one district in Western Cape Province (Wine lands) and two districts in Limpopo Province (Mopani and

Sekhukhune), Pf values/standardised unit on trifoliate orange and rough lemon were not different ($P \leq 0.05$), but were significantly different to those on olive.

Table 4.1 Reproductive factor (RF) of *Tylenchulus semipenetrans* isolates from eighteen citrus-producing district municipalities on olive, trifoliate orange and rough lemon at 120 days under greenhouse conditions (n = 45).

District	Olive		Trifoliate orange		Rough lemon	
Municipality	Pf	RF	Pf	RF	Pf	RF
			Eastern Cape Province			
Amathole	6745[b]	0.67	15475[a]	1.55	20758[a]	2.08
Cacadu	5628[b]	0.56	28313[a]	2.83	32232[a]	3.22
			KwaZulu-Natal Province			
Ilembe	7389[b]	0.74	15558[a]	1.56	23810[a]	2.38
Sisonke	5208[b]	0.52	19746[a]	1.97	30109[a]	3.01
Uthungulu	6523[b]	0.65	20277[a]	2.02	28746[a]	2.87
Zululand	6247[b]	0.62	19633[a]	1.96	21128[a]	2.11
			Limpopo Province			
Capricorn	7613[b]	0.76	15413[a]	1.54	14007[a]	1.40
Mopani	7319[c]	0.73	19419[b]	1.94	56506[a]	5.65
Sekhukhune	6928[c]	0.69	16862[b]	1.68	29935[a]	2.99
Vhembe	8048[b]	0.80	21117[a]	2.11	40144[a]	4.01
Waterberg	6203[b]	0.62	19403[a]	1.94	34436[a]	3.44
			Mpumalanga Province			
Ehlanzeni	6639[b]	0.66	31649[a]	3.17	34490[a]	3.45
Nkangala	5835[b]	0.58	29930[a]	2.99	32246[a]	3.22
			Northern Cape Province			
Diamond Veld	6234[b]	0.62	27749[a]	2.77	33352[a]	3.33
			North-West Province			
Rustenburg	4841[b]	0.48	18727[a]	1.87	21110[a]	2.11
			Western Cape Province			
Cape Wine lands	6608[c]	0.66	17994[b]	1.80	19099[a]	4.91
Overberg	6127[b]	0.61	29520[a]	2.95	26837[a]	2.68
West Coast	6459[b]	0.65	28812[a]	2.88	39934[a]	3.99

Row means followed by the same letter were not different ($P \leq 0.05$) according to Fisher's least significant difference test.

Table 4.2 Classification of *Tylenchulus semipenetrans* biotypes as modified from Inserra *et al*. (1980).

Differential Host	*Tylenchulus semipenetrans* biotype		
	Citrus biotype	Mediterranean biotype	Poncirus biotype
Citrus	+ +	+ +	+ +
Poncirus trifoliata	+ +	+	+ +
Olive	+ +	— —	— —

++ and + reproduces highly and poorly, respectively; — — does not reproduce at all.

Table 4.3 Relative penetration index (RPI) of *Tylenchulus semipenetrans* isolates from 18 citrus-producing district municipalities in South Africa on olive, trifoliate orange and rough lemon seedling rootstocks under greenhouse conditions at 120 days after inoculation (n = 45).

District municipality	Olive				Trifoliate orange				Rough lemon			
	Total$_{Pf}$	Root$_{Pf}$	Soil$_{Pf}$	RPI	Total$_{Pf}$	Root$_{Pf}$	Soil$_{Pf}$	RPI	Total $_{Pf}$	Root$_{Pf}$	Soil$_{Pf}$	RPI
Eastern Cape Province												
Amathole (32º 30'S, 27º30'E)	6745	27	6718	-1	15475	7601	7874	-0.03	20758	12078	8680	0.39
Cacadu (33º 57'S, 25º65'E)	5628	25	5603	-1	28313	24520	7393	2.32	32232	17307	14925	0.16
KwaZulu-Natal Province												
Ilembe (29º 20'S, 31º17'E)	7389	17	7362	-1	15558	11473	4085	1.81	23810	15000	8810	0.70
Sisonke (30º 08'S, 30º04'E)	5208	23	5185	-1	19746	14100	5646	1.50	30109	15969	14140	0.13
Uthungulu (28º 44'S, 32º05'E)	6523	29	6494	-1	20277	16580	3717	3.46	28746	17111	11634	0.47
Zululand (28º 19'S, 31º25'E)	6247	15	6232	-1	19633	5302	14331	-0.63	21128	13309	7819	0.70
Limpopo Province												
Capricorn (23º53'S, 29º26'E)	7613	21	7592	-1	15413	12348	3065	3.03	14007	8823	5184	0.70
Mopani (23º53'S, 29º26')	7319	22	7297	-1	19419	13817	5602	1.47	56506	35599	20907	0.70
Sekhukhune (24º 50'S, 29º 50'E)	6928	12	6917	-1	16862	12603	4259	1.96	29935	18860	11050	0.71
Vhembe (22º 56'S, 30º 28'E)	8048	19	8029	-1	21117	15288	5829	1.62	40144	25291	14853	0.70
Waterberg (24º 42'S, 28º24'E)	6203	17	6186	-1	19403	11803	7600	-0.63	34436	24695	9741	1.54
Mpumalanga Province												
Ehlanzeni (25º45'S, 30º98'E)	6639	16	6623	-1	31649	23408	6241	2.75	34490	24729	9761	1.53
Nkangala (25º45'S, 29º25'E)	5835	13	5822	-1	29930	16919	13011	0.30	32246	17315	14931	0.16
Northern Cape Province												
Frances Baard (25º27'S, 30º59'E)	6234	20	6214	-1	27749	14301	13718	0.04	33352	21012	12340	0.70
North-West Province												
Bojanala (25º25'S, 27º15'E)	4841	8	4833	-1	18727	10218	8509	0.20	21110	11299	9811	0.15
Western Cape Province												
Cape Wineland (33º30'S, 19º40E)	6608	9	6599	-1	17994	15583	2411	5.46	19099	15032	4067	2.70
Overberg (34º30'S, 20º 40'E)	6127	22	6105	-1	29520	20564	8956	1.30	26837	22907	3930	4.83
West Coast (32º30'S, 18º45'E)	6459	19	6440	-1	28812	12951	15861	-0.18	39934	25159	14775	0.70

[z]Row means with the same letter were not different according to Tukey test at 5% level of probability. [y]Each district municipality constituted a separate experiment.

Table 4.4 Standardisation of *Tylenchulus semipenetrans* isolates from 18 citrus-producing district municipalities in South Africa using nematodes per gram of roots in olive, trifoliate orange and rough lemon seedling rootstocks at 120 days after inoculation (n = 45).

District municipality	Olive			Trifoliate orange			Rough lemon		
	Nematode per total root	Total root (g)	Nematode per (g) root	Nematode per total root	Total root (g)	Nematode per (g) root	Nematode per total root	Total root (g)	Nematode per (g) root
Eastern Cape Province									
[y]Amathole (32° 30'S, 27°30'E)	27	83.27	0.32 ≈ 0[cz]	7601	24.81	306[a]	12078	75.23	161[b]
Cacadu (33° 57'S, 25°65'E)	25	69.28	0.36 ≈ 0[c]	24520	56.62	433[a]	17307	69.28	250[b]
KwaZulu-Natal Province									
Ilembe (29° 20'S, 31°17'E)	17	54.70	0.31 ≈ 0[c]	11473	30.80	373[a]	15000	87.47	160[b]
Sisonke (30° 08'S, 30°04'E)	23	71.98	0.32 ≈ 0[c]	14100	52.08	271[a]	15969	92.77	172[b]
Uthungulu (28° 44'S, 32°05'E)	29	81.67	0.36 ≈ 0[c]	16580	48.74	340[a]	17111	77.50	221[b]
Zululand (28° 19'S, 31°25'E)	15	50.01	0.30 ≈ 0[b]	5302	61.41	86[a]	13309	94.03	142[a]
Limpopo Province									
Capricorn (23°53'S, 29°26'E)	21	59.35	0.35 ≈ 0[b]	12348	89.12	139[a]	8823	111.62	79[a]
Mopani (23°53'S, 29°26'E)	22	64.96	0.34 ≈ 0[c]	13817	60.86	227[b]	35599	73.83	482[a]
Sekhukhune (24° 50'S, 29° 50'E)	12	69.42	0.17 ≈ 0[b]	12603	54.33	232[b]	18860	76.06	248[a]
Vhembe (22° 56'S, 30° 28'E)	19	40.07	0.38 ≈ 0[c]	15288	57.73	265[b]	35291	89.92	392[a]
Waterberg (24° 42'S, 28°24'E)	17	55.76	0.30 ≈ 0[c]	11803	51.50	229[b]	31695	55.13	575[a]
Mpumalanga Province									
Ehlanzeni (25°45'S, 30°98'E)	16	45.83	0.35 ≈ 0[c]	23408	61.69	379[b]	24729	60.90	406[a]
Nkangala (25°45'S, 29°25'E)	13	59.82	0.22 ≈ 0[c]	16919	46.42	364[a]	17315	106.25	163[b]
Northern Cape Province									
Frances Baard (25°27'S, 30°59'E)	20	38.33	0.52 ≈ 0[b]	14301	58.65	244[a]	21012	85.13	247[a]
North-West Province									
Bojanala (25°25'S, 27°15'E)	8	24.87	0.32 ≈ 0[b]	10218	62.64	163[a]	11299	74.73	151[a]
Western Cape Province									
Cape Wineland (33°30'S, 19°40'E)	9	58.90	0.15 ≈ 0[b]	15583	89.12	175[a]	15032	64.67	232[a]
Overberg (34°30'S, 20° 40'E)	22	63.27	0.35 ≈ 0[b]	20564	79.13	260[a]	22907	84.98	270[a]
West Coast (32°30'S, 18°45'E)	19	39.01	0.49 ≈ 0[c]	12951	90.13	144[b]	25159	101.39	248[a]

[z]Row means with the same letter were not different according to Tukey test at 5% level of probability. [y]Each district municipality constituted a separate experiment.

4.4 Discussion

In all 18 citrus-producing district municipalities, RPI values on olive were negative, while those on rough lemon and trifoliate orange were predominantly positive except for isolates from Amathole, Zululand and West Coast on *P. trifoliata* (Table 1). Using root standardisation of one gram, nematode numbers in olive were equivalent to zero; while in other host plants they were significantly higher than zero (Table 2). Negative RPI values suggested that nematode numbers were higher in the soil than inside the root system (Kaplan and Davis; 1987; Pofu and Mashela, 2011). This observation provided clear evidence that olive has pre-infection nematode resistance to *T. semipenetrans* isolate in South Africa. Pre-inflectional nematode resistance is common in nematology (Kaplan and Davis; 1987; Pofu and Mashela, 2011). Also Storey (2010) noted that in root samples from commercial olive orchards in South Africa, the citrus nematode could hardly be identified, which further confirmed pre-infectional nematode resistant status in this plant. Observations in our study confirmed those of other values that there could be zero infection on olive when Poncirus biotype is involved. In baseline studies, where reproductive factor values were erroneously used to determine the biotypes (Kwaye *et al*., 2008; Mashela *et al*., 2012), one could not tell where high numbers of the citrus nematode were situated in olive plants.

In this study, standardisation of root systems demonstrated that *T. semipenetrans* isolates reproduced in *P. trifoliata* and rough lemon, while they

failed to reproduce in olive. Generally, nematode population growth is a factor of environmental factors, host plant and nematode species (Seinhorst, 1965). In our study, the higher nematode numbers in *P. trifoliata* than in rough lemon was probably due to differences in genetic make-up of the rootstocks. Apparently, in *P. trifoliata,* the population growth of the isolates was slower than in rough lemon, resulting in a situation where the saturation phase was earlier in rough lemon than in *P. trifoliata* (Salisbury and Ross, 1992). In contrast, at 120 days the nematode population growth in rough lemon was already in the declining phase – since nematode subscribe to density-dependent growth patterns (Salisbury and Ross, 1992).

Using the citrus nematode biotype classification (Inserra *et al*., 1980), the South African *T. semipenetrans* biotype was therefore, validated as the Poncirus biotype, since it reproduces profusely in *P. trifoliata,* but not at all in olives. Findings in this and other studies (Kwaye *et al*., 2008; Mashela *et al*., 2012), did not support the repeated views that the country had the Mediterranean biotype (Cohn, 1976; Inserra *et al*., 1980, 2009; O'Bannon and Esser, 1985). However, it is vital to note that Poncirus and the Mediterranean biotypes have a thin distinction line between differential hosts, as both reproduce on the standard differential hosts, except olive (Inserra *et al*., 2009). Incidentally, the separating host between the two biotypes is *P. trifoliata* with the Poncirus biotype reproducing profusely in this host, while the Mediterranean biotype reproduces poorly (Inserra *et al*., 2009).

The citrus industry had as early as the 1990s noted that *P. trifoliata* was not suitable for the South African condition (Rabe and Von Broemben, 1991). This was linked to the high sensitivity of *P. trifoliata* and the increasing status of poor quality irrigation water in various citrus producing- areas (Levy and Syvertsen, 2004). Incidentally, results of this study provide another justification of abandoning *P. trifoliata* by the South African citrus industry. Poncirus biotype is believed to have originated in Japan, but it is also present in California, USA (Inserra *et al*., 1980). Thus, the citrus industry in South Africa could learn more about managing Poncirus biotype using nematode genotypes from their counterparts in the two-mentioned citrus-producing countries. In conclusion, empirically-derived evidence *T. semipenetrans* isolates from18 district municipalities in South Africa validated earlier observations that the citrus nematode biotype in this country is the Poncirus biotype (Kwaye *et al*., 2008; Mashela *et al*., 2012). This information is indispensable in choices of resistant genotypes for nematode-resistant rootstock programmes, management decisions in citrus replant incidents and regulatory services in the management of the citrus nematode in South Africa.

CHAPTER 5
SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Introduction

Two experiments were conducted to investigate aggressiveness of *Tylenchulus semipenetrans* isolates from Mpumalanga and Limpopo Provinces. In contrast, 18 greenhouse experiments, each comprising isolates from citrus-producing district municipalities with at least 100 000 fruit-bearing trees, were conducted to investigate *T. semipenetrans* biotype in South Africa.

5.2 Summary

The Mpumalanga (CCE) isolate had significantly higher reproductive potentials than the Limpopo (MCE) isolate in both Carrizo and rough lemon rootstocks (Chapter 3). Incidentally, the CCE isolate induced more damage on the seedling rootstocks than the MCE isolate. Consequently, the CCE isolate was more aggressive than the MCE isolate.

Empirical results of *T. semipenetrans* isolates from 18 district municipalities all showed that the isolates did not reproduce on olive, but reproduced on other two hosts (Chapter 4). Consequently, *T. semipenetrans* biotype in South Africa was Poncirus and not the previously much publicised Mediterranean biotype (Cohn, 1976).

5.3 Conclusion

Generally, features of aggressiveness and differential host-status are genetic in nature. The aggressive study suggested that there was variation between isolates from Limpopo and Mpumalanga Provinces of South Africa. In contrast, a biotype study using isolates from 18 district municipalities using relative penetration indices and standardised reproductive potentials conclusively demonstrated that *T. semipenetrans* biotype in South Africa was Poncirus. In conclusion, results of this study demonstrated the complexity of managing the citrus nematode using nematode resistance when the existing biotype had not been empirically-verified.

5.4 Recommendations

The fact that the South African citrus nematode biotype is Poncirus, structural adjustments in new plantings would be necessary. However, since this would include adjustments in rootstock breeding, it would be necessary to subject isolates from different citrus-producing district municipalities to molecular markers since the initial study (Chapter 3) suggested that there were differences in aggressiveness of isolates from adjacent provinces.

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APPENDICES

Appendix 3.1 Analysis of variance of reproductive factors of *Tylenchulus semipenetrans* isolates from Mpumalanga and Limpopo Provinces on Carrizo citrange seedlings.

Source	DF	SS	PERCENT	F-VALUE	P ≤ 0.05
Replication	5	0.30889	3.94	1.62	0.18
Isolate (A)	1	1.414553	18.02	37.06	0.1
Nematode (B)	3	4.45823	56.81	38.93	0.01
A× B	3	0.33030	4.21	2.88	0.05
Error	35	1.33604	17.02		
Total	47	7.84799	100		

Appendix 3.2 Analysis of variance of reproductive factors of *Tylenchulus semipenetrans* isolates from Mpumalanga and Limpopo Provinces on rough lemon seedlings.

Source of variation	DF	P	PERCENT	F-VALUE	P ≤ 0.05
Replication	5	1.37047	3.49	0.84	0.53
Isolate (A)	1	16.0083	40.76	49.02	0.01
Nematode (B)	3	6.72256	17.12	6.86	0.01
A × B	3	3.74153	9.53	3.82	0.02
Error	35	11.4293	29.10		
Total	47	39.2722	100		

Appendix 3.3 Analysis of variance of the of the plant height (cm) of *Tylenchulus semipenetrans* isolates from Limpopo and Mpumalanga citrus growing area on two citrus seedling rootstocks.

Source of variation	DF	SS	PERCENT	F-value	P ≤ 0.05
Replication (A)	5	3278.65	1.50	3.89	0.01
Nematode (B)	4	810.499	0.40	1.20	0.32
Isolate (C)	1	166242	76.02	985.54	0.01
Rootstock (D)	1	27211.6	12.44	161.32	0.01
B × C	4	1242.01	0.57	1.84	0.13
B × D	4	2144.82	0.98	3.18	0.02
C × D	1	285.332	0	1.69	0.20
B × C × D	4	1435.41	0.65	2.13	0.08
Error	95	16024.7	7.33		
Total	119	218675	100		

Appendix 3.4 Analysis of variance of the dry shoot mass (g) of *Tylenchulus semipenetrans* isolates from Limpopo and Mpumalanga citrus growing area on two citrus seedling rootstocks.

Source	DF	SS	PERCENT	F-value	P ≤ 0.05
Replication (A)	5	206.580	1.03	1.25	0.29
Nematode (B)	4	75.3766	0.38	0.57	0.69
Isolate (C)	1	14749.3	73.57	445.19	0.01
Rootstock (D)	1	925.519	4.62	27.94	0.01
B × C	4	42.4482	0.21	0.32	0.86
B × D	4	282.938	1.41	2.14	0.08
C × D	1	395.525	1.97	11.94	0.01
B × C × D	4	221.638	1.11	1.67	0.16
Error	95	3147.41	16		
Total	119	20046.7	100		

Appendix 3.5 Analysis of variance dry root mass (g) of *Tylenchulus semipenetrans* isolates from Limpopo and Mpumalanga citrus growing area on two citrus seedling rootstocks.

Source	DF	SS	PERCENT	F-value	P ≤ 0.05
Replication (A)	5	974.882	2.10	2.08	0.07
Nematode (B)	4	526.143	1.13	1.40	0.24
Isolate (C)	1	24314.1	52.01	258.90	0.01
Rootstock (D)	1	8448.31	18.10	89.96	0.01
B × C	4	334.850	0.72	0.89	0.47
B × D	4	1254.10	2.69	3.34	0.01
C × D	1	1247.27	2.67	13.28	0.01
B × C × D	4	665.546	1.43	1.77	0.14
Error	95	8921.88	19.11		
Total	119	46687	100		