

A morphological and molecular study of *Heterodera carotae* Jones, 1950 in South Africa

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ABSTRACT

Carrot (*Daucus carota* L.), an important vegetable crop, is cultivated worldwide for the fresh market and processing industry. In South Africa carrot is one of the four major root and tuber vegetables consumed. The objectives of this study were to 1) determine the different life stages of *Heterodera carotae* Jones, 1950 on a weekly basis and the number of life cycles completed per growing season, and 2) characterize the carrot cyst nematode population from the Tarlton area, Gauteng Province (South Africa) morphologically and molecularly. No formal morphometric or molecular identifications have been performed on *H. carotae* for the African continent, neither have soil tolerance limits been determined. Tap root and corresponding rhizosphere samples were obtained from a one-hectare carrot trial block in the Tarlton area during the 2016-2017 summer growing season. This was done weekly for 18 weeks. Nematodes were extracted from the samples using the sieving centrifugal flotation and Seinhorst cyst elutriator methods, depending on the life stages required. Accumulation of day degrees, counting and identifying nematode of life stages was done to determine the number of life cycles and the length of the life cycle of the Tarlton population. Infective second-stage juveniles (J2), females, cysts and males were used for morphological and molecular identification using light microscopy (LM) and scanning electron microscopy (SEM). For molecular identification, deoxyribonucleic acid (DNA) extraction and the polymerase chain reaction (PCR) were used for the internal transcribed spacer (ITS)-rDNA region. Interestingly, J2 and young (yellow cysts) were present in as early as the first week and cysts during the second week of sampling. This may have been attributed to remnants of the previous carrot crop still persisting in the soil. Two life cycles of *H. carotae* were recorded for the 18-week study period. Morphological and morphometrical characterization of *H. carotae*, using LM and SEM, suggested the Tarlton population to be conspecific with *H. carotae* populations described from Europe and Canada. Observed differences between the morphometrics of various life stages of the Tarlton population lay within the range of

intraspecific variation compared to that of the aforementioned, described *H. carotae* populations. Morphometrics and morphological identification revealed that J2 of the Tarlton population closely resembles *H. carotae*, except for 1) the slightly shorter body length of some specimens compared to that of the European and Canadian populations, 2) the smaller head diameter of the Tarlton J2 specimens compared to that of European populations, 3) slightly smaller cysts from Tarlton compared to those from European and Canadian populations, 4) shorter underbridge of cysts from Tarlton compared to that of European populations and 5) slightly longer spicules of males from Tarlton than those for both European and Canadian specimens. Nonetheless, the vulval slit length of Tarlton cyst specimens compared well with that of cysts from European and Canadian populations. Molecular results indicated a 99 % similarity of the Tarlton population to three *H. carotae* populations selected from the NCBI Genbank database. Based also on this molecular analysis, the Tarlton population is considered to be conspecific with described *H. carotae* populations. This study added novel and valuable information about a South African carrot cyst nematode population: a species that is of great economic importance for local producers.

Key words: Classification, carrot cyst nematode, extraction methods, preservation methods, day degrees.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Carrot (*Daucus carota* L.) is an important vegetable crop that is cultivated worldwide for the fresh market and processing industry. In South Africa carrot is one of the four major root and tuber vegetables consumed. According to the South African Plant-Parasitic Nematode Survey (SAPPNS¹) database, one genus of cyst nematode (*Heterodera*) containing three species: *Heterodera carotae* Jones, 1950, *Heterodera schactii* Schmidt, 1871 and *Heterodera trifolii* Goffart, 1932 have been reported from carrot fields in South Africa. *Heterodera carotae* was first recorded in South Africa during 1996 near Philippi (Western Cape Province). Thereafter the nematode was identified from infested carrot fields in the Tarlton area, Gauteng (2008-present) and was again recorded during 2002 from a carrot field in Philippi. Despite the limited distribution of *H. carotae* worldwide, the carrot cyst nematode seems to be causing significant economical losses to carrot production in Europe (Greco & Brandonisio, 1986). In Switzerland, Greco (1986) established a tolerance limit of carrot to *H. carotae* of about 40 cysts/250 cm³ of soil. This means a tolerance limit of about 16 cysts/100 cm³ soil. In South Africa, Jones *et al.* (2017) listed yield losses of carrot production during 1989 at 9.3% (Keetch, 1989) due to damage caused by a wide range of plant parasitic nematodes. More recent figures are not available for carrot in South Africa. Except for the limited information contained in Kleynhans *et al.* (1996), no other data on carrot cyst nematodes in South Africa have been published. Also, no formal morphometric or molecular identification have been performed on *H. carotae* for the African continent. During the present study these issues are addressed, especially as a taxonomist and scientist, it is essential to know the identity of the organism one is working with.

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1.2. Literature review

1.2.1. Overview of cultivated carrot [*Daucus carota* Subsp. *sativus* (Hoffm.) Schubl. & G. Martens]

Carrot is a root vegetable crop of which the colour can range from orange, purple, black, yellow and white, depending on the cultivar. Fast growing cultivars mature within three months (90 days) and slower maturing cultivars are harvested at four months or 120 days of age (Anon, 2017a). The carrot has been domesticated for many years (Zukauska *et al.*, 2017). The plant originated in Persia and was cultivated for its leaves and seed (Anon, 2017a). Carrot is a biennial plant, it grows vegetatively in the first season and in the second season it matures and produces flowers and seed. Today the taproot is the most eaten part of the plant, meaning that the carrot plant never matures when cultivated (Anon, 2017a). The vegetable can be eaten raw or cooked in both sweet and savoury dishes. Large quantities are processed alone or in mixture with other vegetables by canning, freezing or dehydration (Zukauskas *et al.*, 2017). Carrot are among the top 10 most economically important vegetable crops in the world in terms of production and market value, and is rich in vitamin C, vitamin B1, vitamin B2 and also in carotene (DAFF, 2016).

1.2.2. Classification and basic anatomy and morphology

The carrot belongs to the Apiaceae family, which includes vegetables such as parsley, celery and parsnips (Anon, 2017a). The taxonomic hierarchy (classification) is as follows (Anon, 2017b):

Kingdom Plantae – Plants

Subkingdom Tracheobionta – Vascular plants

Superdivision Spermatophyta – Seed plants

Division Magnoliophyta – Flowering plants

Class Magnoliopsida – Dicotyledons

Subclass Rosidae

Order Apiales

Family Apiaceae – Carrot family

Genus *Daucus* L. – wild carrot

Species *Daucus carota* L. – Queen Anne's lace

Subspecies: *Daucus carota* Subsp. *sativus* (Hoffm.) Schubl. & G. Martens, a trinomial name.

1.2.3. Cultivation practices

Carrot crops are cultivated worldwide for the fresh and processing industry (Wesemael & Moens, 2008). The carrot is a climate crop that can be sown in early spring in temperate climates and in the autumn or winter in the subtropical areas (Anon, 2017c). In South Africa carrot are planted during summer or in all seasons in the warmer areas of the country (Jones *et al.*, 2017). Carrot is only propagated by seed and the soil should be well tilled and level in order to obtain good germination. The soil must be ploughed to at least 30 cm in depth. Planting density of 150 to 160/m² for double rows and a density of 100/m² in a single row are recommended (DAFF, 2017). Carrot is sensitive to moisture during root enlargement and seed germination (Starke Ayres, 2014). Irrigation water should be applied once or twice a day and the soil should never be allowed to dry out. Soil cultivation between the rows and thinning should be carried out when the soil is moist (DAFF, 2017).

1.2.4. Production

Reports from the Food & Agriculture Organisation (FAO, United Nations) stated that 37,226,640 Metric tonnes (Mt) of carrot was produced worldwide during 2013, with China as the largest producer with 16,929,000 Mt (Anon, 2017d). In South Africa carrot production is concentrated in the Western Cape, Gauteng, Free State, North West and Mpumalanga provinces. Carrot production in this country was estimated at approximately 180,000 million Mt during 2013 (Jones *et al.*, 2017).

1.2.5. Diseases and pests of carrot

Carrot is attacked by several fungal, bacterial and nematode diseases (Anon, 2017e). They can cause poor plant growth, and reduced yield and quality of the product. The most important carrot disease is *Alternaria* leaf blight and powdery mildew, which are widespread and may cause significant yield and quality loss (Anon, 2017e). Cutworms, millipedes and false wireworms also cause damage to the roots (Anon, 2017e). Root-knot nematodes (*Meloidogyne* spp.) are the most economically damaging pest, causing serious losses to the crop. They attack the lateral rootlets and the taproot causing unmarketable thickened, split and forked taproots (Anon, 2013f).

1.2.6. Plant parasitic nematodes associated with carrot and their economic impact on the crop

More than 90 plant-parasitic nematode species from several genera have been associated with umbelliferous (carrot) crops, the most damaging being *Meloidogyne* spp., *Pratylenchus* spp., *Longidorus* spp., *Paratylenchus* spp., *Belonolaimus* spp., *Paratrichodorus* spp., *Rotylenchus* spp., *Ditylenchus* spp. and *Hemicycliophora saueri* Brzeski, 1974 that are causing significant crop loss in carrot production worldwide (Hay *et al.*, 2005).

Meloidogyne spp. have been described as the most important economic pest of carrot production. *Meloidogyne* damage has been associated with the forking of the taproot, forming galls on roots, proliferation of fibrous roots giving the carrot a bearded appearance and stunting of the plants. Infection of carrot by *Meloidogyne* may cause losses in market yield of up to 77% (Hay *et al.*, 2005). According to Subbotin *et al*

(2010b), *Heterodera carotae* Jones, 1950 was first reported by Triffit in Wiltshire, UK as *Heterodera schachtii sensu lato* and Jones again reported the carrot cyst nematode as part of several strains of *Heterodera schachtii sensu lato*. Jones (1950a) described *H. carotae* officially as a new species.

According to the South African Plant-Parasitic Nematode Survey (SAPPNS¹), 18 genera containing 53 plant-parasitic nematode species from carrot fields have been reported. However, in South Africa their impact on carrot have not been established or studied in depth (Kleynhans *et al.*, 1996).

1.2.7. Cyst forming plant parasitic nematodes

According to Turner & Subbotin (2013) the cyst forming nematodes or cyst nematodes for short, belong to the family Hoplolaimidae Filipjev, 1934 under the subfamily Heteroderinae Filipjev & Schuurmans Stekhoven, 1941. About 114 valid species are recognized within the subfamily (Turner & Subbotin, 2013). They are a group of plant parasitic nematodes that cause economically important yield losses to crops such as cereals, pulses and vegetables in many countries worldwide (Turner & Subbotin, 2013). The cyst nematodes feed within the root system of the host and are thus called endoparasites of plants. They are defined by their capacity to retain eggs inside the swollen female body, which is transformed into a resistant, tanned cyst that protects the embryonated eggs at the completion of the female life cycle (Subbotin *et al.*, 2010a). This allows the succeeding generations to survive for extended periods until a suitable host is introduced. This survival mechanism also contributes to the economic importance of these nematodes in agriculture (Turner & Subbotin, 2013). In Switzerland in 1980, R. Vallotton estimated a tolerance limit of carrot to the carrot cyst nematode of 40 cysts/250 cm³ soil (Greco, 1986). This count amounts to 16 cysts/100 cm³ soil, 100 cm³ being the amount of soil used for soil sample extraction using the Seinhorst Cyst Elutriator (Swart & Marais, 2017). As this is very important for the carrot farmer, especially when a field has to be evaluated on the number of cysts/100 soil cm³, an estimate of the tolerance limit under South African field conditions is a necessity.

1.2.8. The life cycle of cyst forming nematodes.

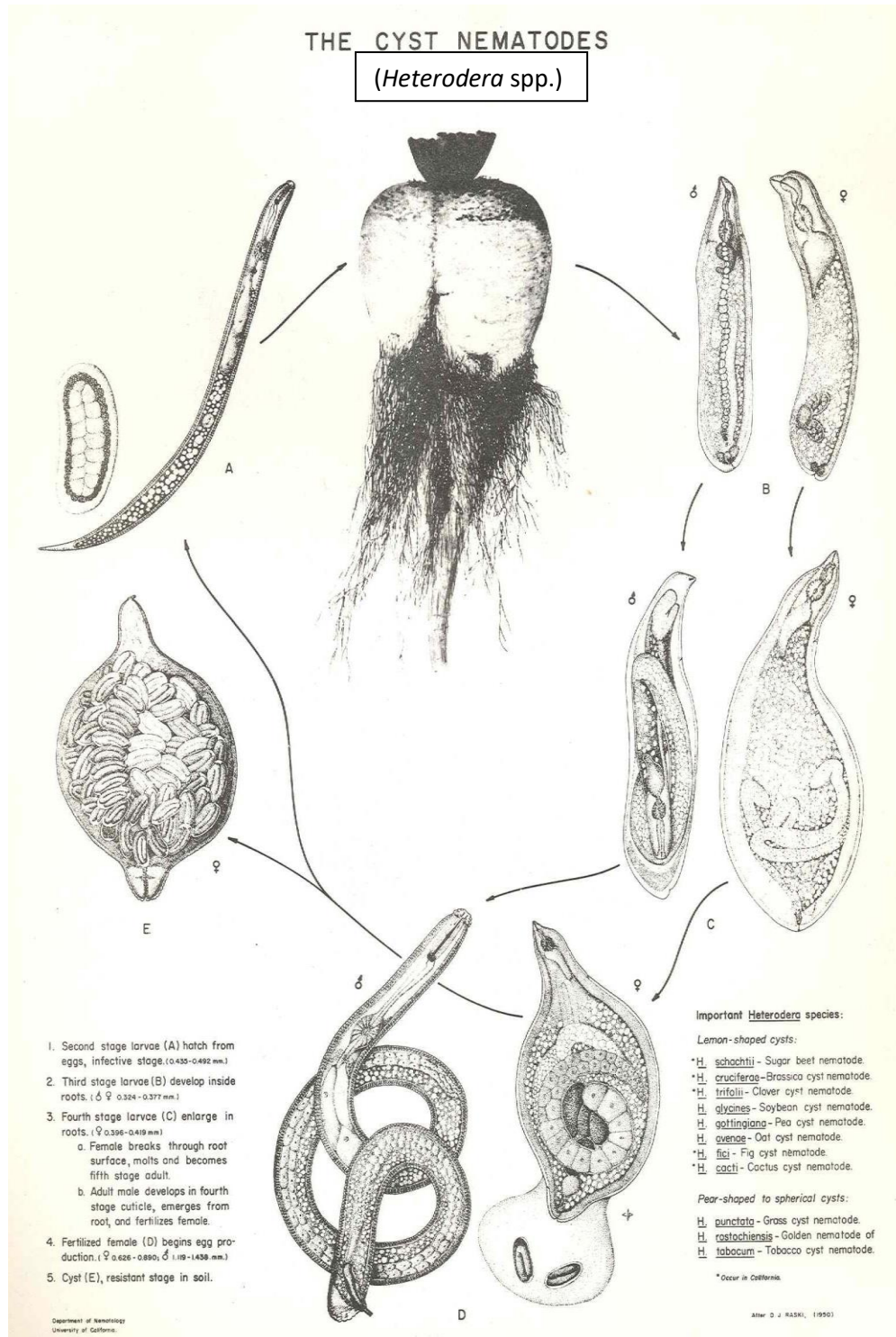


Figure 1.1. Illustration of the life cycle of the genus *Heterodera* by Charles S. Papp (Ferris, 1999)

The life cycle of cyst nematodes (Fig. 1.1) is comprised of the egg, four juvenile stages and one adult stage (female or male) (Subbotin *et al.*, 2010a). Mature females are capable of producing 500 up to 700 eggs (Subbotin *et al.*, 2010a). According to Sharma & Sharma (1998) the nematodes retain their eggs within a cyst that is formed from the cuticle of the mature female by a polyphenol tanning process. This is the dormant stage of the life cycle (Turner & Subbotin, 2013). The active part starts when the second stage juvenile (J2) hatches from the egg while within the cyst or the gelatinous matrix that comprises the egg sac (Subbotin *et al.*, 2010a). Once hatched, the J2 leaves the cyst via the natural openings, such as the fenestral region or at the neck where the female has broken away from the root. Second stage juveniles released into the soil immediately begin to search for a suitable host. As a survival strategy, not all J2 hatch at the same time (Turner & Subbotin, 2013).

Because of the combination of diapause and quiescence in cyst nematodes they can survive in the soil for more than 20 years in the absence of a host plant (e.g., *Globodera rostochiensis* Wollenweber, 1923) (Subbotin *et al.*, 2010a). The J2 enters the root system, migrates to the pericycle and proceeds to select a suitable cell from which a feeding site (syncytium) will be formed (Turner & Subbotin 2013). After establishing the feeding site, the J2 undergoes three stages to reach the adult stage (Subbotin *et al.*, 2010a). Cyst forming nematodes are sexually dimorphic (Subbotin *et al.*, 2010a) meaning that the male and female exist in two clearly separable forms (Abercrombie *et al.*, 1992). Second stage juveniles may develop into a male or a female adult. It takes approximately 7 days, depending on the temperature, for the J2 to moult into the third stage juvenile (Turner & Subbotin, 2013). At the fourth stage the young female ruptures the cortex of the root and the formation of the vulva gives the male access to her reproduction system. The vermiform males develop at a similar rate as the female, but males are non-feeding and live for only a short time in the soil. The complete life cycle from egg to egg of cyst forming nematodes can be completed in about 30 days but depends upon environmental conditions, as well as the synchronization of the specific cyst nematode species with its plant host (Turner & Subbotin, 2013).

1.2.9. Classification of cyst forming nematodes

For clarification, the definitions of the following frequently used terms, are given:

- Taxonomy is the science of naming, describing and classifying organisms and includes all plants, animals and microorganisms of the world (Anon, 2017g).
- Classification is the arrangement of animals and plants in taxonomic groups according to their observed similarities including at least Kingdom, Phylum, Class, Order, Family, Genus and Species (Anon, 2017h).
- Systematics is the study of the diversification of living forms, both past and present and the relationships are visualized as evolutionary trees (Anon, 2017i).

According to Turner & Subbotin (2013), the classification of the cyst forming nematodes is as follows:

Phylum: Nematoda Potts, 1932

Class: Chromadorea Inglis, 1983

Subclass: Chromadoria Pearse, 1942

Order: Rhabditida Chitwood, 1933

Suborder: Tylenchina Thorne, 1949

Infraorder: Tylenchomorpha De Ley & Blaxter, 2002

Superfamily: Tylenchoidae Orley, 1880

Family: Hoplolaimidae Filipjev, 1934

Subfamily: Heteroderinae Filipjev & Schuurmans Stekhoven, 1941

Genera: *Heterodera* Schmidt, 1871 (Type Genus)

Globodera Skarbilovich, 1959

Punctodera Mulvey & Stone, 1976

Cactodera Krall & Krall, 1978

Dolichodera Mulvey & Ebsary, 1980

Betulodera Sturhan, 2002

Paradolichodera Sturhan, Wouts & Subbotin, 2007

Vittatidera Bernard, Handoo, Powers, Donald & Heinz, 2010

1.2.9.1. Systematic position of the genus *Heterodera* Schmidt 1871

The genus *Heterodera* Schmidt 1871, being the type genus of the subfamily Heteroderinae Filipjev & Schuurmans-Stekhoven, 1941, is believed to have evolved in plants of tropical, subtropical and temperate regions worldwide (Evans & Rowe, 1998).

According to Subbotin *et al.* (2010b) the systematic genus outline of *Heterodera* is as follows:

Genus *Heterodera* Schmidt, 1871

=*Tylenchus* (*Heterodera*) Schmidt, 1871

=*Heterodera* (*Heterodera*) Schmidt, 1871

=*Heterobolbus* Railliet, 1896

=*Bidera* Krall & Krall, 1978

=*Ephippiodera* Shagalina & Krall, 1981

=*Afenestrata* Baldwin & Bell, 1985

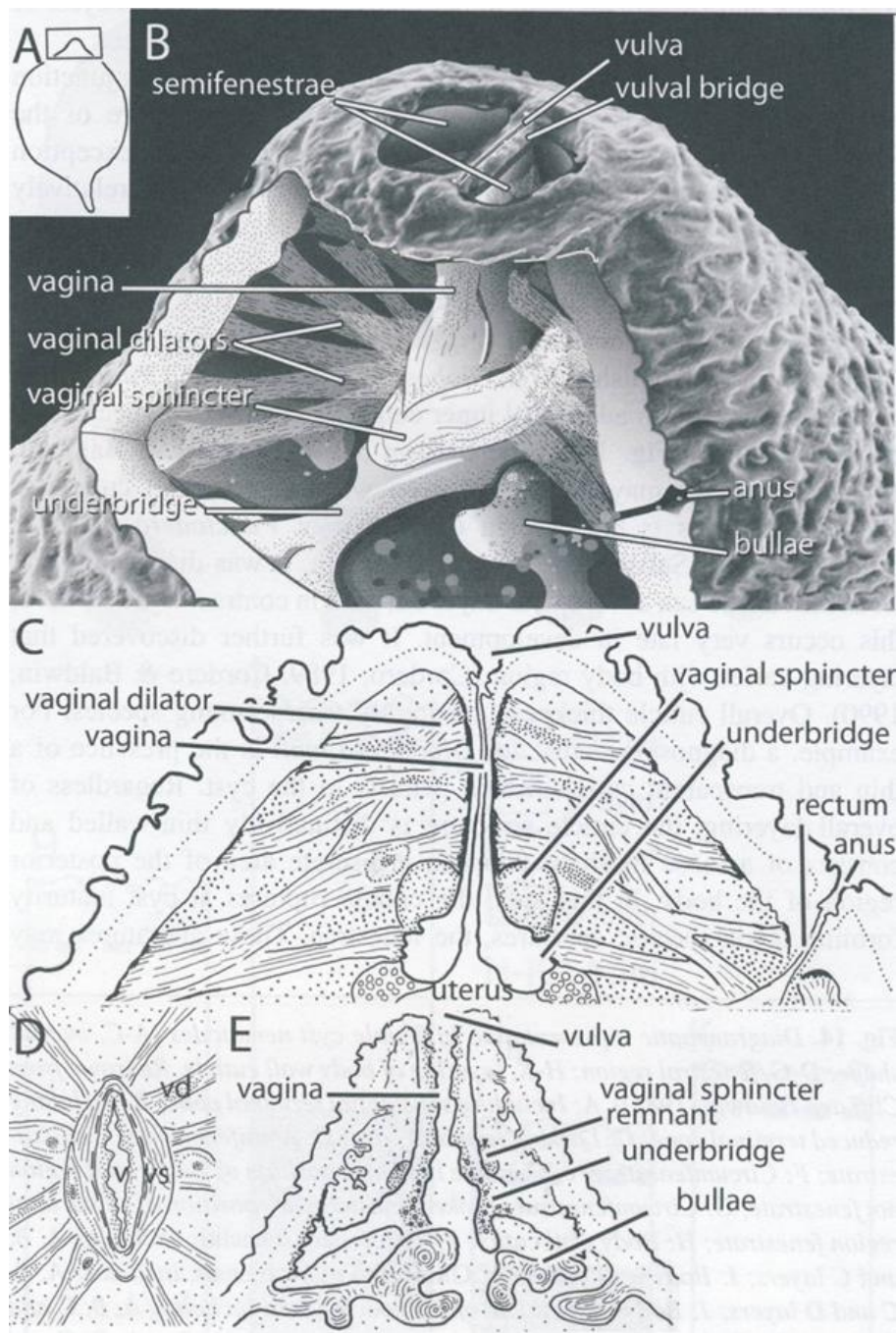
=*Afrodera* Wouts, 1985

=*Brevicephalodera* Kaushal & Swarup, 1989

1.2.9.2. Diagnosis of the genus *Heterodera* Schmidt 1871

1.2.9.2.1 Morphological diagnosis

The species in the genus *Heterodera* have a similar morphology and are often distinguished from each other by small details. Mature females and cysts are lemon shaped as they possess a protruding anterior end (head) and posterior end that are ending in a cone (Fig. 1.2) carrying the vulva, anus and fenestrae (Turner & Subbotin, 2013). The cuticle surface of the females and cysts has no annulation, but has a zigzag or lace like pattern (Subbotin *et al.*, 2010b). Wouts & Baldwin (1998) stated that the vulva lips are amalgamated into a vulval cone, the anus is subterminal on the dorsal side of the vulva cone with or without fenestration and the bullae, and underbridge are present or absent. The J2 are vermiform with an offset, dome shaped head and tail tapering to a point. The cuticle is regularly annulated (Turner & Subbotin, 2013) and is marked by three to four lateral field incisures and punctiform phasmids (Subbotin *et al.*, 2010b). Vermiform males are rare or absent in parthenogenetic forms. The male life cycle lasts about 10 days, they are found in the soil, especially at the roots near young females. The male body length is between 900-1600µm, the cuticle is annulated with three to four lateral incisures. (Subbotin *et al.*, 2010a).



1.2.9.2.2. Molecular diagnosis

Because of the similar morphology and small details that often distinguish the species in the genus *Heterodera* (Turner & Subbotin, 2013), the polymerase chain reaction-internal transcribed spacer-restriction fragment length polymorphism (PCR-ITS-RFLP) has been developed to differentiate between different species. Molecular identification often requires PCR by sequencing the ITS-rRNA genes (rRNA = ribosomal ribonucleic acid) with specific species developed primers. At present the *Heterodera* molecular diagnostic outline has been generated for 40 species, but another 40 known species have not been molecularly characterised as yet (Waeyenberge, *et al.*, 2009). Applying both morphological and molecular approaches are recommended to achieve the identification of the cyst nematodes of the genus *Heterodera* (Subbotin *et al.*, 2010b). Additionally, phylogenetic trees can be constructed by using molecular data to represent the historical relationship between groups of organisms or taxa, thereby reconstructing the genealogical ties between organisms, and to estimate the time of divergence between them, i.e. when they last shared a common ancestor. According to Jacobs (2005) these trees can be based primarily on morphological differences, but with the advances made by molecular studies, it is now possible to use DNA and protein sequences to reconstruct phylogenetic relationships.

1.2.10. The carrot cyst nematode *Heterodera carotae* Jones, 1950

According to Baldwin & Mundo-Ocampo (1991) the carrot cyst nematode belongs to the Goettingiana group [no bullae, underbridge poorly developed or absent, vulval slit more than 30 µm, ambifenestrate (two semifenestrae) or bifenestrate (two round fenestrae)] of the genus *Heterodera*. In 1950 *H. carotae* or carrot cyst nematode, was first described in England by Jones (Greco, 1986). Thereafter the carrot cyst nematode has been reported throughout the carrot growing areas of European and other countries (Subbotin *et al.*, 2010b). In Europe it has been reported from Cyprus, Denmark, France, Germany, Hungary, Italy, Ireland, the Netherlands, Portugal, Poland, Russia, Serbia, Slovenia, Slovakia, Sweden, Switzerland and the United

Kingdom (Subbotin *et al.*, 2010b). From outside Europe, *H. carotae* has been reported from the United States of America (Subbotin *et al.*, 2010b) and recently from Canada (Yu *et al.*, 2017). A report from India needs to be confirmed. From Africa, *H. carotae* has only been reported from South Africa (Subbotin *et al.*, 2010b). *Heterodera carotae* damage has severe negative effects on carrot production. In the field the symptoms appear as small, circular patches but may extend to the entire crop (Fig. 1.3 & Fig. 1.4). Carrot infected by *H. carotae* become stunted and have chlorotic or reddish foliage (Subbotin *et al.*, 2010b).



Figure 1.3 Above ground damage symptoms by carrot cyst nematodes, showing patches in an infested field where no plants are growing (Photo by A. R. Shubane).



Figure 1.4 Above ground damage symptoms by carrot cyst nematodes, showing dead and chlorotic foliage of infested plants (Photo by A. R. Shubane).

The tap roots are small and unmarketable (Fig. 1.5), and in heavily infested soil complete failure of the crop may occur (Greco, 1986). *Heterodera carotae* has a very limited host range, compared to other *Heterodera* spp. and can feed and reproduce on cultivated carrot and also its wild counterparts (Umbelliferae, such as *Trills leptophylla*, *T. arvensis* and *T. japonica*) (Greco, 1986).



Figure 1.5 Below-ground damage symptoms of carrot cyst nematodes (Photo by A.R. Shubane).

1.2.11. Management of cyst forming nematodes in carrot fields

Cyst nematodes are causing major damage to cultivated crops worldwide, mainly due to the genera *Globodera* and *Heterodera*. (Turner & Subbotin, 2013). They produce eggs inside the female body, which, upon death becomes a hardened protective wall, resistant to invasion by potential parasites and chemicals. This presents a unique problem in their management strategies. In cyst nematodes with a narrow host range, crop rotation has proven to be an important component in managing these nematodes (Turner & Subbotin, 2013). Cultivar resistance has also become the most economical practice in managing cyst nematodes. Nematicides were very effective in controlling these nematodes until it was withdrawn because of health and safety concerns (Turner & Subbotin, 2013). In South Africa only, furfural is registered for the use on carrot (Jones *et al.*, 2017). Application of a nematicide in the soil of seed beds/fields before planting the crop may reduce the potential impact of cyst nematodes.

1.3. Motivation for this study

- Except for limited information contained in Kleynhans *et al.* (1996), no other data on carrot cyst nematodes in South Africa have been published. Also, no formal morphometric or molecular identification have been performed on *H. carotae* for the African continent. As a taxonomist and a scientist, it is essential to know the identity of the organism one is working with.
- As the carrot cyst nematode in particular has proven to be a problematic species on carrot in the Tarlton area (Gauteng), it was decided to focus on the biology and systematics of this nematode in this particular area.

1.4. Objectives of the study

- To study the carrot cyst nematode population in the Tarlton area morphologically and molecularly, thereby determining if the South African *H. carotae* is conspecific with *H. carotae* worldwide.
- To study the different life stages of *H. carotae* on a weekly basis for taxonomic purposes and determine the number of life cycles the nematode completes per growing season. This is compared to that of its European counterpart.

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CHAPTER 2

MATERIALS AND METHODS

2.1. Introduction

Various techniques have been developed to extract cyst nematodes from soil and roots of plants. The nematodes in a sample must truly represent the prevailing population at a given time and several sampling patterns have been put in place to accomplish this. In the present study, root and corresponding rhizosphere samples were obtained from a carrot (*Daucus carota* L., cultivar Soprano) field in the Tarlton area (Gauteng Province of South Africa). The samples were taken in a zigzag formation to provide maximum coverage throughout the trial field (Haydock & Perry, 1998). In the Nematology discipline, morphology has been the basis for identification of species for a long time (Golden, 1986) and included light microscope and scanning electron microscope (SEM) studies. The latter has been especially useful in showing surface details, which provided a sounder basis for identification. At present molecular identification of cyst nematodes, using the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) and other techniques, has proven to be preferred by many scientists and may become the technique of choice for cyst nematode diagnostics (Fleming & Powers, 1998).

The purpose of the present study was to study the carrot cyst nematode, *Heterodera carotae* Jones, 1950 in the Tarlton area morphologically and molecularly, thereby determining if the South African *H. carotae* is conspecific with *H. carotae* worldwide. The different life stages of *H. carotae* were also extracted from soil and root samples on a weekly basis to study the morphology of the different life stages for taxonomic purposes. The number of life cycles completed by the nematode per growing season was compared with that of its European counterpart. The accumulation of day degrees were also compared with nematode life stages in the roots of carrot at 20 cm below the soil surface (See also Chapter 3).

This chapter describes the different techniques used to i) extract cyst nematodes from soil and root samples, ii) fix and mount different stages of the nematodes for light microscopy, SEM and molecular analysis (DNA extraction and PCR), and iii) study the biology of the cyst nematode infecting carrot.

2.2. Extraction of nematodes from soil samples and carrot tap root samples

Carrot tap root samples with corresponding rhizosphere soil samples were collected on a one-hectare trial block in the Tarlton area, Krugersdorp, Gauteng Province, South Africa during the 2016-2017 summer growing season (Fig. 2.1, Fig. 2.2 and Fig. 2.3). This was done on a weekly basis for 18 weeks, using the zigzag pattern of collecting samples. The soil in this field was classified as sandy loam (sand: 76, 2 %; loam: 13, 5 %; clay: 10, 3 %) with a pH of 6.41 and 0.72 % organic matter. The carrot field was irrigated by using drag lines and conventional tillage was used. The trial site received about 585 mm water from 14/11/2016 – 13/3/2017 (irrigation and rain). No nematicides were used and only two fungicides were administered [Copstar 120 SC® (active substance: Copper hydroxide) and Folicur® 250 EC (active substance: Tebuconazole (triazole))] during the growing season of this carrot crop. The history of the trial block is as follows: A cover crop mix of Babala grass (*Pennisetum glaucum*), Bargazer sorghum (*Sorghum bicolor*), teff (*Eragrostis tef*), beans (*Phaseolus* spp.), Dolichos beans (*Lablab purpureus*), interval turnip (*Brassica rapa*), Barkant turnip and sunhemp (*Crotalaria juncea*) were planted on 23 September 2015. Thereafter carrot were planted and harvested in March 2016. The land was then fallow for seven months, after which the present carrot crop was sown.

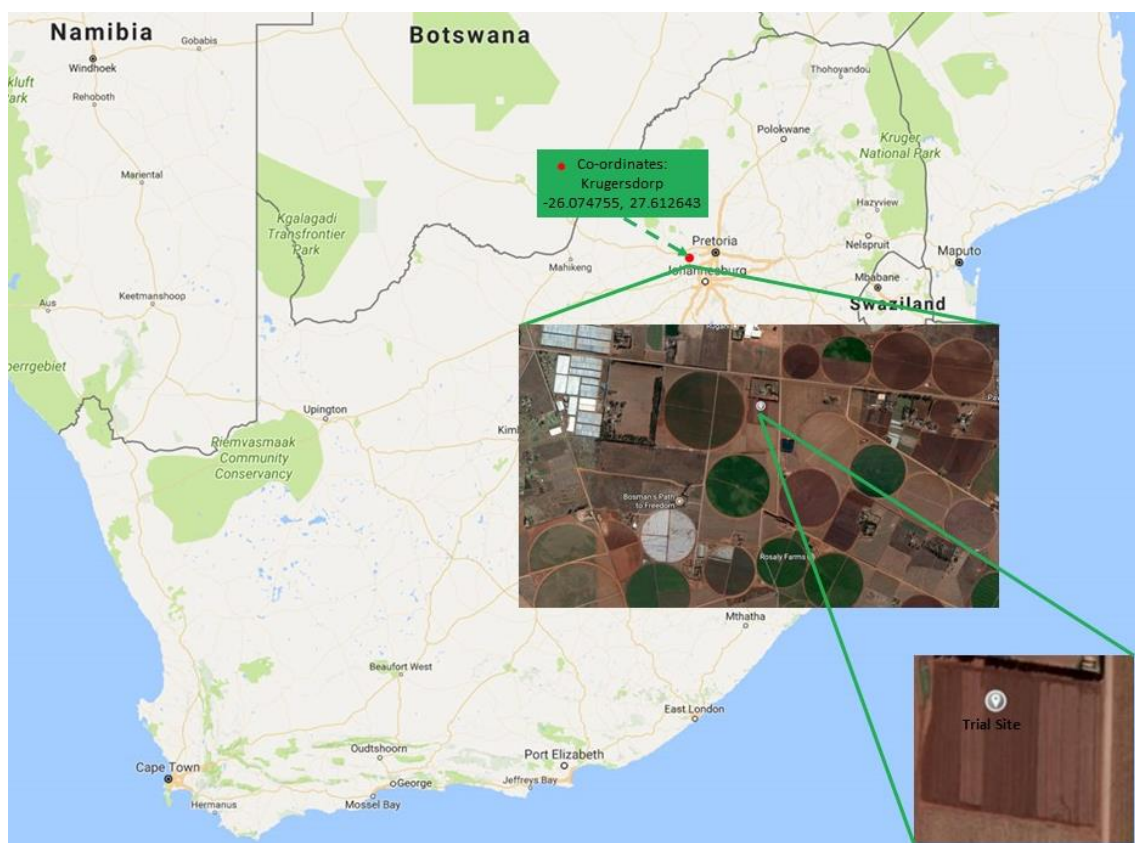


Figure 2.1 The trial site near Tarlton (Gauteng Province of South Africa) where carrot was sampled for the identification of the carrot cyst nematode, *Heterodera carotae* (Map obtained from E. van Niekerk, Graphical Design: ARC-PHP: Photo's by A. Browne).



Figure 2.2 Ms Adoration Shubane at Trial Block S at harvest in a carrot field in the Tarlton area (South Africa) (Photo by K. Tsamwisi).



Figure 2.3 Mr Kudakwashe Tsamwisi (Greenway Farms) taking root and soil samples in Trial Block S at harvest in a carrot field near Tarlton (South Africa) (Photo by A.R. Shubane).

Nematodes were extracted from the soil (250cm³ soil/sample) by the sieving centrifugal flotation method (Fig. 2.4 – Fig. 2.7) according to the protocol of Swart & Marais (2017). The 250 cm³ soil was washed through a course meshed 2 mm aperture sieve into a 5-liter bucket. The suspension was stirred, and then allowed to settle for 30 seconds. The suspension was then poured through a 45 µm aperture sieve. This procedure was repeated two more times, but the settling times were shortened to 20 and 10 seconds, respectively. The residue was then transferred from the 45 µm aperture sieve to two centrifuge tubes, 5 cm³ kaolin was added to the suspension, which were then centrifuged for 7 minutes at 3500 revolutions per minute (rpm). The supernatant was decanted from the tubes and discarded. A sugar solution (450 cm³ sugar/1 l water) was then added to the tubes, carefully mixed and centrifuged for 3 minutes at 3500 rpm. The suspensions were then poured through a 45 µm aperture sieve and collected into a beaker for examination in a De Grisse counting dish (De Grisse, 1969).



Figure 2.4 Sample bag with soil and roots, together with a beaker with 250g soil. This is ready for the start of the sieving centrifugal flotation method (Photo by S.P. Swart).



Figure 2.5 Soil sample washed through a 45 µm sieve during the sieving centrifugal flotation method (Photo by M. Marais).



Figure 2.6 Centrifuge (Photo by S.P. Swart).



Figure 2.7 Centrifuge tubes are filled with the extracted soil sample, ready to be centrifuged for 7 minutes at 3500 revolutions per minute (Photo by S.P. Swart).

Nematodes were extracted from the carrot tap roots by cutting off the rootlets with a scissor into small, ±1-cm pieces. The tap root itself was not used for extraction purposes as *H. carotae* infect only the rootlets of the carrot. The soil was also sieved through a 2-mm aperture sieve to enable picking up all rootlets by using a tweezer. This method was applied (and discovered during the study) to make sure that all rootlets were collected for extraction. The rootlets were weighed to make up 20 g. Ten ml of 4 % sodium hypochlorite (NaOCl) was added to 1 l of water, and added to the

rootlets and blended using a fruit blender at a medium speed for 45 seconds. NaOCl is used to dissolve the egg masses, thereby setting the eggs free to be counted. The carrot-water suspension was poured through nested 1000 μm , 45 μm and 38 μm aperture sieves.

The residue on the top sieve (1000 μm aperture) was discarded, and the residues on the other sieves collated into centrifuge tubes, kaolin was added and they were centrifuged for 7 minutes at 3500 rpm. The supernatant was decanted from the tubes and discarded. A sugar solution (450 cm^3 sugar/1 l of water) was added to the residue in the tubes, thoroughly mixed and centrifuged for 3 minutes at 3500 rpm. The suspension was then poured through the 38 μm aperture sieve and was rinsed from the sieve into a beaker for examination in a De Grisse counting dish (Swart & Marais, 2017).

2.2.1. Extraction of cyst nematodes

The nematode cysts were extracted from 100 cm^3 dried soil samples by using the Seinhorst cyst elutriator (Seinhorst, 1964). The Seinhorst elutriator works by using an upward current of water to gather the dried cysts in a 150 μm aperture sieve. First the clamp on the lower downpipe is closed and then the cylinder is closed with a rubber bung. The speed of the upward current was adjusted to the desired setting of about 4 cm water/second and both downpipes were placed in a nest of 1000 μm - and 150 μm aperture sieves. A sample of 100 cm^3 dried soil was washed through a 2-mm aperture sieve into the top bowl of the apparatus. The water (and debris) rised in the cylinder and spilled over the collar into the nest of sieves. After about 30 seconds the lower downpipe was opened and the debris (with cysts) was carefully washed through the nested sieves. The debris in the 1000 μm aperture sieve was gently washed through on to the 150 μm aperture sieve. The rest of the debris on the 1000 μm aperture sieve was discarded and the cysts collected into the 150 μm aperture sieve. The content of this sieve was transferred, with the aid of a wash bottle, to a funnel lined with a round Whatman filter paper (185 mm in diameter). The filter paper was folded into a funnel shape before fitted into the funnel. When the water had drained from the funnel, the filter paper was removed, opened to form a full circle, dried further on a plastic disc

(plate) and examined for cysts using a dissection microscope with light from above (Swart & Marais, 2017). See Fig. 2.8 – Fig. 2.19 for the cyst extraction method.



Figure 2.8 The Seinhorst cyst elutriator (Photo by S.P. Swart).



Figure 2.9 The soil sample containing cysts being washed through the 2-mm aperture sieve (Photo by S.P. Swart).



Figure 2.10 The water (and debris) spills over the collar through the upper downpipe aperture sieve (Photo by S.P. Swart).



Figure 2.11 Debris and cysts collected on a 150 μm aperture sieve (Photo by S. P. Swart).



Figure 2.12 Debris and cysts were transferred from the 150 μm aperture sieve to the filter paper that fitted snugly in the plastic funnel into the funnel (Photo by S.P. Swart).



Figure 2.13 Sample in the funnel, draining into the wash trough (Photo by S.P. Swart).



Figure 2.14 Sample drained into the wash trough with the ring of cysts and debris formed on the inner sides of the filter paper funnel (Photo by S. P. Swart).



Figure 2.15 The filter paper funnel opened into a circle and dried on a paper towel (Photo by S. P. Swart).



Figure 2.16 The filter paper, still damp, put on a round, plastic plate (Photo by S.P. Swart).



Figure 2.17 The filter paper on the plastic plate is put on the stage of a dissection microscope to be viewed (Photo by S. P. Swart).



Figure 2.18 The sample being viewed with light from above with the circle of cysts and debris clearly visible (Photo by S.P. Swart).



Figure 2.19 The funnel in preparation, the Whatman filter paper already folded in quarters to be put into the funnel (Photo by S.P. Swart).

2.3. Fixing and Mounting of infective juveniles (J2)

After the nematodes were collected in a Syracuse dish, most of the water was drawn off using a syringe. FPG fixative (100 ml formalin 40 %, 10 ml propionic acid, 890 ml distilled water) was drawn into a pipette and put into a test tube. Boiling water (80 ml) was poured into a small glass beaker and the test tube held in the boiling water until it reached 60-70 °C. The hot fixative was poured onto the live nematodes. The Syracuse dishes were then placed in a petri dish (60 x 15 mm), closed and placed in a desiccator. The desiccator was placed in an incubator for a minimum of three days at 38-40 °C. Seinhorst (1959), Solution 1 (200 ml 95 % alcohol, 10 ml glycerol, and 790 ml distilled water) were added after half of the FPG fixative was drawn off. The Syracuse dishes were left open and were placed back into the desiccator for about 12 hours. Seinhorst (1959), Solution 2 (950 ml 95% alcohol, 50 ml glycerol) was added to the nematodes after half of Solution1 was drawn off. The dish with the nematodes suspended in the fixative solution were slightly covered for slow evaporation and put

back into the incubator for 2-3 days or until all the alcohol has evaporated. This was placed in a desiccator (with silica for dehydration) at room temperature for about one day. After that, the nematodes were ready for mounting (Swart & Marais, 2017).

The fixed specimens were mounted on a handmade aluminum Cobb slide (Fig. 2.20). Firstly, a wax ring was made in the middle of a 24 x 24 mm square coverslip (the wax must match the nematode's thickness). A small drop of glycerol was placed in the center of the wax ring. Four to five fixed specimens were placed in the glycerol drop and were gently pressed down onto the surface of the coverslip. A 15 μ m thick, 19 mm round coverslip was briefly heated over a spiritus flame and lowered onto the glycerol drop. The coverslips were put on an electrothermal slide drying bench for the wax to dissolve and seal the coverslips. The coverslip containing the nematode individuals was then put into the aluminum slide, carton squares were put on each side of the coverslip and the aluminum was bent into shape to form a finished Cobb slide (Swart & Marais, 2017). Twenty juveniles were mounted on Cobb slides for morphologic and morphometric study. Slides were kept in slide storage boxes.

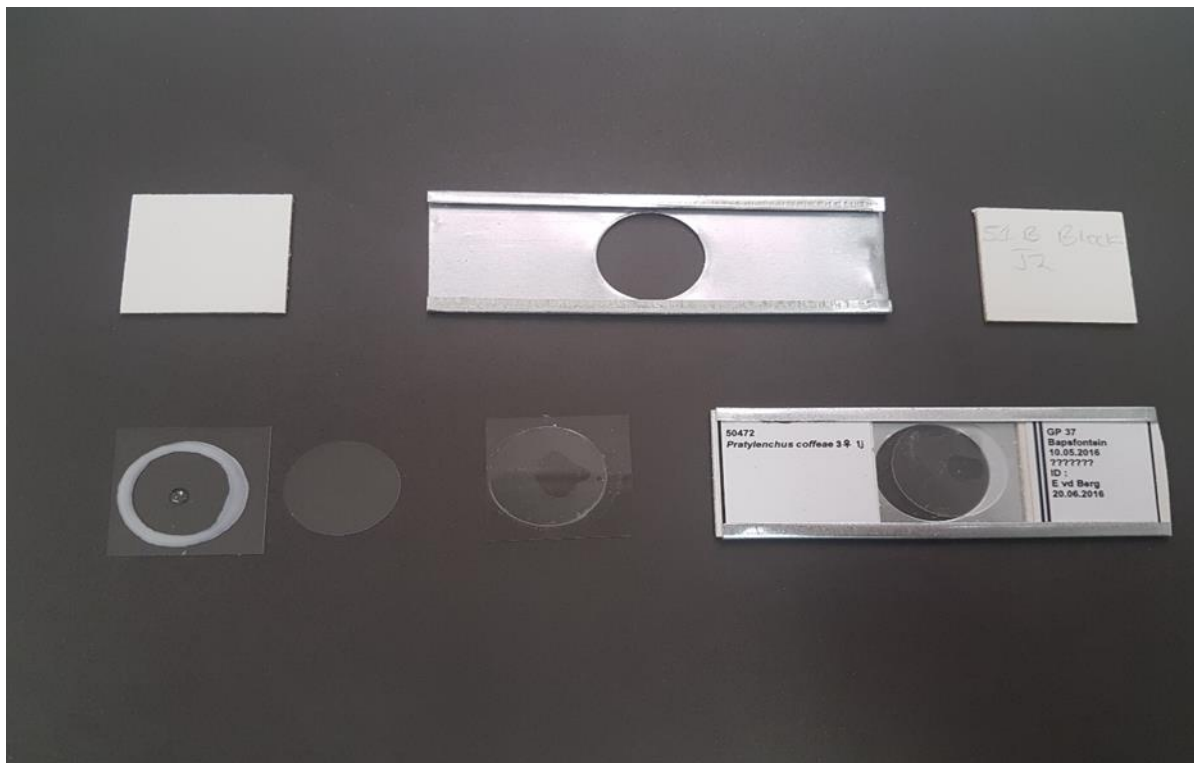


Figure 2.20 Making a Cobb slide (Photo by A.R. Shubane).

2.4. Fixing and mounting of females

Females were fixed in cold lactophenol and incubated at 40 °C for three days (Knoetze *et al.*, 2017). The female body was transferred into a small drop of 100 % lactic acid, punctured behind the neck area by using a scalpel blade and cut into half with an insulin syringe. The anterior half of the body, containing the head and neck structures, were placed in a glycerol drop and gently pressed down onto the surface of a glass microscope slide. A round coverslip was applied and sealed onto a microscope slide with glyceel (Marais *et al.*, 2017).

2.5. Fixing and mounting of males

After the nematodes were collected in a Syracuse dish, most of the water was drawn off. Cold TAF fixative (7ml formalin 40 %, 2ml triethanolamine, and 91 ml distilled water) were drawn into a pipette and poured onto the nematodes. The specimens were immediately mounted in a small drop of cold TAF, which were placed into the center of a glass microscope slide. Four to five specimens were placed in the drop and were gently pressed down onto the surface of the slide. A round coverslip was applied and sealed onto a glass microscope slide with glyceel (Kleynhans, 1991).

2.6. Mounting of the terminal pattern of cyst nematodes

A cyst was placed in a drop of water on a cavity slide on the stage of a dissecting microscope. The cyst was then transferred to a cavity slide with lactophenol and heated until the lactophenol was fuming. It was then transferred to 100 % lactic acid. Using an insulin syringe, the posterior end of cyst was cut off and the body content removed with a dissecting needle without disturbing the structures in the vicinity of the vulva. The cuticle around the vulval area was then carefully trimmed away so that only the vulval cone was left. The vulval cone was transferred to water, then to 96 % alcohol and then to xylene for clearing. A piece of glycerol-gelatine jelly was placed on a glass slide and gently heated to 55 - 60 °C. The vulval cone was transferred to the melted jelly by using a dissecting needle and then its position was corrected (vulval area facing directly upwards) by using a dissecting needle. Three glass rods were placed around the vulval cone to avert it from collapsing during the steps that follow. Air bubbles were removed from the mounted vulval cone. After the jelly has solidified, a

heated 15 µm thick coverslip was gently placed on top and again melted down a bit. The coverslip was fastened to the slide by using glyceel. Glass slides carrying the mounted terminal region were kept in slide storage boxes (Swart & Marais, 2017).

2.7. Fixation and mounting of cysts, J2, females and males for SEM

2.7.1 External and internal morphology of cysts

To study the external morphology of the cysts with SEM, whole cysts were fixed in 4 % formalin (12, 5 ml formalin, 87, 5 ml distilled water) for a minimum of 48 hours. After 48 hours, they were transferred into PCR tubes and submitted to ultrasound in a Sonorex GT 120 transistor device for 10 minutes to be cleaned of any debris. They were rinsed in double distilled water and placed on filter paper inside a petri dish in a desiccator to dry at room temperature for 24 hours. The dried cysts were then mounted on aluminum SEM stubs, with carbon double-sided adhesive tape, in different positions to facilitate the taking of photomicrographs of the anterior and posterior ends of the cysts (Lax & Doucet, 2002).

To study the internal morphology of cysts, individual cysts were placed in a cavity slide with lactic acid (45 % solution), cut/trimmed along the mid-regions with an insulin syringe and cleaned internally with a dissecting needle without disturbing the structures in the vicinity of the vulva (Swart & Marais, 2017). They were transferred into a clean solution of 45 % lactic acid for 24 hours. After 24 hours, the cones were transferred to micro centrifuge tubes, submitted to ultrasound in a Sonorex GT 120 transistor device for 10 minutes to be cleaned and then transferred to 4 % formalin for 24 hours. They were rinsed in double distilled water and placed on filter paper inside a petri dish in a desiccator to dry at room temperature for 12 hours. The dried vulval cones were then mounted on aluminum stubs, with carbon double-sided adhesive tape, so that the cones could be viewed on the inside (Lax & Doucet, 2002).

2.7.2. External morphology of J2, females and males

After the nematodes were collected in a Syracuse dish, most of the water was drawn off. TAF fixative solution (distilled water, 40 % formalin and triethanolamine) were poured over the specimens and they were left at room temperature for a week. The specimens were put into an especially handmade holder (Fig. 2.21) consisting of a horizontally cut Eppendorf tube (with its lid), forming a small holder, and two round filter papers snugly fitting between the tube and its lid. A hole was cut into the lid to let liquids pass through the filter papers. A small drop of TAF was placed onto the bottom filter paper and the specimens were transferred with a needle into the TAF. Next, the drop of TAF was very carefully drawn off, and the second filter paper carefully lowered onto the specimens. The two filter papers with the specimens between them were then sealed by pressing the cylinder part of the Eppendorf tube into the lid. This makes a perfect little holder to carry the specimens through the next steps until they were critically point dried. The specimens were transferred into 30 % ethanol overnight and then processed three times through an ethanol series [50 %, 70 %, 80 %, 90 % and 96 %] with an interval of 30 minutes in each. It was then placed in 100 % ethanol and critical point dried with carbon dioxide as intermediate fluid. After this step, the Eppendorf holder was opened to have access to the specimens on the filter papers.

To make it easier to locate the specimens, a drop of toluidine blue stain (1 mg toluidine blue in 5 ml absolute ethanol) was put on the filter papers so that the paper stained blue and the specimens showed up white. The specimens were picked up with a needle and mounted on a SEM stub with double sided carbon tape. They were coated with gold-palladium (21 nm) and examined with a FEI Quanta FEG 250 scanning electron microscope at 5-10 KV. The method described above is based on that of Marais *et al.* (2017). The staining of the filter papers to show up the specimens was discovered during the transference of the critical point dried specimens to the SEM stub.

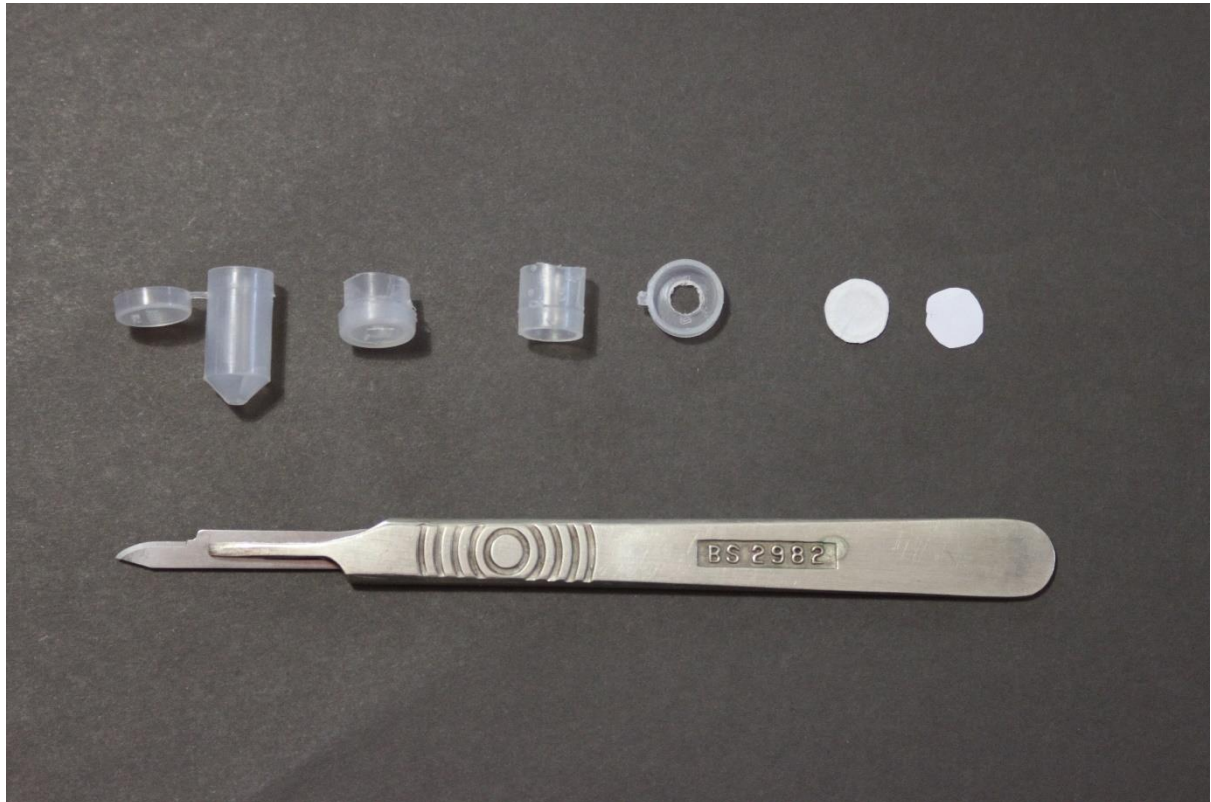


Figure 2.21 Especially handmade holder made from an Eppendorf tube that is used to prepare cyst specimens for scanning electron microscopy (Photo by A. Swart).

2.8. DNA extraction and polymerase chain reaction

Cysts were cut open and the J2 removed. Individual J2 were hand-picked with the hair of a brush attached to a dissecting probe and placed in 5 μ l PCR reaction buffer (16 mM $(\text{NH}_4)\text{SO}_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20) containing 60 $\mu\text{g ml}^{-1}$ Protein K in a sterile PCR tube. The J2 were cut into small pieces with the sterile needle of an insulin syringe.

The tube was incubated at 60 °C for 30 minutes, and a further 5 minutes at 95 °C. The tubes were stored at -20 °C until further use (Knoetze & Swart, 2014).

The ITS region was amplified using the primers 18S (TTG ATT ACG TCC CTG CCC TTT) and 26S (TTT CAC TCG CCG TTA CTA AGG). PCR amplification were carried out in the same tube containing 5 μ l of nematode lysate together with 0.5 μM of each primer, dNTPs each at 200 μM final concentration, 1 x Taq reaction buffer, 1.5 mM MgCl_2 and 1 U Taq polymerase (KAPA2G Robust HotStart readymix, KAPA Biosystems). The cycling conditions were 30 cycles of 94 °C for 20 seconds, 55 °C for

30 seconds, and 72 °C for 45 seconds, with a final extension at 72 °C for 2 minutes (Knoetze & Swart, 2014).

2.9. Biology of the carrot cyst nematode

The study of the biology of the carrot cyst nematode under South African field conditions was undertaken by comparing the accumulation of day degrees with nematode life stages in the roots of carrots at 10- and 20 cm below the soil surface. These temperatures were taken by using an Aquacheck probe as part of soil moisture monitoring by Irricheck, a company that specialises in scheduling irrigation in crop fields (www.irricheck.co.za). The daily maximum and minimum temperatures were used to calculate the degree days that accumulated during the first 10 weeks of the growing season, using DegDay version 1.01, which can be downloaded at <http://biomet.ucdavis.edu/DegreeDays/DegDay.htm>. Calculations in this application are described by Zalom *et al.* (1983). A basal development temperature of 10 °C, as determined by Greco & Brandisio (1986) was used. Unfortunately, temperature data was only recorded up to week 10 and therefore, we could not calculate accumulated day degrees for the entire growing season of the crop.

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CHAPTER 3

STUDY OF THE LIFE CYCLE OF *HETERODERA CAROTAE* JONES, 1950 UNDER FIELD CONDITIONS

3.1. Introduction

The host range of the carrot cyst nematode, *Heterodera carotae* Jones, 1950 is rather narrow and only cultivated and wild carrot (*Daucus carota* L.) are infected (Yu *et al.*, 2017). The infective, second-stage juveniles (J2) invade only carrot feeder roots, mainly at the tips. Although the nematode can develop in the early growth stage of the main root, no invasion or development occurs in the taproots. The plant reacts to the invasion of the J2 by producing new rootlets, which in turn can also be invaded. The taproot of an infected plant therefore often has a heavily bearded appearance. According to observations by European scientists, field infestations usually appear in patches of stunted plants on which the foliage become reddish before they die (Subbotin *et al.*, 2010b). Taproots are greatly reduced in size and are unmarketable. Complete failure of the crop may occur in heavily infested soil. In Switzerland, a tolerance limit of carrot to the carrot cyst nematode was estimated at 40 cysts/250 cm³ of soil (Greco, 1986).

Initial investigations into the life cycle of *H. carotae* were made by Greco & Brandonisio (1986), who determined the basal development temperature of *H. carotae* at 10 °C. These authors found that *H. carotae* required 260 and 360 degree days to develop females and cysts at 20 °C, respectively, and that it can complete two life cycles under Italian environmental conditions. A more recent study by Colagiero & Ciancio (2011) was done by modelling changes in future temperature increases regarding its effect on *H. carotae*, also in Italy. These authors demonstrated that this cyst nematode can complete two to three life cycles under increasing temperatures of 1.5 to 2 °C and that additional generations could be expected during Italian winter seasons.

For clarity, a degree day (DD), also known as a growing degree day, a heat unit or a thermal unit, is the measurement of the amount of heat that accumulates above a specified base temperature, which is 10 °C for *H. carotae* as determined by Greco & Brandonisio (1986), during a 24-hour period (Herms, 2004). According to this author,

one DD accumulates for each degree the average temperature remains above the base-temperature over the 24 hours.

Thus, several degree days can accumulate during this period (24 h). Another term is also used: Cooling Degree Days (CDD), which is a measure of how much (in degrees) and for how long (in days) the temperature was higher than a specific base temperature (Anon, 2017). In essence, DD are used to accurately predict the development of an organism and is essential for effective pest management (Herms, 2004).

Limited work on *H. carotae* has been done to date. Greco & Brandonisio (1986) found that hatching of J2 from eggs was negligible at 25 °C and that two generations of the nematode can be completed within one growing season under Italian conditions. Mugniery & Bossis (1988) investigated the relationship between temperature and the length of different life stages of *H. carotae* in France. Later Colagieri & Ciancio (2011) used this data as an example of modelling changes induced by global warming on *H. carotae*. Although this cyst nematode does cause problems to carrot producers in South Africa, no research has been done under local environmental conditions to determine the duration of its life cycle. Therefore, this study was focused on elucidating the duration of the life cycle of *H. carotae* under field conditions in a carrot production area in South Africa.

3.2. Materials and methods

3.2.1. Study area

For the purpose of this study, one hectare of a field in the Tarlton area, Gauteng Province, South Africa, infested with *H. carotae* was sown with carrot (cultivar Soprano) on 7 November 2016. The soil in this field was classified as sandy loam (sand: 76, 2 %; loam: 13, 5 %; clay: 10, 3 %) with a pH of 6, 41(H₂O). The carrot crop was irrigated by using draglines and only two fungicides were administered [Copstar 120 SC® (active substance: Copper hydroxide) and Folicur® 250EC (active substance: Tebuconazole (triazole))] during the growing season of this carrot crop. The same cultivar was sown in an adjacent field on the same date, where a full Integrated Pest Management (IPM) program was followed. This included the administration of two

nematicides, with oxamyl and fenamiphos as the respective active ingredients, according to registration (Van Zyl, 2013). The field in which the life cycle of *H. carotae* was studied, was planted with a cover crop mix of Babala grass (*Pennisetum glaucum*), Bargazer sorghum (*Sorghum bicolor*), teff (*Eragrostis tef*), beans (*Phaseolus* spp.), Dolichos beans (*Lablab purpureus*), interval turnip (*Brassica rapa*), Barkant turnip and sunhemp (*Crotalaria juncea*) (*Crotalaria juncea* L.) on 23 September 2015. Thereafter carrot was planted, which was harvested in March 2016. The land was then left fallow for seven months, after which the present carrot trial, representing the study plot for this study, was sown.

3.2.2. Soil temperatures

Soil temperatures were measured at 20 cm depth and 20 cm apart throughout the 1-hectare study plot, using an Aquacheck probe (<http://www.aquachecktech.com/>). Temperature measurements done this way forms part of soil moisture monitoring by the company Irricheck (www.irricheck.co.za) who specializes in the scheduling of irrigation for agricultural crops. Unfortunately, temperature data was only recorded up to week 10, therefore inhibiting our ability to calculate accumulated day degrees for the entire growing season of the crop.

3.2.3. Day Degrees

The daily maximum and minimum temperatures were used to calculate the degree days that accumulated during the first 10 weeks of the growing season, using DegDay, Version1.01, which can be down loaded at <http://biomet.ucdavis.edu/DegreeDays/DegDay.htm>. Calculations used in this application are based on the protocol developed by Zalom *et al.*, (1983). A basal development temperature of 10 °C for *H. carotae*, as determined by Greco & Brandisio (1986), was used for this study.

3.2.4. Sampling

Soil samples were collected weekly from emergence to harvest, representing 18 weeks, at a depth of approximately 20 cm from the soil surface. A zigzag pattern, as described by Swart & Marais (2017) was followed during sampling from week 1 to week 6 only soil samples and from week 7 onwards, carrot taproots were also included in the samples.

Measurements of the length of carrot taproots were done on a weekly basis and pictures of *H. carotae* infected and healthy/non-infected tubers were also taken weekly for the observation of the accumulating symptoms. These photographs are included in Appendix 1.

3.2.5. Extraction of nematodes

Nematodes were extracted from the soil (250 cm³) by the centrifugal flotation method as described in Chapter 2. Nematodes were also extracted from the carrot taproots from week 7 onwards as described in Chapter 2. Nematode cysts were extracted from 100 cm³ dried soil samples by using the Seinhorst cyst elutriator (Seinhorst, 1964), as described in Chapter 2. This method has been adapted specifically for extracting cysts from soil. Afterwards, the water suspensions containing the nematodes were examined, individuals classified into their developmental stage and counted in a De Grise counting dish (De Grise, 1969).

3.2.6. Light microscopy

Nematodes were counted and classified according to their developmental stages using a Wild Heerbrugg 173700 dissection microscope and cold light source Leica KL 1500 LCD. The life- or developmental stages were identified by studying the drawings such as that of (Ferris, 1999) depicted in Chapter 1 (Fig. 1.1).

3.3. Results

3.3.1. Soil temperatures

Figure 3.1. plots the fluctuations in soil temperatures that occurred during the first 10 weeks of this study, taken at a depth of 20 cm at the study location in Tarlton, Gauteng, South Africa.

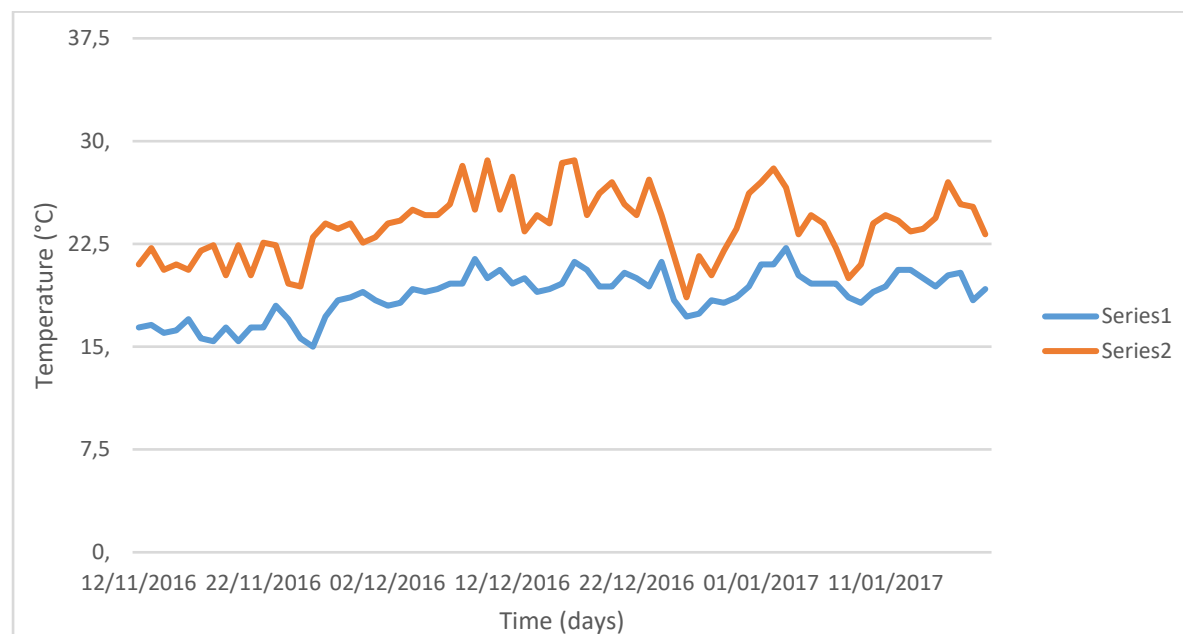


Figure 3.1. Minimum (blue/Series1) and maximum (red/Series2) daily temperatures recorded at a depth of 20 cm in a carrot field near Tarlton (Gauteng Province, South Africa) where samples were obtained for species identification and duration of a life-cycle for *Heterodera carotae* Jones, 1950.

During this period, a maximum temperature of 28.6 °C and a minimum temperature of 15.0 °C were recorded. Soil temperatures recorded across the study plot during the first 10-week of this study constantly remained above the basal development temperature of 10 °C (Greco & Brandisio, 1986), leading to a relatively rapid increase in accumulated CDD.

3.3.2. Accumulated Cooling Day Degrees

Figure 3.2 shows the accumulation of CDD calculated from maximum and minimum temperatures measured at the trial location for the first 10 weeks of the growing season. In this period 742.4 CDD accumulated.

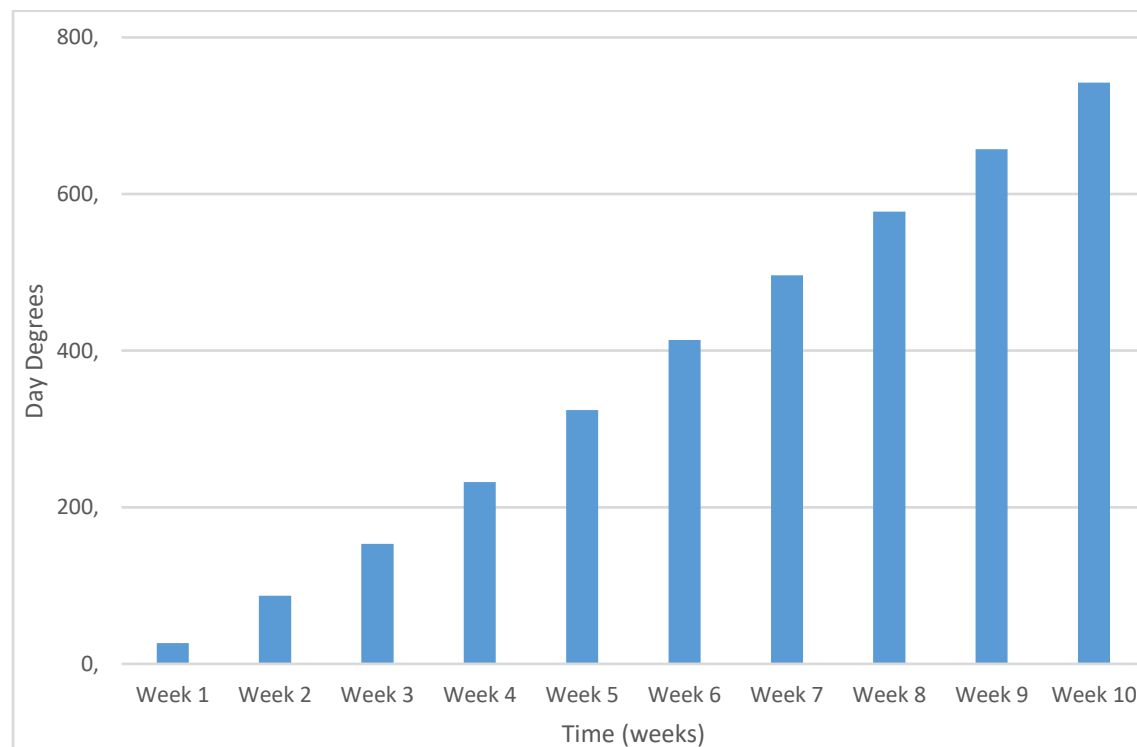


Figure 3.2 Accumulation of Day Degrees during a 10-week period in a carrot field near Tarlton (Gauteng Province, South Africa) where a study on the identification and duration of the life cycle of the carrot cyst nematode, *Heterodera carotae*, Jones, 1950 was conducted.

3.3.3. Nematode life stages found in soil samples

Figure 3.3 depicts the numbers of different life stages detected in a 250 cc soil sample, taken at weekly intervals at the study location for the duration of the growing season. Second stage juveniles (J2) were detected in samples for the first 7 weeks, disappearing in week 8, only to reappear again in week 13. Third (J3) and fourth juvenile (J4) stages were observed in the soil from week 7, but their origin are thought to be from small tap roots that were included in soil samples and from which it could have been dislodged during the extraction process

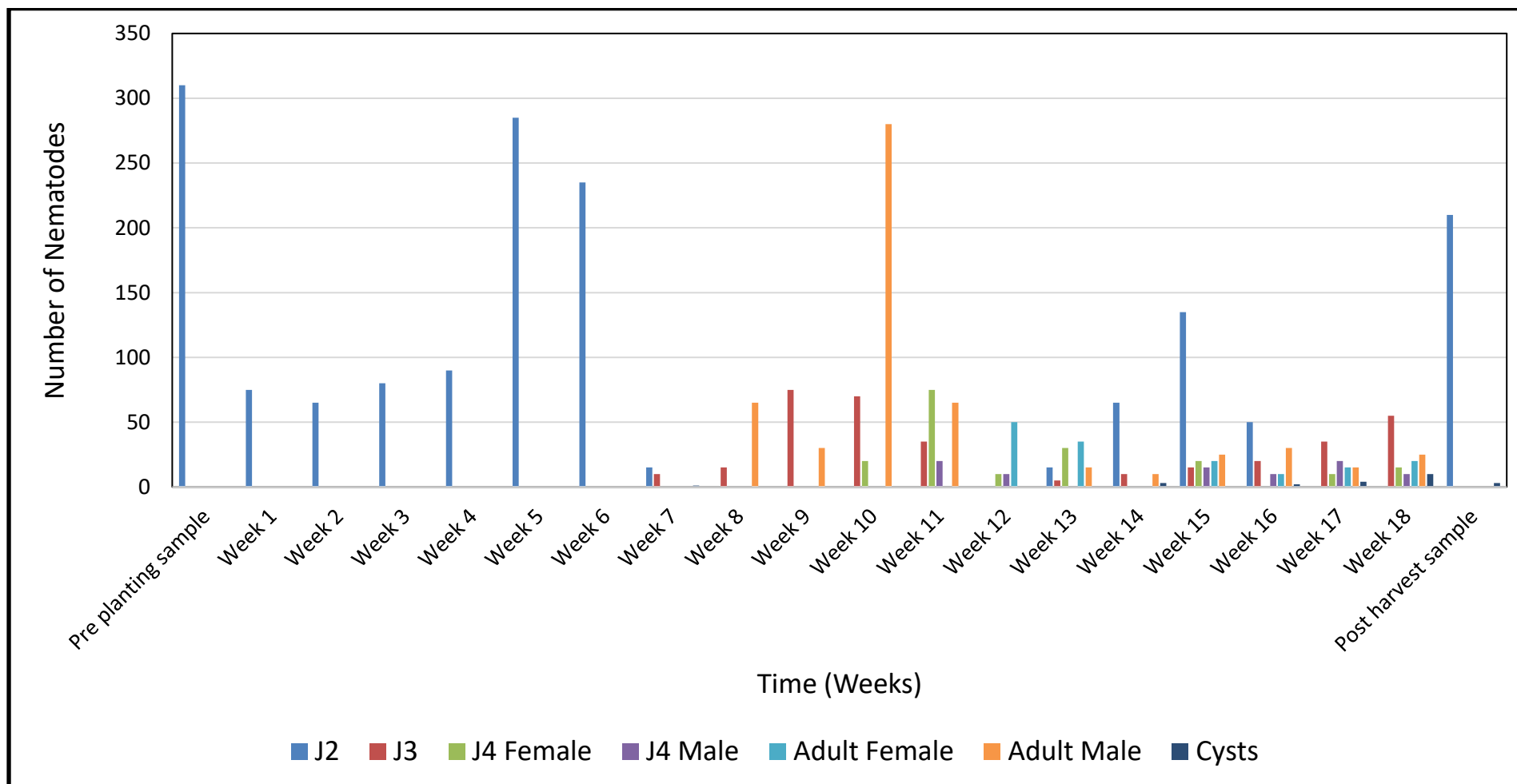


Figure 3.3 Numbers of the different life stages of a *Heterodera carotae* Jones, 1950 population detected in soil samples, taken at weekly intervals for 18 weeks, after planting from a carrot field near Tarlton (Gauteng Province, South Africa).

Adult females first appeared in the soil during Week 12 and the first adult female with an egg sac was observed in Week 15. Adult males were first observed in the soil in week 8 (577 CDD). The greatest number of J2 was present in soil samples obtained during Weeks 5 and 6 (285 CDD and 235 CDD, respectively), while the greatest number (12) adult females were found during Week 12. A very high number (280) of adult males were found in Week 10 (742 CDD).

3.3.4. Nematode life stages found in root samples

Figure 3.4 shows the different nematode stages found in the roots from Week 7 onwards. As expected of all life stages, the highest numbers for either J3 or J4 stages were found in most of the root samples, because *H. carotae* is a sessile endoparasite, which occur inside the roots. The highest numbers of J3 were recorded during week 9 (140) while J4 male numbers peaked during week 16 (50). Surprisingly few adult females and cysts were detected in root samples, but it is suspected that these life stages may have dislodged when the roots were removed from the soil during sampling.

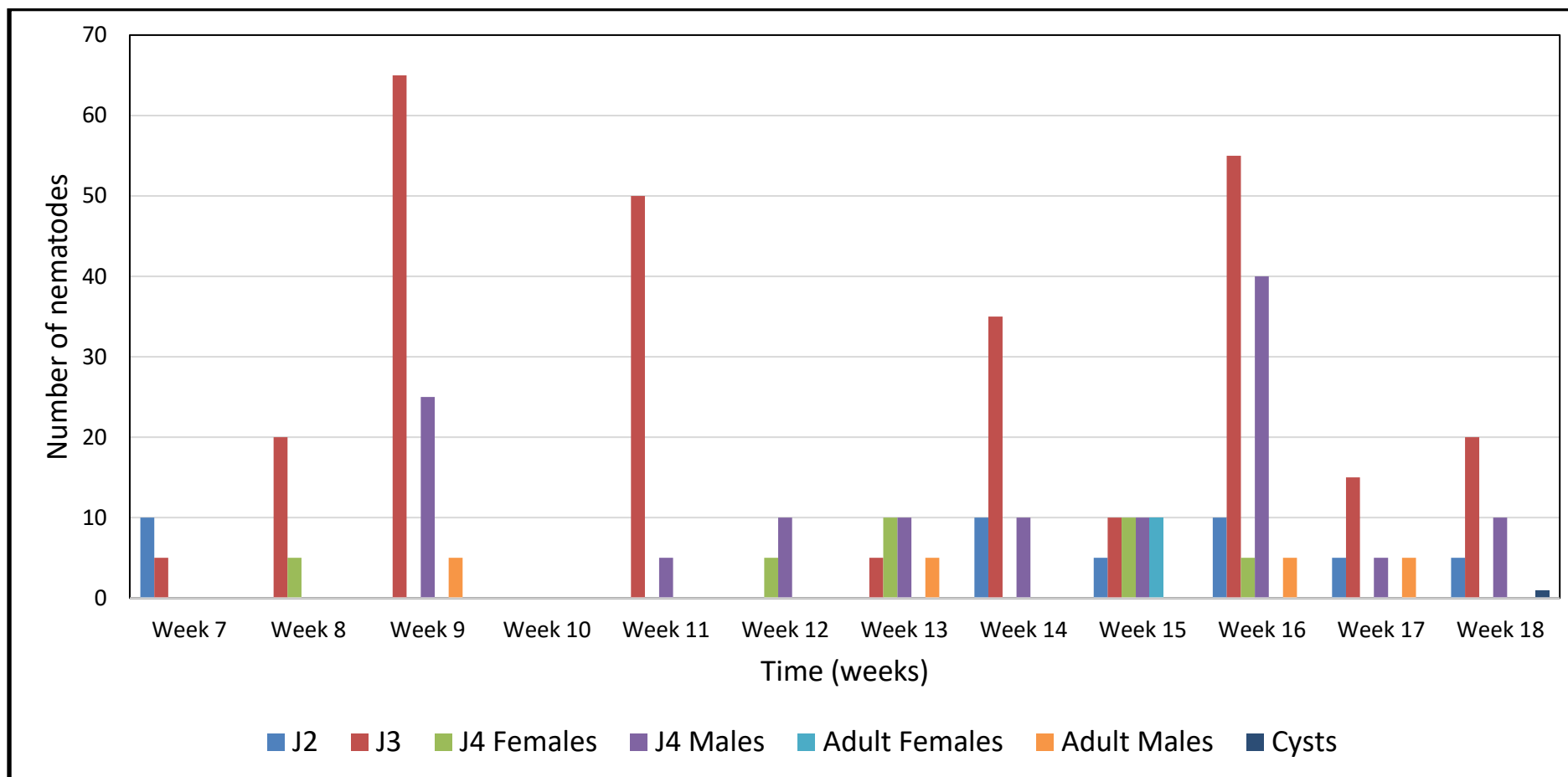


Figure 3.4 Numbers of the different life stages of a *Heterodera carotae* Jones, 1950 population detected in root samples taken at weekly intervals from a carrot field near Tarlton (Gauteng Province, South Africa).

3.3.5. Cysts found in soil samples

Figure 3.5 shows the number of yellow (new) cysts and brown (older) cysts that were detected in soil samples taken weekly and extracted using the Seinhorst elutriator. Yellow cysts were found in the soil as early as week 2, which is surprising considering that only 87 CDD has accumulated by then. Also, no J3, J4 or adults were detected prior to that. After Week 7(496 CDD), no brown cysts were detected until Week 12, when they started appearing again.

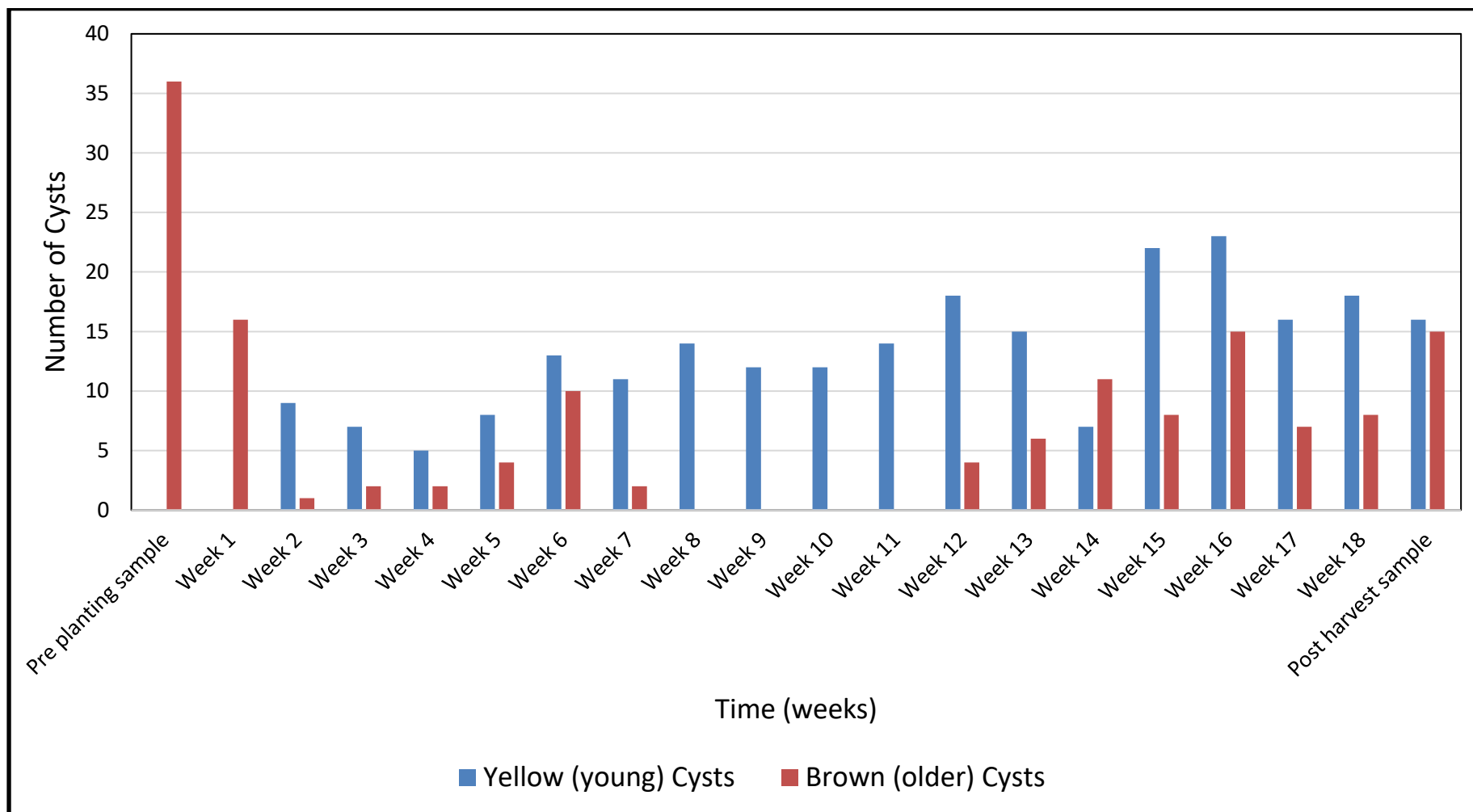


Figure 3.5 Numbers of *Heterodera carotae* Jones, 1950 cysts detected in soil samples at weekly intervals from a carrot field near Tarlton (Gauteng Province, South Africa).

3.3.6. Symptoms caused by parasitizing *Heterodera carotae* individuals on carrot tubers

Taproots from the trial field were severely stunted when compared with tubers from the adjacent field where an IPM approach, including the application of nematicides, was followed (Fig. 3.6A). Symptoms observed on severely infected carrot tubers included bearding, deformities and stunting (Fig. 3.6B). Appendix I contains photographs with corresponding measurements taken weekly during the trial period indicating the severe effect *H. carotae* infection had on carrot yield and quality.

Carrot tubers from the nematicide-treated and untreated adjacent field differed significantly in terms of their lengths. Those from the nematicide-treated field had a mean length of 25 cm, while the mean length of those from the untreated field were 17 cm.



Figure 3.6A Healthy tubers at harvest (18 weeks) from an adjacent nematicide-treated carrot field near Tarlton (Gauteng Province, South Africa) (Photo by A.R. Shubane).



Figure3.6B Severe symptoms of *Heterodera carotae* Jones, 1950 infestation, resulting in heavily infected tubers at harvest (18 weeks) from the trail field near Tarlton (Gauteng Province, South Africa) (Photo by A.R.Shubane).

3.4. Discussion

This study represents the first for *H. carotae* on carrot in South Africa and hence the generation of novel data for this nematode species in terms of the duration of its life cycle. The availability of limited and fragmented information on life cycle studies of this nematode species worldwide, for which three literature studies could be found, further accentuates the value of data obtained during this study.

An unexpected result obtained was that J2 as well as young, yellow female cysts were present in soil samples during the second and first weeks of sampling, respectively. This indicates that some factor was present in the field before sowing, causing not only hatching of J2 from eggs, but also development and completion of the life cycle of this local *H. carotae* population that resulted in adult cysts being detected in soil samples prior to sowing of the follow up crop. This can only be attributed to two things: Either a weed was present in the field which could sustain a population of *H. carotae* or that remnants of the previous carrot crop still persisted in the soil. The latter scenario is experienced with tuber crops of which some or part of the tubers remain in the soil after harvesting and then serve as a reservoir for nematode infection and development. It is suggested that the J2 observed in soil samples during Weeks 1 to 6 of our study were present as a direct result of the emergence of the current crop that were planted for the purpose of the study. The J2 present during weeks 12 to 18 are hence suggested to be those of a second generation of the developing nematode population. Thus, the young, yellow cysts detected from week 12 to 18 developed from the J2 that emerged during that time. More investigations into the survival of this nematode species in fields where fallow is practiced are thus necessary for the successful management of the pest. The history of the trial block is as follows: A cover crop mix of Babala grass, Bargazer sorghum, teff, beans, Dolichos beans, Interval turnip, Barkant turnip and sunhemp were planted on 23 September 2015. Thereafter carrot were planted and harvested in March 2016. The land was then fallow for seven months, after which the present carrots were sown.

Greco & Brandonisio (1986) found that adult males and females of *H. carotae* were found in soil samples after 210 and 260 DD, respectively, have accumulated. In this study, however, the first adult males were detected after 577 CDD have accumulated (week 8) and the first adult females only during week 12 for which no CDD could be calculated. It is suggested that the substantially longer time period experienced in detecting adult life stages of *H. carotae* in our study, opposed to that by Greco & Brandonisio (1986), can be attributed to the development of the *H. carotae* population being slowed down at temperatures above 25 °C. The latter has been reported by Mugniery & Bossis (1988) who found that mortality of life stages of a French population

of *H. carotae* increased at 27 °C, effectively slowing down the duration of the life cycle. In our study, a mean maximum temperature of 28.6 °C, well above the threshold of 25 °C reported by Mugniery & Bossis (1988), was recorded that supports this assumption. Nonetheless, the maximum temperature above which no development of *H. carotae* takes place, is not defined in South African conditions, making the use of DD less accurate as an indication of temperature units needed for the optimal development of *H. carotae*. This is substantiated since Greco & Brandonisio (1986) calculated the amount of DD to indicate the presence of certain life stages of *H. carotae* in a controlled environment at a constant temperature of 20 °C. At this relatively low maximum temperature, no inhibition of the developmental stages of the nematode is expected to have taken place.

It is evident from data of this study that *H. carotae* males emerged before females and were present before the first females were detected. Under adverse environmental conditions it is believed that more males will develop in a *H. carotae* population (Greco, 1986). This further accentuates the possibility that the higher soil temperatures (25 °C, with a mean of 28.6 °C) experienced in this South African field study may have inhibited the optimal development of *H. carotae*. Greco & Brandonisio (1986) also found that J2 hatching from eggs was negligible at 25 °C in both glasshouse and field experiments in Italy. However, the presence of J2 and older cysts late in the season (after week 18) in our study does indicate that another generation (in other words, three population) of *H. carotae* can possibly occur under these conditions. Unfortunately, temperature data was not obtained after Week 10 of this study to link it with the occurrence of these stages of *H. carotae*. The occurrence of two life cycles of this South African population of *H. carotae* is, however, in agreement with that by Greco & Brandonisio (1986) who reported that two life cycles of *H. carotae* were completed under Italian environmental conditions. Also, our results are partly in agreement with those by Colagiero & Ciancio (2011) who predicted, based on results by Greco & Brandonisio (1986) and historic temperature data, that two to three generations of *H. carotae* could be expected to occur in Italy in future with 1.5 to 2 °C temperature increases. These authors predicted that additional *H. carotae* generations may occur in future, especially during the Italian winter.

The ultimate aim of nematode research is to link nematode population densities or damage to yield. The total projected yield for the carrot plot infected (and not treated) with *H. carotae* was 36 t/ha of which 78 % were marketable, while the estimated yield of the nematicide-treated field was 57 t/ha, with 73 % of the crop being marketable. The projected percentage of marketable produce did not differ significantly, which could be attributed to the fact that 52 % of the marketable carrots in the trial plot were deemed small (16 cm in length). This could indicate the effect of this cyst nematode on feeder roots, causing carrots to be stunted or small. The bottom line is that the nematicide-treated field yielded 21 t/ha more carrot tubers than the untreated trial plot, resulting in ZAR 273000.00/ha more income for a carrot grower in the Tarlton area after deduction of the costs of the nematicides applied.

3.5. Conclusions

Under South African summer field conditions two life cycles of *H. carotae* were completed during the carrot-growing season of 18 weeks. Greco & Brandisio (1986) found this under European conditions (France), also with a growing season of about 18 weeks. Moreover, Colagiero & Ciancio (2011) predicted, based on modelling changes in future temperature increases, that two to three generations of *H. carotae* could be expected to occur in under Italian environmental conditions.

According to this study, the infective J2 was present in the soil from weeks 1-7 and again from weeks 12-18 after planting. This may help the farmer to decide on the time to administer nematicides or apply other strategies as part of an IPM.

At soil temperatures greater than 25 °C, it is suggested that the development of the South African *H. carotae* population actually slowed down. However, the upper temperature limit of development for this nematode species is not clearly defined. Greco & Brandonisio (1986) found that egg hatch was negligible at 25 °C.

The tolerance limit to the carrot cyst nematode in this trial is about 13 cyst/100 cm³ of soil lower than the European tolerance limit of 40 cyst/250 cm³ of soil, when calculated to 100 cm³ of soil, is 16 cysts (Greco, 1986).

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CHAPTER 4

Morphological and molecular characterization of *H. carotae* in South Africa (Tarlton population)

4.1. Introduction

Morphological observation by means of light microscopy has been used traditionally as the basis for the identification and systematics of cyst nematodes (Golden, 1986). The life cycle of cyst nematodes is comprised of the egg, four juvenile stages and one adult stage (female or male) (Subbotin *et al.*, 2010a). The cyst and second stage juvenile (J2) life stages are of the greatest value in the study of the morphology and identification of these nematodes, especially as they are easily obtainable from soil samples (Golden, 1986). The eggs are also very useful as they can be used in a variety of studies (Golden, 1986). The adult male and female of cyst nematode stages are more difficult to obtain and are not generally used in morphology and identification (Golden, 1986). According to this author it may be of great value to the identification of cyst nematodes if the morphology of adult stages of both sexes can be studied in more detail.

With the introduction of the scanning electron microscope (SEM), examination of the ultrastructure of the surface details of nematode individuals provided a sounder basis for morphological identification (Golden, 1986). SEM has proven to be a valuable tool for understanding the morphological diversity of cyst nematode species, specifically the labial patterns and number of lateral lines of the J2, the spicules of the male and the cone patterns of the female and cyst (Subbotin *et al.*, 2010a). According to these authors, as well as Turner & Subbotin (2013), the molecular approach has been introduced to give more clarity on the identification and systematics of the members of, for instance, the genus *Heterodera* Schmidt, 1871. These techniques represent extra tools to identify species, which often show similar morphology with small details for distinguishing the members in the genus. The internal transcribed spacers, ITS1 and ITS2, located between the repeating array of nuclear 18S and 28S ribosomal RNA (rRNA) genes and separated by the 5.8S rRNA gene, are variable genetic markers, which have been used successfully for the analysis of population structure and

systematics in a wide range of organisms. The polymerase chain reaction and internal transcribed spacer-restriction fragment length polymorphism (PCR-ITS-RFLP) was initially used to differentiate between different species (Szalanski *et al.*, 1997). Further developments in the application of molecular techniques include the sequencing of the ITS regions and the development of specific species-developed primers (Waeyenberge *et al.*, 2009). Applying both morphology and molecular approaches are recommended to achieve the identification of life stages of cyst nematodes (Subbotin, *et al.*, 2010b). Following this approach, the aim of this study was to characterize the Tarlton population of the carrot cyst nematode, *Heterodera carotea* Jones, 1950.

4.2. Morphological and morphometric study

4.2.1. Materials and methods

4.2.2. Nematode population

Populations of the genus *Heterodera* were obtained from the carrot (*Daucus carota* L.) tap root samples with corresponding rhizosphere soil samples collected on a one-hectare trial block in the Tarlton area, Krugersdorp, Gauteng Province, South Africa during the 2016-2017 summer growing season. This was done on a weekly basis for 18 weeks, using the zigzag pattern of collecting samples (Haydock & Perry, 1998). The soil in this field was classified as sandy loam (sand. 76.2 %. loam. 13.5 %. clay. 10.3 %) with a pH of 6.41 and 0.72 % organic matter content. The carrot field was irrigated by using draglines and conventional tillage was practised. The trial site received about 585 mm water from 14/11/2016–13/3/2017 (irrigation and rain). No nematicides were used and only two fungicides were administered [Copstar 120 SC® (active substance: Copper hydroxide) and Folicur® 250 EC (active substance: Tebuconazole (triazole))] during the growing season of this carrot crop.

4.2.3. Extraction of nematodes

Females, males and J2 were extracted from soil (250 cm³ soil/sample) by the sieving centrifugal flotation method. The 250 cm³ soil was washed through a coarse meshed 2 mm aperture sieve into a 5-liter bucket. The suspension was stirred, and then allowed to settle for 30 seconds. The suspension was then poured through a 45 µm aperture sieve. This procedure was repeated two more times, but the settling times were shortened to 20 and 10 seconds, respectively.

The residue was then transferred from the 45 µm aperture sieve to two centrifuge tubes, kaolin (5 cm³) was added to the suspension, which were then centrifuged for 7 minutes at 3500 revolutions per minute (rpm). The supernatant was decanted from the tubes and discarded. A sugar solution (450 cm³ sugar/1 l water) was then added to the tubes, carefully mixed and centrifuged for 3 minutes at 3500 rpm (Swart & Marais, 2017).

The cysts were extracted from 100 cm³ dried soil samples by using the Seinhorst cyst elutriator (Seinhorst, 1964). The Seinhorst elutriator works by using an upward current of water to gather the dried cysts in a 150 µm aperture sieve. First the clamp on the lower downpipe is closed and then the cylinder is closed with a rubber bung. The speed of the upward current was adjusted to the desired setting time of about 4 cm water/second and both downpipes were placed in a nest of 1000 µm- and 150 µm aperture sieves. A sample of 100 cm³ dried soil was washed through a 2-mm aperture sieve into the top bowl of the apparatus. The water (and debris) rised in the cylinder and spilled over the collar into the nest of sieves. After about 30 seconds the lower downpipe was opened and the debris (with cysts) was carefully washed through the nested sieves. The debris in the 1000 µm aperture sieve was gently washed through on to the 150 µm sieve. The rest of the debris on the 1000 µm aperture sieve was discarded and the cysts collected into the 150 µm aperture sieve. The content of this sieve was transferred, with the aid of a wash bottle, to a funnel lined with a round Whatman filter paper (185 mm in diameter). The filter paper was folded into a funnel shape before fitted into the funnel. When the water had drained from the funnel, the

filter paper was removed, opened to form a full circle, dried further on a plastic disc (plate) (Swart & Marais, 2017).

4.2.4. Light microscopy (LM)

Cysts were collected from the soil by using the Seinhorst cyst elutriator (Seinhorst, 1964). A single cyst was placed in a drop of water on a cavity slide on the stage of a dissecting microscope. This cyst was transferred to a cavity slide with lactophenol and heated until the lactophenol was fuming. It was then transferred to a drop of 100 % lactic acid where an insulin syringe needle was used to cut off the posterior end of the cyst. The body content was carefully removed with a dissecting needle without disturbing the structures in the vicinity of the vulva. The cuticle around the vulval area was then carefully trimmed away with the insulin syringe needle so that only the vulval cone was left. The vulval cone was transferred to water, then to a 96 % alcohol solution and then to xylene for clearing. A piece of glycerol-gelatine jelly was placed on a glass slide and gently heated to 55 - 60 °C. The vulval cone was transferred to the melted jelly by using a dissecting needle and then its position was corrected (vulval area facing directly upwards) by using a dissecting needle. Three glass rods were placed around the vulval cone to avert it from collapsing during the steps that follow. Where necessary, air bubbles were removed from the mounted vulval cone with a dissecting needle. After the jelly had solidified, a heated 15 µm thick coverslip was gently placed on top and again melted down a bit. The coverslip was fastened to the slide by using glyceel. This process was described by Swart and Marais (2017).

J2 were fixed in FPG fixative (100 ml formalin 40 %, 10 ml propionic acid, 890 ml distilled water), processed into glycerol by using the Seinhorst (1959) method and permanently mounted in glycerol in the center of a wax ring on a square coverslip fitting into a handmade aluminum Cobb slide (Swart & Marais, 2017). The manufacturing of a Cobb slide is explained in Chapter 2 (Paragraph 2.3.). Males were fixed in cold TAF fixative (7ml formalin 40%, 2ml triethanolamine, and 91 ml distilled water) and immediately mounted in a small drop of cold TAF on a glass microscope slide (Kleynhans, 1991). The females were fixed in cold lactophenol and incubated at 40 °C for three days (Knoetze *et al.*, 2017). The female body was transferred into a

small drop of 100 % lactic acid, punctured behind the neck area by using a scalpel blade and cut into half with an insulin syringe needle. The anterior half of the body, containing the head and neck structures, was placed in a glycerol drop and gently pressed down onto the surface of a glass microscope slide. A round coverslip was applied and sealed onto a microscope slide with glyceel (Marais *et al.*, 2017). Measurements of the mounted specimens were done by means of an Olympus Bx53 compound microscope equipped with a drawing tube. Measurements of curved structures were made along the median line. The images of vulval cones, males and J2 were taken with a Zeiss Axio Imager2 with an AxioCam MRC camera.

4.2.5. Scanning electron microscopy (SEM)

For the external morphology of the cyst, whole cysts were fixed in 4 % formalin (12.5 ml formalin, 87.5 ml distilled water) for a minimum of 48 hours. After 48 hours, they were transferred into PCR tubes and submitted to ultrasound in a Sonorex GT 120 transistor device for 10 minutes to be cleaned of any debris. They were rinsed in double distilled water and placed on filter paper inside a petri dish in a desiccator to dry at room temperature for 24 hours. The dried cysts were then mounted on aluminum SEM stubs, with carbon double-sided adhesive tape, in different positions to facilitate the taking of photomicrographs of their anterior and posterior ends (Lax & Doucet, 2002). For the internal morphology of cysts, individual cysts were placed in a cavity slide with lactic acid (45 %), cut/trimmed along the mid-regions with an insulin syringe and cleaned internally with a dissecting needle without disturbing the structures in the vicinity of the vulva (Swart & Marais, 2017). They were transferred into a clean solution of 45 % lactic acid for 24 hours. After 24 hours, the cones were transferred to PCR tubes, submitted to ultrasound in a Sonorex GT 120 transistor device for 10 minutes to be cleaned and then transferred to 4 % formalin for 24 hours. They were rinsed in double distilled water and placed on filter paper inside a petri dish in a desiccator to dry at room temperature for 12 hours. The dried vulval cones were then mounted on aluminum stubs, with carbon double-sided adhesive tape, so that the cones could be viewed on the inside (Lax & Doucet, 2002).

The J2, females and males were fixed in a TAF fixative solution (distilled water, 40 % formalin and triethanolamine) poured over the specimens and left at room temperature for a week. The specimens were put into an especially handmade holder consisting of a horizontally cut Eppendorf tube (with its lid), forming a small holder, and two round filter papers snugly fitting between the tube and its lid. A hole was cut into the lid to let liquids pass through the filter papers. A small drop of TAF was placed onto the bottom filter paper and the specimens were transferred with a dissection needle into the TAF. Next, the TAF was very carefully drawn off, and the second filter paper (saturated with TAF) carefully lowered onto the specimens. The two filter papers with the specimens between them were then sealed by pressing the cylinder part of the Eppendorf tube into the lid. This makes a perfect little holder to carry the specimens through the next steps until they were critically point dried. The specimens were transferred into a 30 % ethanol solution overnight and then processed through an ethanol series [50 %, 70 %, 80 %, 90 % and 96 %] with an interval of 30 minutes in each. It was then placed in 100 % ethanol and critical point dried with carbon dioxide as intermediate fluid. After this step, the Eppendorf holder was opened to have access to the specimens on the filter papers. To make it easier to locate the specimens, a drop of toluidine blue stain (1 mg toluidine blue in 5 ml absolute ethanol) was put on the filter papers so that the paper stained blue and the specimens showed up white. The specimens were picked up with a dissection needle and mounted on a SEM stub with double-sided carbon tape. They were coated with gold-palladium (21 nm) and examined with a FEI Quanta FEG 250 scanning electron microscope at 5-10 KV. The method described above is based on that of Marais *et al.* (2017). The staining of the filter papers to show up the specimens was discovered by accident when we struggled to transfer the very small J2 to the SEM stub.

4.3. Abbreviations and symbols of morphometric terms used in this study according to Subbotin *et al.* (2010a)

n	= number of specimens
L	= total body length in μm (male, J2)
a	= body length divided by diameter at mid-body (male, J2)
b	= total body length/ distance from anterior end to the junction of pharynx and intestine (male, J2)
c	= body length/ tail length (male, J2)
c'	= tail length /body diameter at anus (male, J2)
DGO	= distance from anterior end to orifice of dorsal gland opening (female, male, 2)
W	= diameter at midbody (male, J2, cyst)

The Tarlton carrot cyst nematode specimens have morphologically and morphometrically been compared to populations of *H. carotae* described from different countries in Europe by Mathews (1975) and Wouts and Weischer (1977). This was done according to Subbotin *et al.* (2010). The specimens were also compared to the recently described population of *H. carotae* from Ontario, Canada (Yu *et al.*, 2017).

4.4. Results of the morphological and morphometric study

***Heterodera carotae* Jones, 1950**

(Figs. 4.1-4.29 and Tables 4.1-4.6)

Measurements: See Tables 4.1-4.6.

4.4.1. Description

4.4.2. Second stage juveniles (J2)

Labial region slightly offset (Fig. 4.1 and Fig. 4.2). Head with three to four annules and medial lips with labial disc, set off from the rest of the body (Fig.4.1 and Fig. 4.2). With SEM, medial lips distinct, each slightly divided (Fig. 4.3). Stylet and stylet knobs well developed .With SEM (Fig. 4.4) the lateral lines start about 7 annuls posterior to the head. Well-developed oval median bulb with distinct valve (Fig. 4.5). Nerve ring situated a short distance posterior to excretory pore. Hemizonid and excretory pore distinct in most of the specimens, 4-5 annuli from each other, 92.4-145 μm from

anterior (Fig. 4.6). Phasmid indistinct in most specimens, tail tapering to finely pointed terminus (Fig. 4.7 and Fig. 4.8). Lateral field with four incisures (Fig 4.9). Tail shape typical of that of *H. carotae*.

4.4.3. Cyst

Mature cysts small, lemon shaped with distinct neck and vulval cone. Yellow to russet brown in colour. Surface pattern consisting of irregular zigzag lines (Fig. 4.10). Of interest is the fact that young, light-yellow cysts, filled with eggs, may have an egg sac attached and still be embedded in the root (Fig. 4.11) Interestingly, this has never been mentioned in literature. Vulval bridge often broken in older cysts (Fig. 4.12). With SEM, no bullae observed (Fig. 4.13). Underbridge bifurcate, slender, unsclerotised (Fig. 4.14). Fenestrae and anus distinct, anus situated in slight roundish depression (Fig. 4.15). Distance between vulva slit and anus distinct (Fig. 4.16).

4.4.4. Female

White, colour changing to yellowish when approaching cyst stage, lemon shaped with large egg sac. Labial region very small with light microscope (LM). With SEM, labial disc conspicuous, oval shaped (Fig. 4.17). Medial and lateral lips amalgamated, first and second annules wide and conspicuous (Fig. 4.17). Labial framework with LM weakly developed. Stylet small. Median bulb large, rounded with a well-developed valve. Subcrystalline layer present in some females (Fig. 4.18). Females were very scarce and although a few specimens could be mounted on slides and were measured, no specimens were good enough to photograph with LM.

4.4.5. Male

Body vermiform, always twisted with a 90° twist in posterior half when killed by gentle heat. The body slender with short, bluntly rounded tail. Labial region offset with 4 - 5 head annules (Fig. 4.19). With SEM, medial lips distinct, each slightly to totally divided, labial disc conspicuous, oval to rectangular in frontal view and raised above lateral lips (Fig. 4.20). Lateral field with four incisures forming three bands, outer lines crenate, outer band areolated (Fig. 4.21 and Fig. 4.22). With SEM (Fig. 4.23) the lateral lines start about 7 annul posterior to head. Lateral lines continue almost to tail tip from where they break up in a few block-like structures at the tip (Fig. 4.24 and Fig. 4.25). Labial

framework robust (Fig. 4.5). With LM well- developed oval median bulb with distinct valve. Hemizonid and excretory pore situated near each other (about six annules apart), located 80 - 90 annuli from the head (Fig. 4.26). Spicules arcuate, gubernaculum distinct, slightly curved (Fig. 4.27). With SEM, spicule tip is bidentate (Fig. 4.28 and Fig. 4.29).

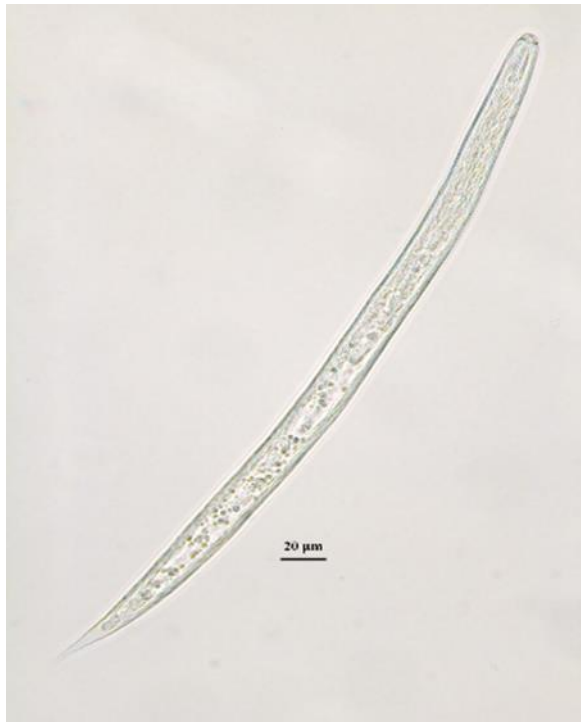


Figure 4.1 Whole body of a *Heterodera carotae* second-stage juvenile (Light Microscope photo by A.R. Shubane)

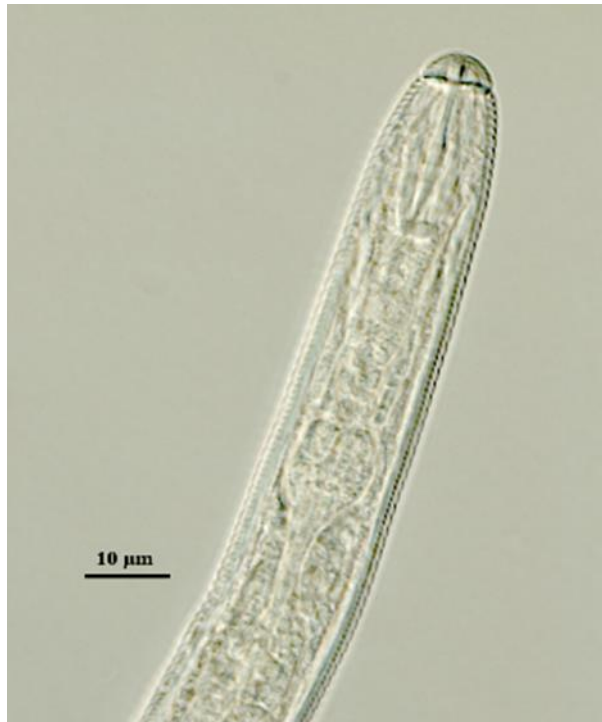


Figure 4.2 Anterior region of a *Heterodera carotae* second-stage juvenile (Light Microscope photo by A.R. Shubane).

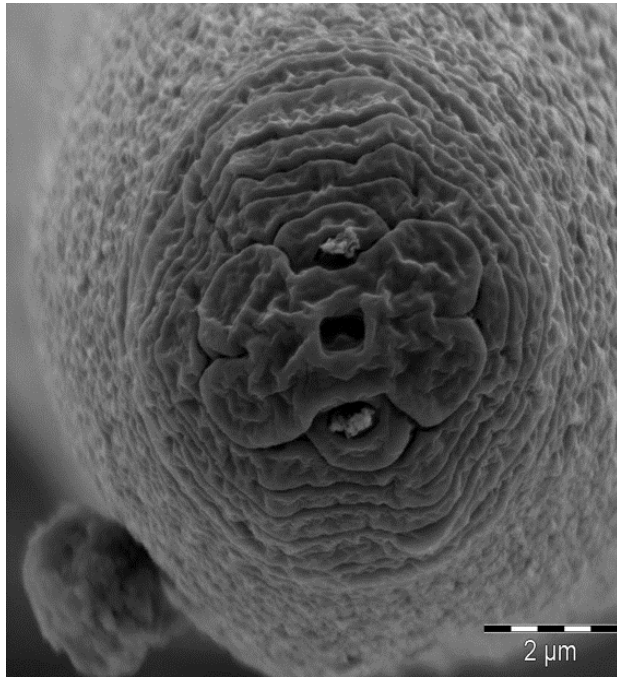


Figure 4.3 En face view of a *Heterodera carotae* second-stage juvenile (Scanning Electron Microscope photo by L.R. Tiedt).

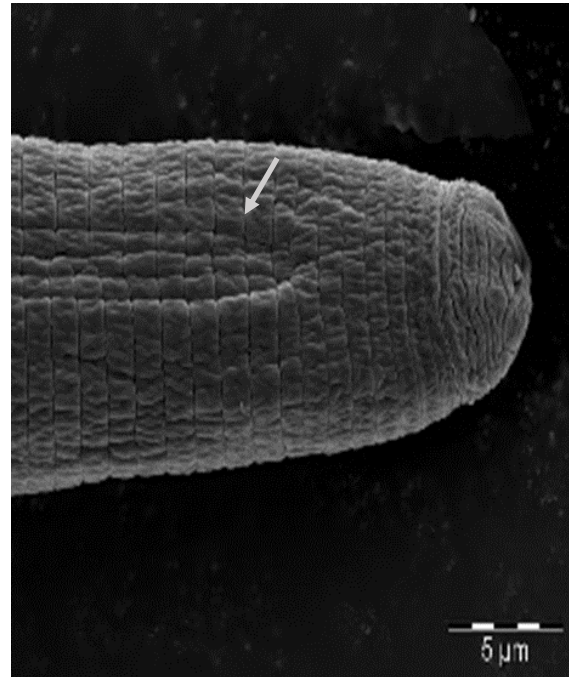


Figure 4.4 Lateral view of the anterior part of a *Heterodera carotae* second-stage juvenile showing the start of the lateral lines (arrow) (Scanning Electron Microscope photo by L.R. Tiedt).



Figure 4.5 Anterior part of a *Heterodera carotae* second stage juvenile, showing the prominent median bulb and valve (black arrow) (Light Microscope photo by A.R. Shubane).

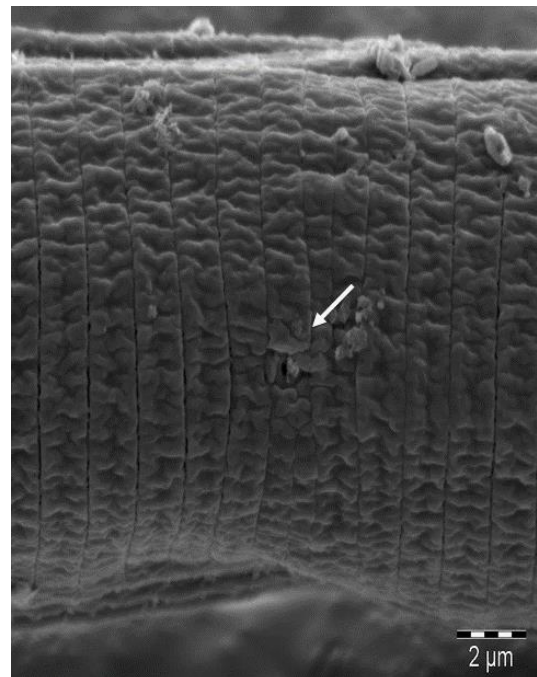


Figure 4.6 Anterior region of a *Heterodera carotae* second-stage juvenile showing excretory pore (arrow) (Scanning Electron Microscopy by L.R. Tiedt).

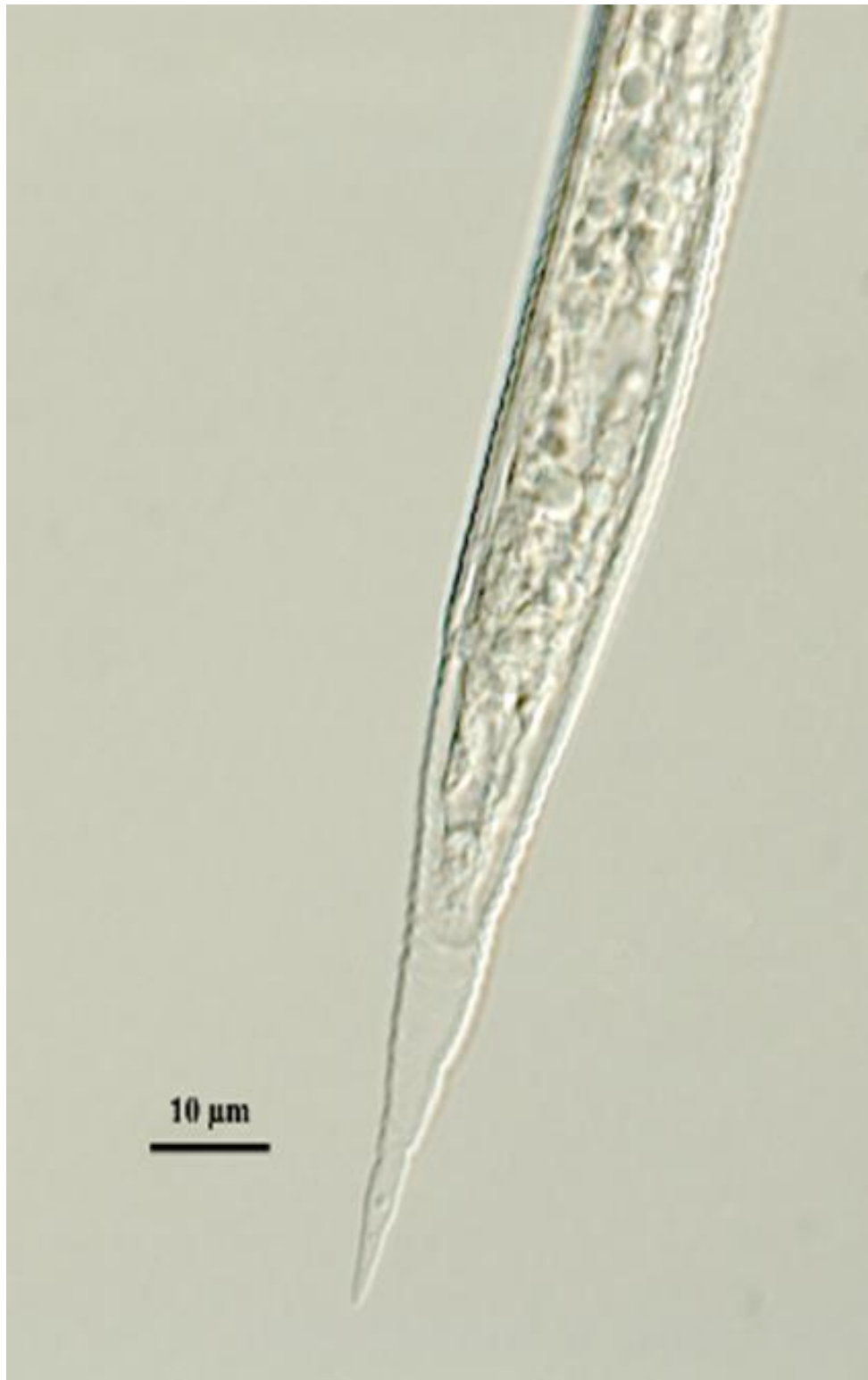


Figure 4.7 Tail region of a *Heterodera carotae* second-stage juvenile (Light Microscope photo by A. R. Shubane).

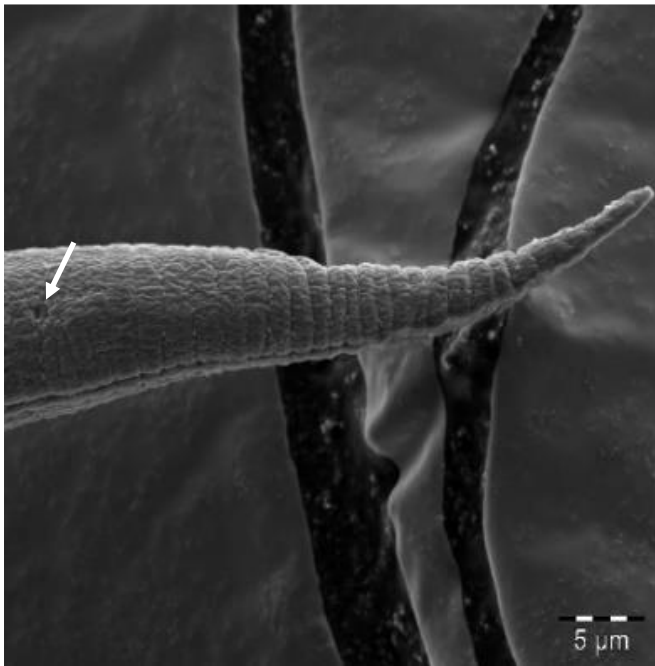


Figure 4.8 Tail tip of a *Heterodera carotae* second-stage juvenile showing the anus (arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

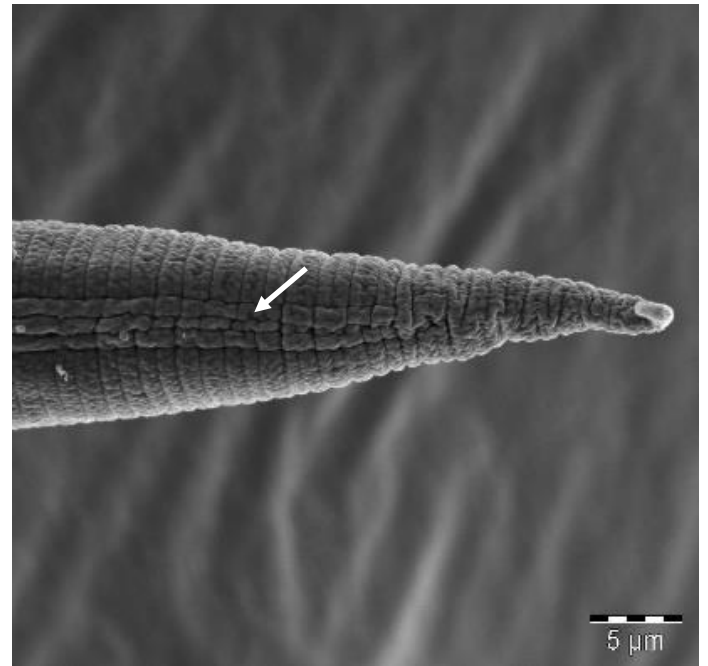


Figure 4.9 Lateral field of a *Heterodera carotae* second-stage juvenile with four incisures at the tail (white arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

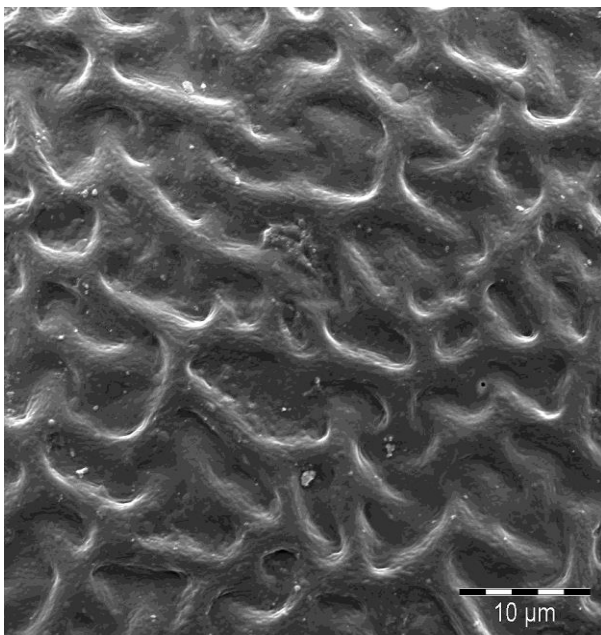


Figure 4.10 Actual ultrastructure of the surface pattern of the *H. carotae* cyst showing up as irregular zigzag lines when viewed with LM (Scanning Electron Microscope photo by L.R. Tiedt).

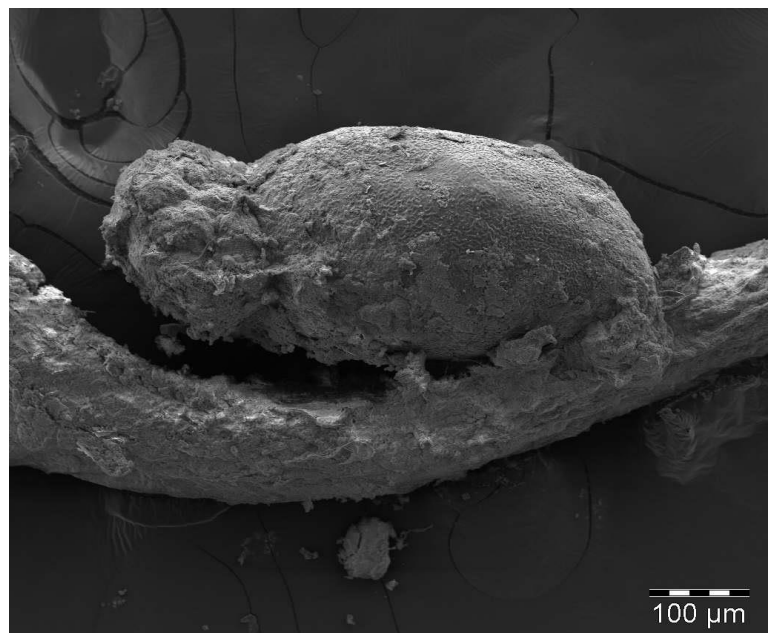


Figure 4.11 Young cyst of *Heterodera carotae* embedded in a carrot root (Scanning Electron Microscope photo by L.R. Tiedt).

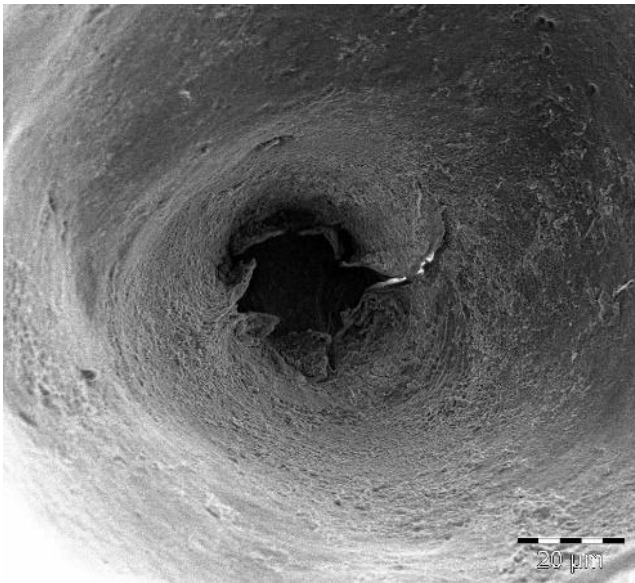


Figure 4.12 Exterior view of vulval cone of *Heterodera carotae* cyst showing the absence of the vulval bridge (Scanning Electron Microscope photo by L.R. Tiedt).

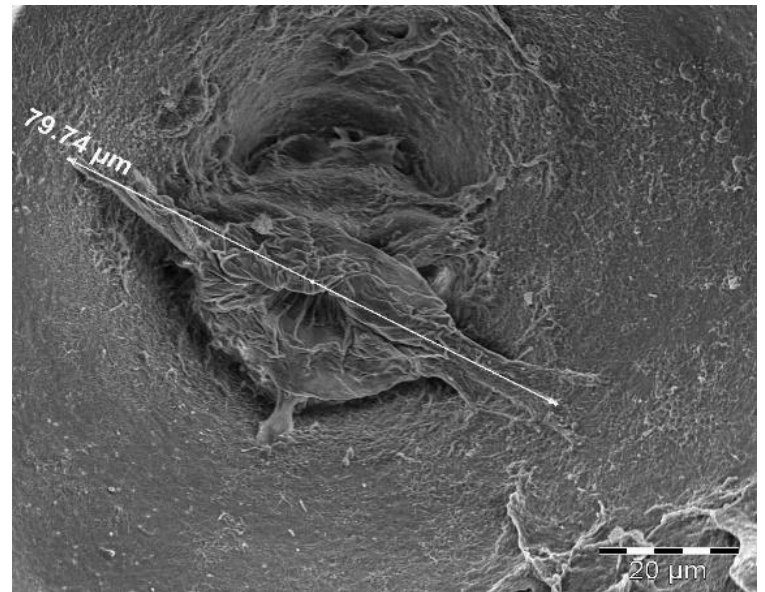


Figure 4.13 Interior view of *Heterodera carotae* vulval cone showing the underbridge with measurement and absence of bullae (Scanning Electron Microscope photo by L.R. Tiedt).

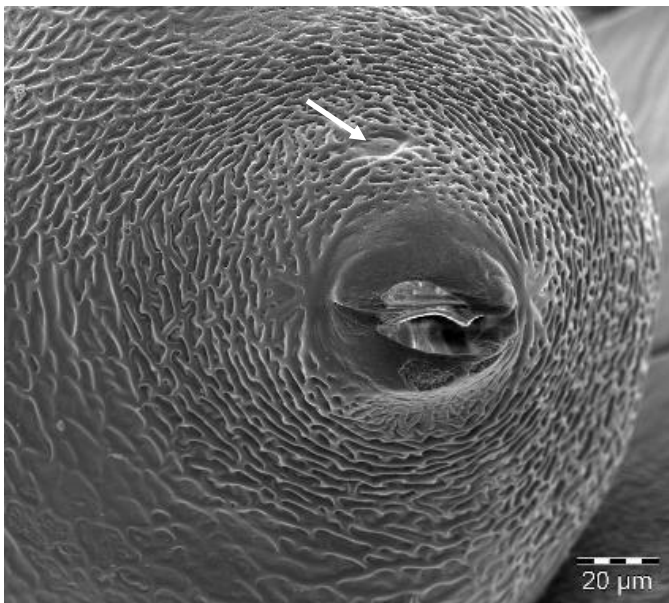


Figure 4. 14 Vulval cone of *Heterodera carotae* cyst showing fenestrae and anus (white arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

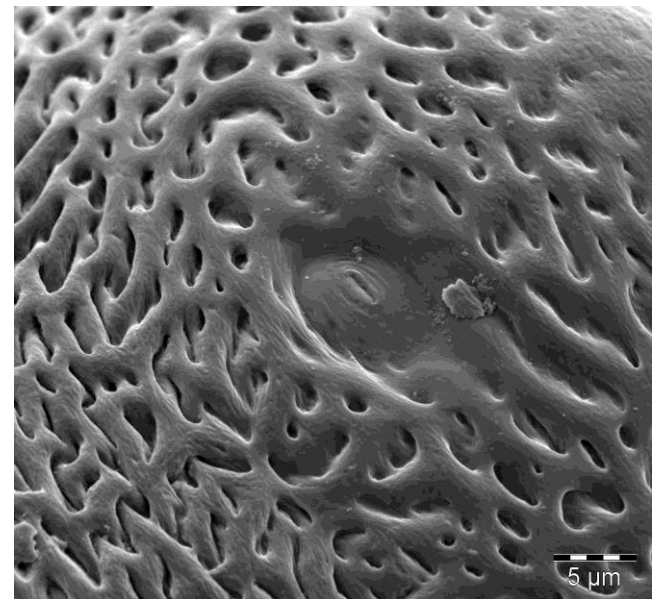


Figure 4.15 Vulval cone of *H. carotae* showing anus located in shallow depression (Scanning Electron Microscope photo by L.R. Tiedt).

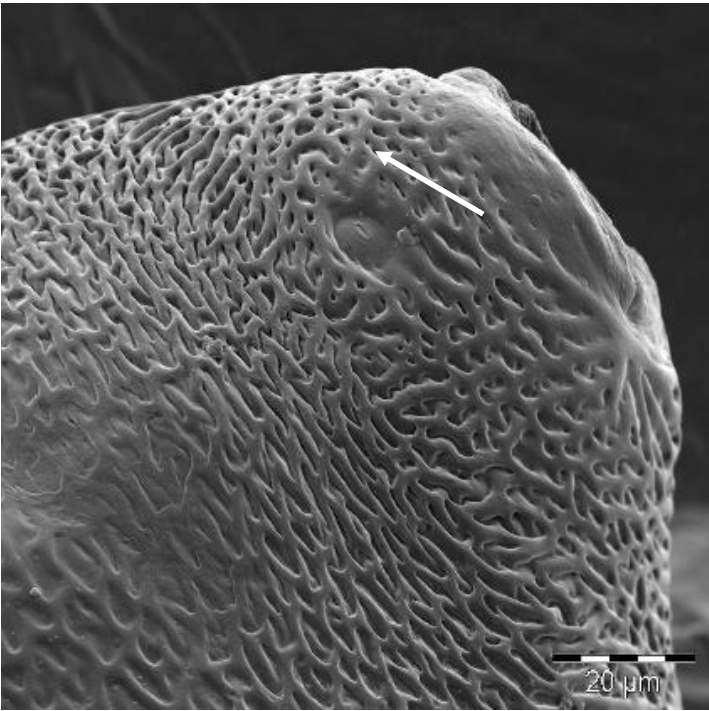


Figure 4.16 Posterior part of *Heterodera carotae* vulval cone showing anus (white arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

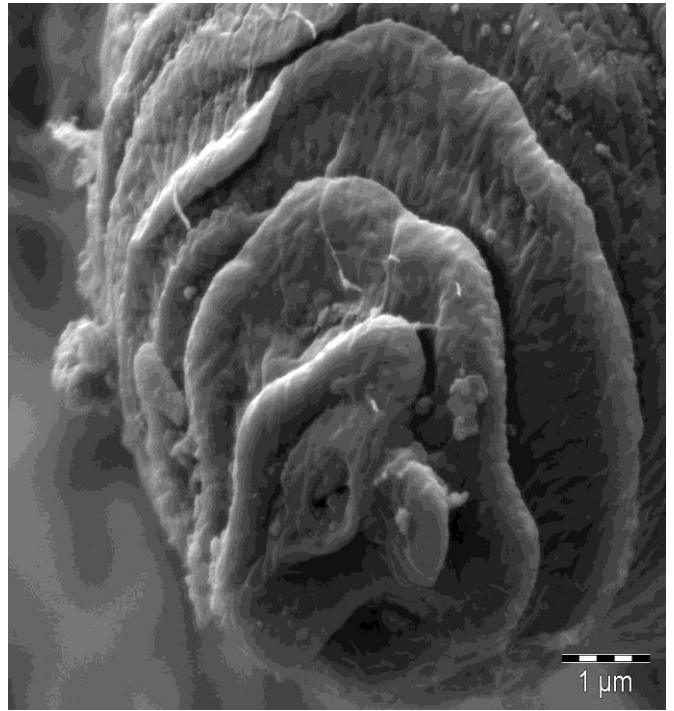


Figure 4.17 En face view of a *Heterodera carotae* female (Scanning Electron Microscope photo by L.R. Tiedt).

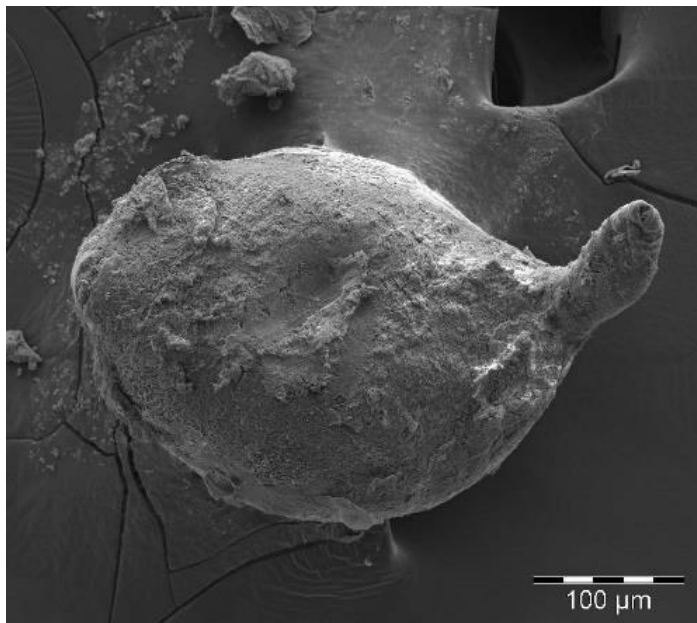


Figure 4.18 Female of *Heterodera carotae* coated with a subcrystalline layer (Scanning Electron Microscope photo by L.R. Tiedt).

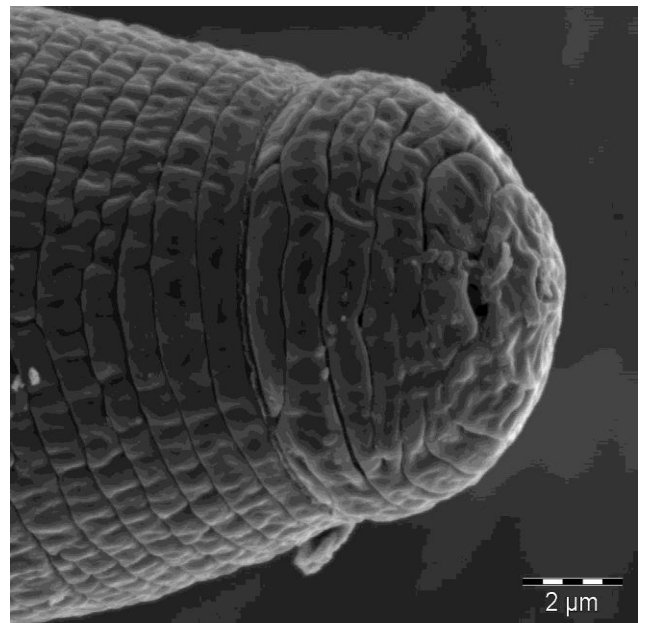


Figure 4.19 Labial region offset in a *Heterodera carotae* male, lateral view (Scanning Electron Microscope photo by L.R. Tiedt).

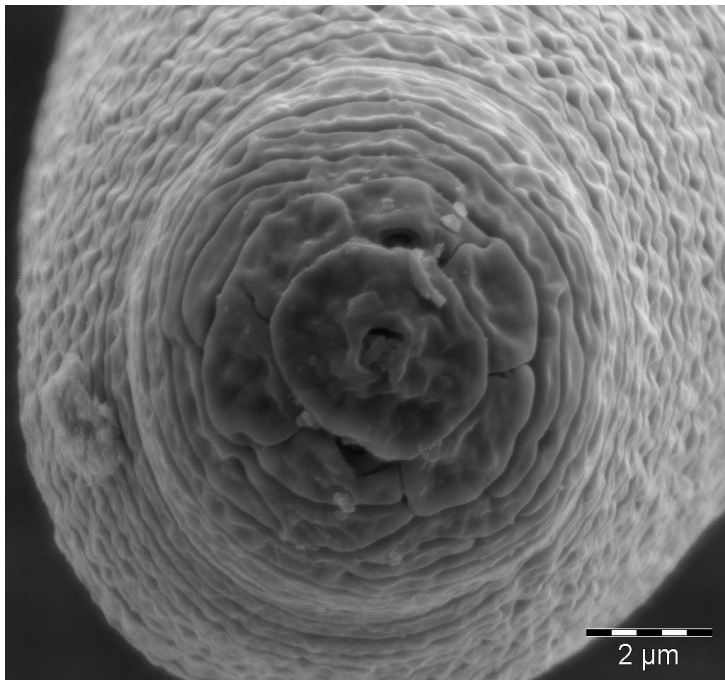


Figure 4.20 En face view of a *Heterodera carotae* male (Scanning Electron Microscope photo by L.R. Tiedt).

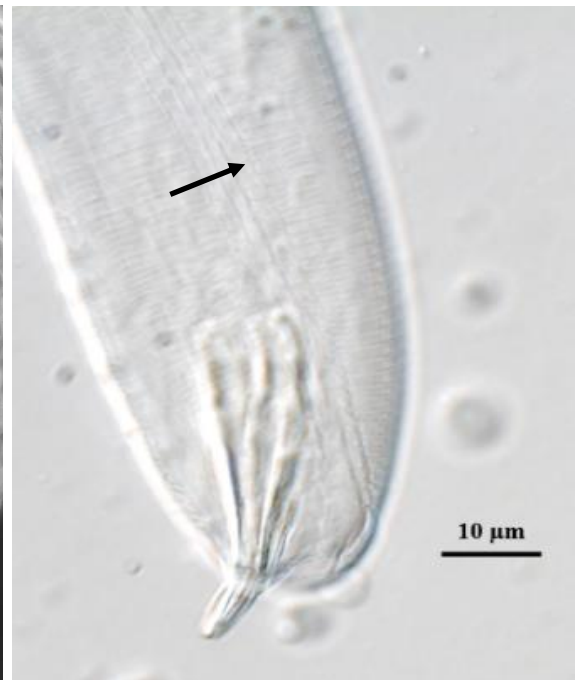


Figure 4.21 Male tail of *Heterodera carotae* in sublateral view, showing the lateral lines (black arrow) (Light Microscope by A.R. Shubane).

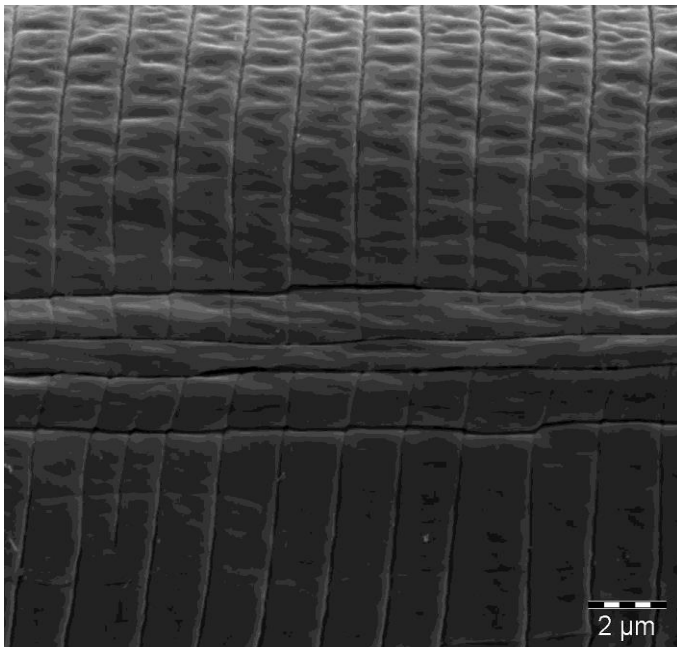


Figure 4.22 Lateral lines of a *Heterodera carotae* male in midbody (Scanning Electron Microscopy by L.R. Tiedt).

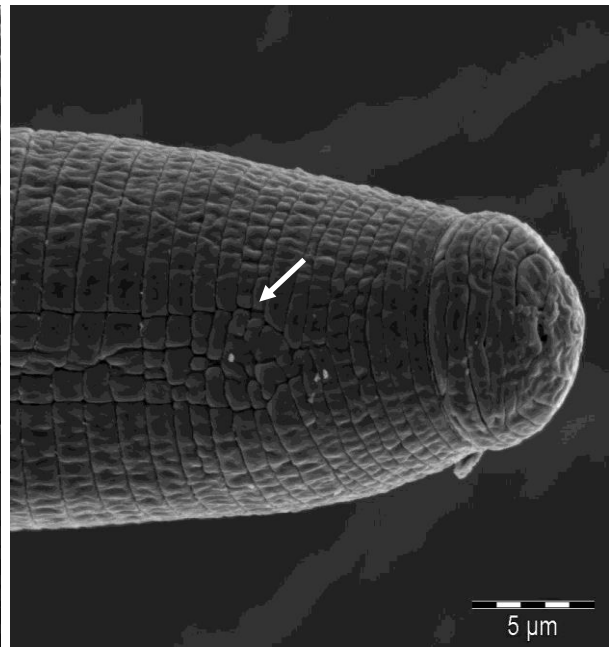


Figure 4.23 Sub-lateral view of the anterior part of a *Heterodera carotae* male, showing the start of the lateral lines (arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

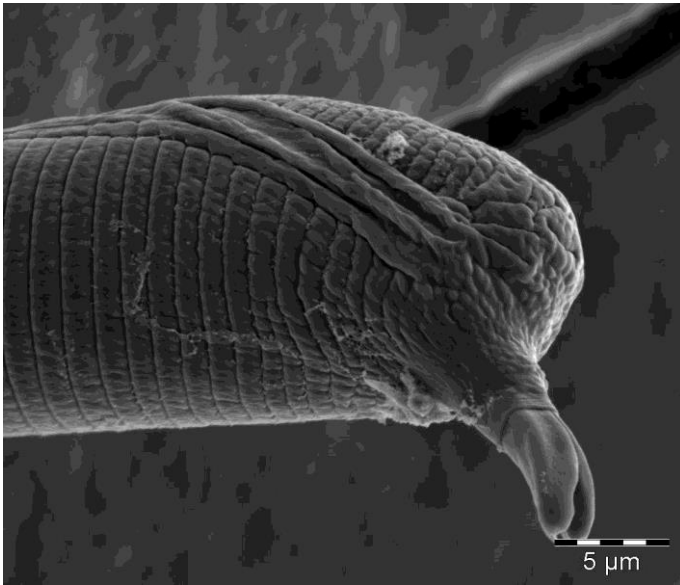


Figure 4.24 Male tail of *Heterodera carotae*, lateral view (Scanning Electron Microscope photo by L.R. Tiedt).

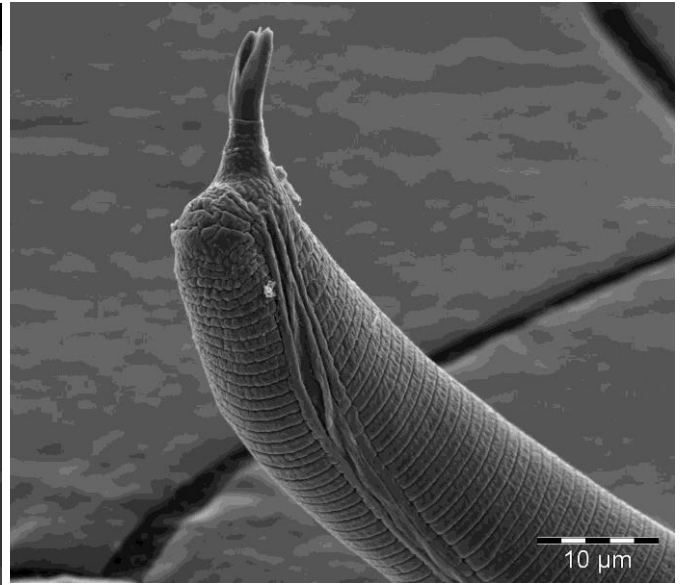


Figure 4.25 Male tail of *Heterodera carotae*, subdorsal view (Scanning Electron Microscope photo by L.R. Tiedt).



Figure 4.26 Anterior part of a *Heterodera carotae* male, showing the excretory pore (arrow) (Light Microscope photo by A.R. Shubane).



Figure 4.27 Male tail of *Heterodera carotae* in lateral view (Light Microscope by A.R. Shubane).

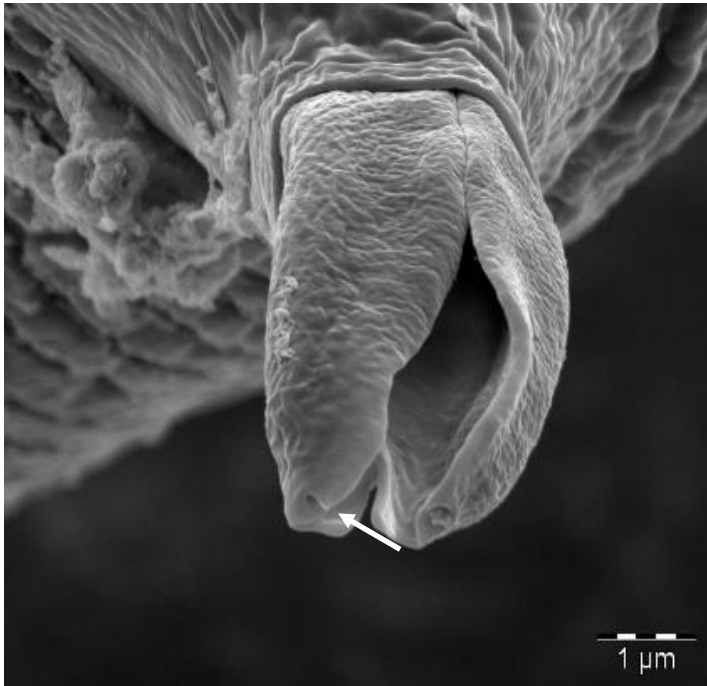


Figure 4.28 Spicule of a *Heterodera carotae* male showing frontal view of bifid terminus (white arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

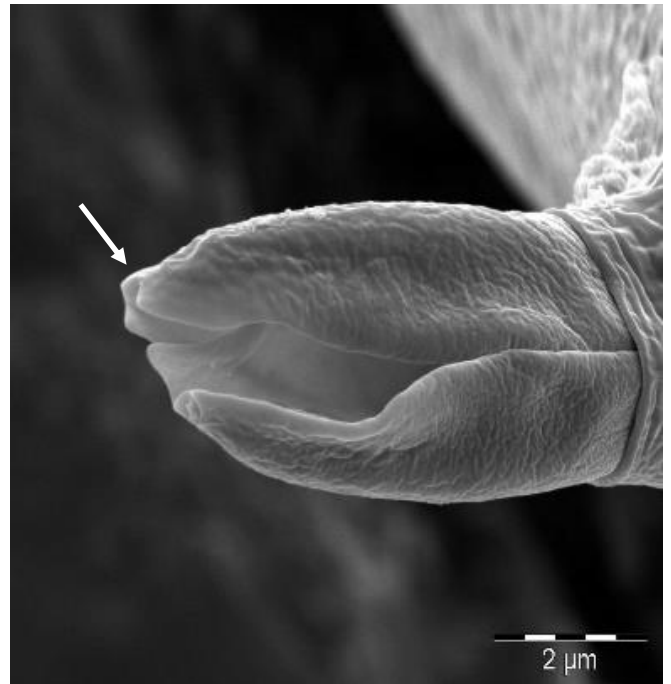


Figure 4.29 Spicule of a *Heterodera carotae* male showing lateral view of the bifid terminus (arrow) (Scanning Electron Microscope photo by L.R. Tiedt).

Table 4.1 Morphometrical data of second-stage juveniles (J2) and males of *Heterodera carotae* (Tarlton population, South Africa). All measurements are in μm and in the form: mean $\pm$ standard deviation (SD) (range)

Character	J2	Male
n	20	4
L	366.2 $\pm$ 60.8 (270.3-434)	1185 $\pm$ 26(1147-1205)
a	21.7 $\pm$ 4.1(14.7 -27.8)	40.3 $\pm$ 2.7 (38.5-44.1
b	3.5 $\pm$ 4.7 (1.8-23.5)	6.6 $\pm$ 1.0 (6.2-7.5)
c	87.8 $\pm$ 2 (5.-11.1)	24.0 $\pm$ 27.7 (49.1)
c'	5.8 $\pm$ 6.8 (3.4-34.5)	4.3 $\pm$ 0.5 (3.8-5)
Lip region width	6.9 $\pm$ 1.0 (4.8-7)	10.38 $\pm$ (8-11.1)
Lip region height	4.6 $\pm$ 0,9 (3.1-6.2)	6.6 $\pm$ 1.1 (5-8)
Nerve ring from anterior	85.3 $\pm$ 14.0 (68.8-142)	70.9-71.4
Oesophagus length	141 $\pm$ 32.5(17.4-167)	177 $\pm$ 13.3 (160-190)
DGO	3.9 $\pm$ 1.3 (1.9-6.2)	5.1 $\pm$ 0.6 (4.3-6)
Excretory pore from anterior	110 $\pm$ 14.9 (92.4-145)	162 $\pm$ 7 (152-168)
Width at mid-body	17.0 $\pm$ 2 (14.9-21.1)	25 $\pm$ 0.4 (24.8-28.4)
Width at anus	10.9 $\pm$ 2.8 (1.2-14.3)	20.1 $\pm$ 2.3 (18.5-23.5)
Median bulb length	14.4 $\pm$ 1.9 (11.8-18.0)	11.8 (n=1)
Median bulb width	10.4 $\pm$ 1.1 (9.3-13.6)	9.3 (n=1)
Head to median bulb valve	66.7 $\pm$ 4.8 (55.8-73.8)	92. $\pm$ 8.4 (80.6 98.5)
Annule width	1.9 $\pm$ 0.4 (1.2-2.5)	2.1 $\pm$ 0.3 (1.9-2.4)
Hyaline region	26.2 $\pm$ 2.2 (22.3-30.4)	-
Lateral field at mid-body	4.6 $\pm$ 2.5 (2.5-11.2)	5.1 $\pm$ 0.6 (4.3-5.5)
Tail length	47.6 $\pm$ 5.9 (37.8-58.3)	7.8 $\pm$ 0.8 (7-9)
Hyaline length as % of tail length	0.5 $\pm$ 2.4 (0.6-0.5)	-
Spicule length	-	38.6 $\pm$ 1.5 (36.5-40.0)
Gubernaculum length	-	11.8 $\pm$ 1.9 (9.0-13)

Table 4.2 Morphometrical data of cysts and female of *Heterodera carotae* (Tarlton population, South Africa). All measurements are in μm and in the form: mean $\pm$ standard deviation (SD) (range).

Characters	Cyst	Female
n	16	1
Length excluding neck (L)	372 $\pm$ 49.0 (294 -450)	387
Width	252 $\pm$ 48.4 (179-341)	139
Length/Width	1.4 $\pm$ 0.2 (1.1-1.7)	2.8
Fenestral length	37.5 $\pm$ 6.6 (26.7-54.6)	-
Fenestral width	26.8 $\pm$ 6.5 (16.7-42.7)	-
Underbridge length	57.6 $\pm$ 8.1 (67.6-79.7)	-
Vulva slit length	40.7 $\pm$ 10.6 (28.3-54.6)	-
Distance between anus/vulva slit	2.9 $\pm$ 5.4 (15.5)	-
Neck length	-	98.2
Stylet length	-	28
Stylet cone length	-	12
Stylet shaft length	-	16
Anterior end to median bulb	-	51.5
Anterior end to excretory pore	-	87.7

Table 4.3 Comparative morphometrical data for *Heterodera carotae* second-stage juveniles from the Tarlton area (South Africa) and those from Canada (Yu *et al.*, 2017), Europe and The United Kingdom (Mathews, 1975), as well as Germany and The Netherlands (Wouts & Weiser, 1977) (all measurements in μm).

Population	Ontario, Canada Yu <i>et al.</i> (2017)	Ireland , England and Italy Mathews (1975)	Munster, Germany Wouts & Weischer (1977)	Paderborn, Germany Wouts & Weischer (1977)	Wageningen, The Netherlands Wouts & Weischer (1977)	Tarlton area (South Africa)
Characters						
n		100	10	10	20	20
L	388-457	422 (375-452)	425 $\pm$ 5.5	429 $\pm$ 4.5	411 $\pm$ 5.7	366.2 $\pm$ 60.8 (270-434)
Body diameter at mid-body		20 (19-21)	19.6 $\pm$ 0.2	18.7 $\pm$ 0.2	20.3 $\pm$ 0.2	17.0 $\pm$ 2 (18.0-21.1)
Stylet length	23-25	24 (22-25)	23.7 $\pm$ 0.3	24.1 $\pm$ 0.2	22.5 $\pm$ 0.2	23.7 $\pm$ 1.1 (21.1-25.4)
Labial region height		(4-6)	4.73 $\pm$ 0.08	4.67 $\pm$ 0.08	4.43 $\pm$ 0.08	4.6 $\pm$ 0.9 (3.1-6.2)
Labial region width		10 (9-10)	9.55 $\pm$ 0.2	9.0 $\pm$ 0.1	9.38 $\pm$ 0.1	6.9 $\pm$ 1.0 (5.8-8.7)
Anterior end to valve of median bulb		66 (61-72)	71.3 $\pm$ 0.8	69.7 $\pm$ 0.9	69.3 $\pm$ 1.0	66.7 $\pm$ 4.8 (55.8-73.8)
Anterior end to excretory pore		99				110 $\pm$ 14.9 (92.4-145)
Tail length	45-56	51.8 (43.5-58.6)	49.7 $\pm$ 0.6	53.2 $\pm$ 0.9	49.2 $\pm$ 1.0	47.6 $\pm$ 5.9 (37.8-58.3)
Length of hyaline region	25-33	28.3 (20.3-31.8)	27.9 $\pm$ 0.5	30.7 $\pm$ 0.8	27.9 $\pm$ 0.6	26.2 $\pm$ 2.2 (22.3-30.4)

Table 4.4 Comparative morphometrical data of *Heterodera carotae* cysts from the Tarlton area compared with that of Canadian (Yu *et al.*, 2017), European and United Kingdom counterpart species Mathews (1975) (all measurements in μm).

Population	Yu <i>et al.</i> (2017) (Ontario, Canada)	Mathews (1975) (Ireland, England and Italy)	Tarlton area (South Africa)
Characters			
n	-	150	16
Length excluding neck	348-520	408 (218-625)	372 $\pm$ 49.0 (294 -450)
Width	260-465	309 (165-500)	252 $\pm$ 48.4 (179-341)
Fenestral length	33-56	31 (27-36)	37.5 $\pm$ 6.6 (26.7-54.6)
Fenestral width	35-56	39 (35-42)	26.8 $\pm$ 6.5 (16.7-42.7)
Underbridge length	-	90	57.6 $\pm$ 8.1 (67.6-79.7)
Vulva slit length	20-43	47 (43-51)	40.7 $\pm$ 10.6 (28.3-54.6)

Table 4.5 Comparative morphometrical data of *Heterodera carotae* females from the Tarlton area compared with that of European and United Kingdom counterpart species (Mathews, 1975) (all measurements in μm) No morphometrical data of *Heterodera carotae* females were published for the Canadian populations (Yu *et al.* (2017)

Population	Mathews (1975) (Ireland, England and Italy)	Tarlton area (South Africa)
Characters		
n	10	1
L	451(375-513)	387
W	203 (168-238)	139
Stylet length	27 (25-31)	28
Anterior end to excretory pore	81-119	87,7

Table 4.6 Comparative morphometrical data for *Heterodera carotae* males from the Tarlton area (South Africa) and those from Europe and The United Kingdom (Mathews, 1975), and Canada (Yu *et al.*, 2017) (all measurements in μm).

Populations	Yu <i>et al.</i> (2017) (Ontario, Canada)	Mathews (1975) (Ireland, England and Italy)	Tarlton area (South Africa)
Characters			
n	-	40	4
L	1137-1149	1154 (1090-1220)	1185 $\pm$ 26(1147-1205)
Midbody width	28-29	30 (27-33)	25 $\pm$ 0.4 (24.8-28.4)
Stylet length	33-44	35 (31-38)	29.6 $\pm$ 0.8 (28.5-29.6)
Labial region height	-	7	6.7 $\pm$ 1.1 (5.6-8.0)
labial region width	-	11	10.4 $\pm$ 1.4 (8.7-11.8)
Anterior end to median bulb	-	90 (85-105)	92.9 $\pm$ 8.4 (80.6-98.6)
Anterior end to excretory pore	-	153 (148-161)	163 $\pm$ 7.0 (152-168)
Spicule length -	33-37	34 (31-36)	38.6 $\pm$ 1.5 (36.5-40.0)
Gubernaculum length -	13-14	12 (10-13)	11.8 $\pm$ 1.9 (9-13)

4.5. Molecular study

4.5.1 Materials and methods

4.5.2. DNA extraction and PCR

Cysts were cut open and the J2 removed. Individual J2 were hand-picked with the hair of a brush attached to a dissecting probe and placed in 5 μl PCR reaction buffer (16 mM $(\text{NH}_4)\text{SO}_4$, 67 mM Tris-HCl pH 8.8, 01% Tween-20) containing 60 $\mu\text{g ml}^{-1}$ Protein K in a sterile PCR tube. The nematodes were cut into small pieces with the sterile needle of an insulin syringe. The tube was incubated at 60 $^{\circ}\text{C}$ for 30 minutes, and a further 5 minutes at 95 $^{\circ}\text{C}$. The tubes were stored at -20 $^{\circ}\text{C}$ until further use (Knoetze & Swart, 2014).

4.5.3. Amplification of ITS-rDNA

Two PCR amplification primers that amplify the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes were used.

The rDNA1 primer (5'-TTGATTACGTCCCTGCCCTTT-3') and rDNA2 primer (5'-TTTCACTCGCCGTTACTAAGG-3') have been described by Vrain *et al.* (1992) for amplification of the ITS regions. The primers were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). PCR amplifications were carried out using 5 µl of nematode lysate together with 0.5 µM of each primer, dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 1 × Taq reaction buffer, 1.5 mM MgCl₂ and 1 U Taq polymerase. The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94 °C for 20 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 45 seconds, repeated for 30 cycles.

A two minute incubation period at 72 °C followed the last cycle in order to complete any partially synthesised strands.

4.5.4. DNA sequencing

PCR products of the ITS-rDNA regions of five cysts from the Tarlton population were cleaned up and sequenced by Inqaba Biotechnical Industries (Pty) Ltd, using an ABI 3500xL Genetic Analyzer with 24 capillaries. A BigDye version 3.1 dye terminator cycle sequencing kit from Applied Biosystems was used for the reactions. Sequence assembly and editing was performed on the CLC DNA Workbench (<http://www.clcbio.com>). Sequence data derived from all the cysts was used to construct a consensus sequence for the Tarlton population.

4.5.5. Sequence analysis

The consensus sequence was compared to known sequences in the public databases by means of the Basic Local Alignment Search Tool, or BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequence was aligned with selected sequences of other species from the Heteroderidae from the GenBank sequence database. *Globodera capensis* n. sp was used as outgroup species. Sequences were aligned using CLC DNA Workbench (<http://www.clcbio.com>). The GenBank accession numbers of the sequences used are indicated in the phylogenetic trees.

4.5.6. Phylogenetic analysis

Phylogenetic analyses to resolve the relationships between the isolates and selected representatives of the Heteroderidae were conducted in MEGA6 (Tamura *et al.*, 2013). The Maximum Parsimony method (MP) was used to obtain a consensus tree using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000), in which the initial trees were obtained by the random addition of sequences (10 replicates). All ambiguous positions were removed for each sequence pair. The confidence intervals for the various branching patterns in the trees was measured with the bootstrap test (BS) with 100 replicates (Felsenstein, 1985).

4.5.8. Amplification of ITS regions

Amplification of ITS1+2 regions of the isolates with primers rDNA1 and rDNA2 yielded a single fragment of approximately 1200 bp (Fig 4.30). The ITS-rDNA sequence from the Tarlton population of *H. carotae* was submitted to the GenBank database under the accession number MG97690.

4.6. Results of molecular study

4.6.1. Sequence analysis

BLAST results indicated a 99 % similarity of the Tarlton isolate with three isolates (KX436295, KX436296 and KX436297) of *H. carotae* in the Genbank database from Canada (Yu *et al.*, 2017).

4.6.2. Phylogenetic relationships

Phylogenetic relationships of selected species of the Heteroderidae inferred from ITS-rRNA sequences using the Maximum Parsimony method (MP) are presented in (Figure 4.31.) The bootstrap tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. In the consensus tree, all the main groups of *Heterodera* are presented as clades (Subbotin *et al.*, 2001). The isolate from Tarlton grouped together in a clade with *H. carotae*, *Heterodera cruciferae* Franklin 1945 and *Heterodera urticae* Cooper 1955, but resolution amongst these species is unclear due to low bootstrap support.

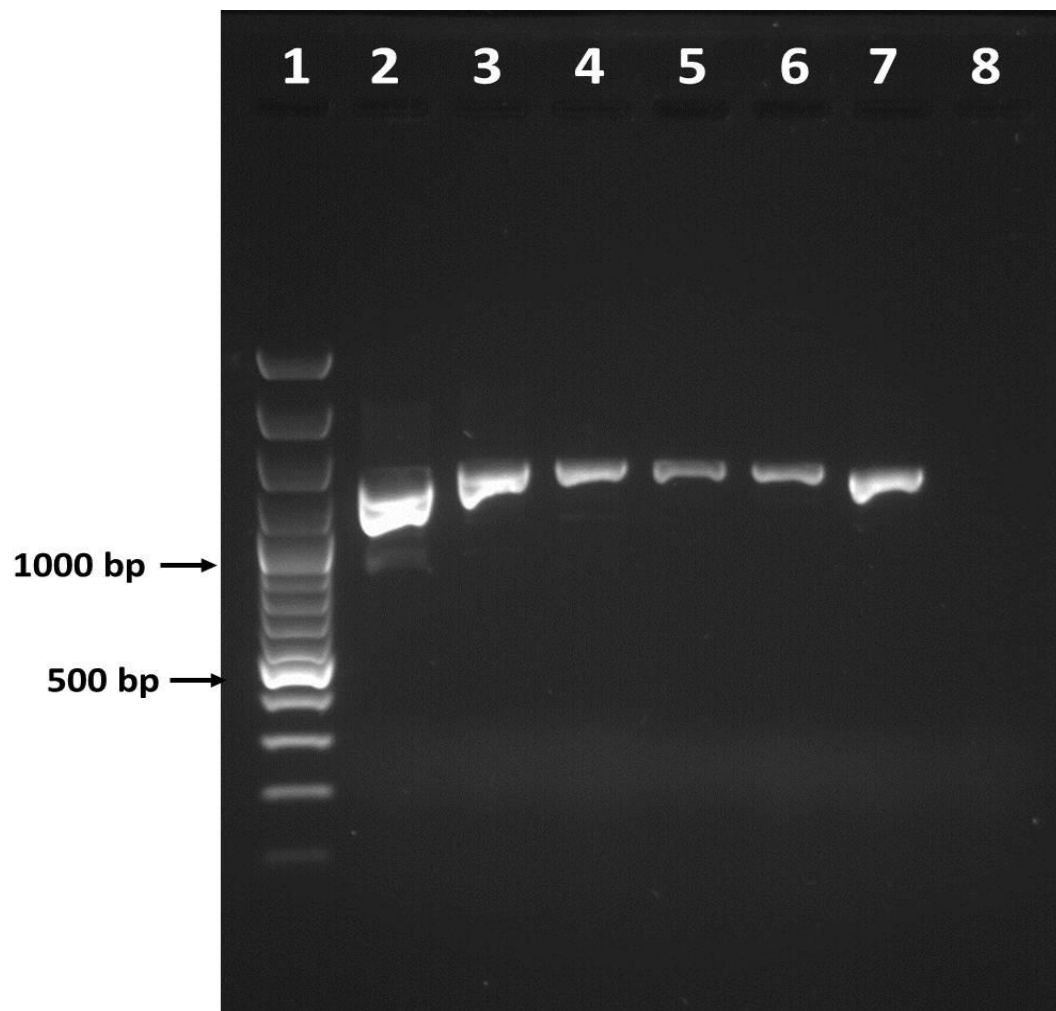


Figure 4.30 ITS Amplification products from five *Heterodera carotae* second stage juveniles. Lane 1 = 1000bp marker; lane 2 = *Globodera rostochiensis* positive control (1000bp); lanes 3 to 7 = Tarlton samples (1000bp); lane 8 = H₂O control.

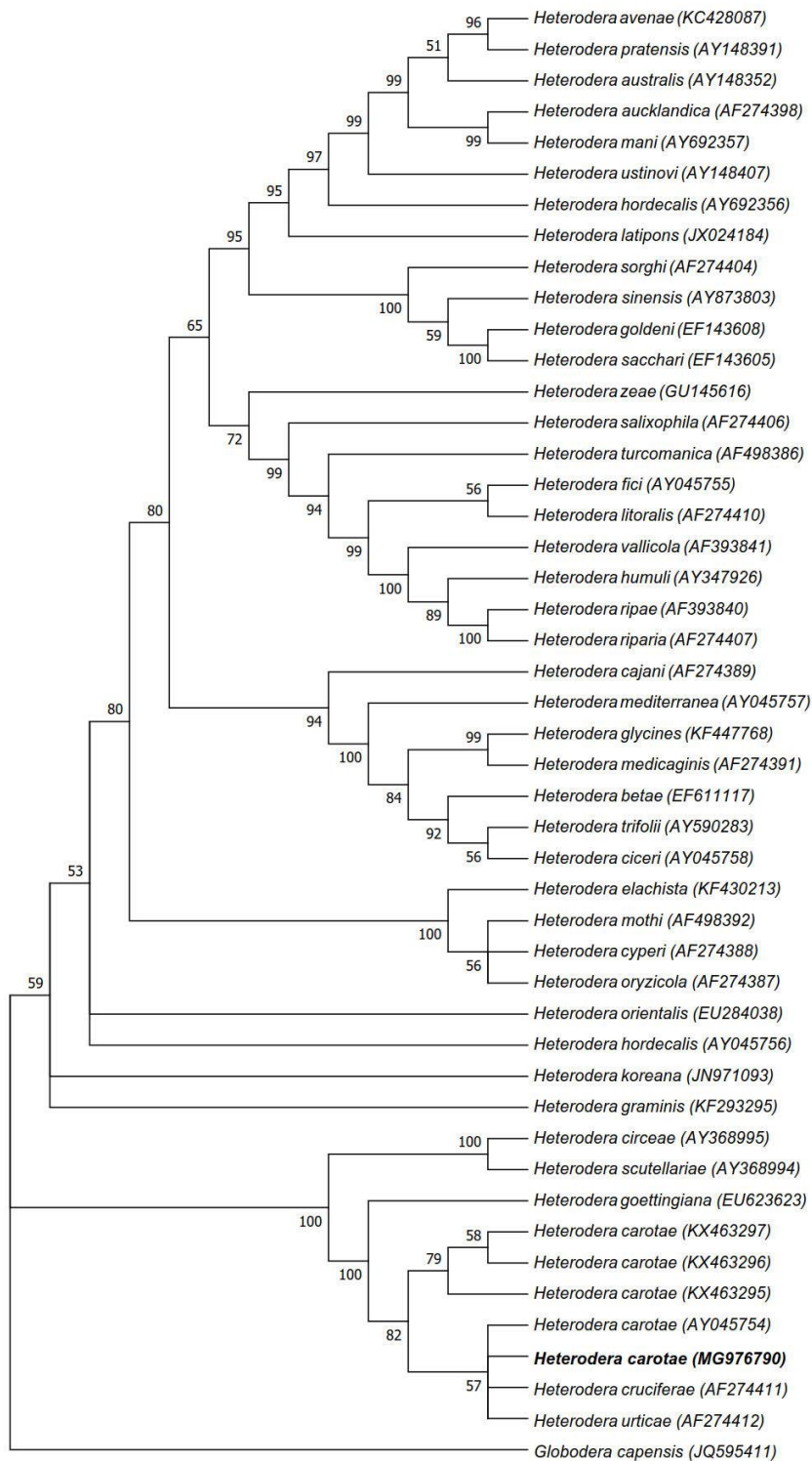


Figure 4.31 Phylogenetic relationships of selected species of the Heteroderidae inferred from ITS-rRNA sequences using the Maximum Parsimony method are in the consensus tree, all the main groups of *Heterodera* are presented as clades. The isolate from Tarlton grouped together in a clade with *H. carotae*, *H. cruciferae* and *H. urticae*, but resolution amongst these species are unclear due to low bootstrap support.

4.7. Discussion

The J2 of the Tarlton population closely resembles *H. carotae* except for the slightly shorter body of some specimens compared to that described from Europe and Canada (270.3-434 μm vs 375-452 μm from Europe and 388-457 μm from Canada) (Subbotin *et al*, 2010 b. & Yu *et al*, 2017). The head diameter of the Tarlton J2 specimens is also smaller than that of specimens described from Europe (4.3-8.7 μm vs 9-10 μm from Europe). Cysts from Tarlton is slightly smaller [L (excluding neck) x diameter = 294-450 μm x 179-341 μm vs 218-625 μm in Europe and 348–520 μm in Canada] and the underbridge is shorter in the Tarlton specimens compared to that of their European counterparts (about 80 μm vs 90 μm). The vulval slit length in the Tarlton specimens compares well with that of the cysts in Europe and Canada (28.3 -54.6 μm vs 43-51 μm and 20-43 μm) but that of the European specimens is much longer (43-51 μm). The spicules of the males from Tarlton are slightly longer than that of both European and Canadian specimens (36.5-40.0 μm vs 31-36 μm and 33-37 μm , respectively).

Based on the similarities of the Tarlton population to those described by Mathews (1975) in the C.I.H. Descriptions Set 5, No.61, as well as the Canadian descriptions of their populations, we consider the Tarlton population to be conspecific with *H. carotae*. We consider the observed differences as lying within the range of intraspecific variation of *H. carotae*. Of interest is the fact that we were able to take SEM photo micrographs of the females, males, J2 and cysts, which have never been published before. We therefore consider the SEM study as a good contribution to the morphology of *H. carotae*.

Resulting from phylogenetic analysis, the carrot cyst nematode population from Tarlton grouped together in a clade with *H. carotae*, *H. cruciferae* and *H. urticae* population extracted from NCBI Genbank. BLAST results indicated a 99 % similarity with the latter *H. carotae* population. Molecular results hence complemented morphological and morphometrical data by means of which the Tarlton population is suggested to resemble *H. carotae*. This study represents the first description of a local carrot cyst nematode population, adding valuable and novel information to both the local and international Nematology fraternities.

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CHAPTER 5

Conclusion and recommendations

The carrot cyst nematode (*Heterodera carotae* Jones, 1950) was reported as the second most economically important pest in carrot production worldwide (Berney & Bird 1992). Carrot is one of the four major root and tuber vegetables consumed in South Africa. The carrot crop therefore contributes much to the gross value of agricultural production in the country. No data on carrot cyst nematodes in South Africa and Africa have been published except the limited information contained in Kleynhans *et al.* (1996). Therefore, the present study that was conducted in the Tarlton area, Gauteng Province (South Africa) focused on the following objectives: (1) to determine the different life stages of *H. carotae* on a weekly basis for taxonomic purposes, and the number of life cycles the nematode complete per carrot growing season, (2) to determine the threshold level of cysts/100 cm³ soil and (3) to compare the number of life cycles/growing season of the species under South African conditions compared to that of its European counterpart and (4) to determine if the South African *H. carotae* is conspecific with *H. carotae* worldwide (morphologically and molecularly). The outcome of the third objective represents very important information to South African farmers as it gives them an estimate of the severity of the impact of *H. carotae* on the carrot crop. Along the same vein Mugniery & Bossis (1988) investigated the relationship between temperature and the length of different life stages of *H. carotae* in France. Later Colagieri & Ciancio (2011) used this data as an example of modelling changes induced by global warming on *H. carotae* under Italian environmental conditions.

During the present study scanning electron microscope (SEM) photo micrographs of the females, males, J2 and cysts, were taken which have never been published before. It is thus considered that the SEM study is a worthy contribution to the morphology of *H. carotae* worldwide. The following conclusions were made on the morphology, morphometrics and molecular study: The J2 closely resembles *H. carotae* except for the slightly shorter body of some Tarlton-specimens compared to that described from Europe and Canada (270.3-434 µm vs 375-452 µm from Europe and 388-457 µm from Canada). The head diameter of the Tarlton J2 specimens is also smaller than that of specimens described from Europe (4.3-8.7 µm vs 9-10 µm from Europe).

Cysts from Tarlton are slightly smaller [L (excluding neck) x diameter = 294 -450 μm x 179-341 μm vs 218-625 μm in Europe and 348 – 520 μm in Canada] and the underbridge is shorter in the Tarlton cyst specimens compared to that of their European counterparts (about 80 μm vs 90 μm). The vulval slit length in the Tarlton specimens compares well with that of the cysts in Canada (28.3-54.6 μm vs 20-43 μm), while that of the European specimens is much longer (43-51 μm). The spicules of the males from Tarlton are slightly longer than that of both European and Canadian specimens (36.5-40.0 μm vs 31-36 μm and 33-37 μm). Based on the similarities of the Tarlton population to those described by Mathews (1975) in the C.I.H. Descriptions Set 5, No.61, as well as the Canadian descriptions (Yu et al., 2017) of their population, the Tarlton population is considered to be conspecific with *H. carotae*. The observed differences are within the range of intraspecific variation of *H. carotae*. Molecularly the isolate from Tarlton is grouped together in a clade with *H. carotae*, *H. cruciferae* Franklin 1945 and *H. urticae* Cooper 1955. BLAST results indicated a 99 % similarity of the Tarlton population with *H. carotae*.

The way forward includes a glasshouse study to investigate the life stages of *H. carotae*, especially the time when the adult female turns into a cyst. This has to be done since it was challenging to determine during this study which specimens were females and which were young cysts because young cysts were, like females, attached to the root and also like females, carried an egg sac. More research also needs to be done on the number of life cycles of *H. carotae* under field conditions in all four seasons (summer, autumn, winter and spring). It would also be interesting to find out how many degree days *H. carotae* requires to develop females and cysts at the different soil temperatures associated with the different seasons. Such interventions will enable researchers to predict the severity of infestation on a seasonal basis. Of special interest is the fact that the number of cysts at planting was almost the same as their number at harvest. This can only be accounted for if the soil temperature rose above the level where no J2 hatching occurs. Greco & Brandonisio (1986) found that J2 hatch was negligible at 25 °C. In the period during the first 10 weeks of the growing season when the soil temperature was measured, a maximum temperature of 28.6 °C and a minimum temperature of 15 °C were recorded for this study. This also warrants further seasonal investigation.

Resulting from this study, two popular articles were published: *Farmers Weekly*, 4 August 2017 and *Landbou Weekblad*, 29 September 2017. Also, one oral presentation was given by the author of this dissertation at the 21st Symposium of the Nematological Society of Southern Africa (NSSA) held from 7 – 11 May at Zimbali, Balito, Kwa-Zulu Natal, South Africa. Full details about these contributions are listed in Appendix A, B and C.

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Appendix A: Measurements and length carrot taproots over 18 weeks.



Figure 1. Measurements and length of the carrot taproots in Week 7 (Photo by A.R. Shubane).



Figure 2. Measurements and length of the carrot taproots length in Week 8 (Photo by A.R. Shubane).



Figure 3. Measurements and length of the carrot taproots in Week 9 (Photo by A.R. Shubane).



Figure 4. Measurements and length of the carrot taproots in Week 10 (Photo by A.R. Shubane).



Figure 5. Measurements and length of the carrot taproots in Week 11 (Photo by A.R. Shubane).



Figure 6. Measurements and length of the carrot taproots in Week 12 (Photo by A.R. Shubane).



Figure 7. Measurements and length of the carrot taproots in Week 13 (Photo by A.R. Shubane).



Figure 8. Measurements and length of the carrot taproots in Week 14 (Photo by A.R. Shubane).

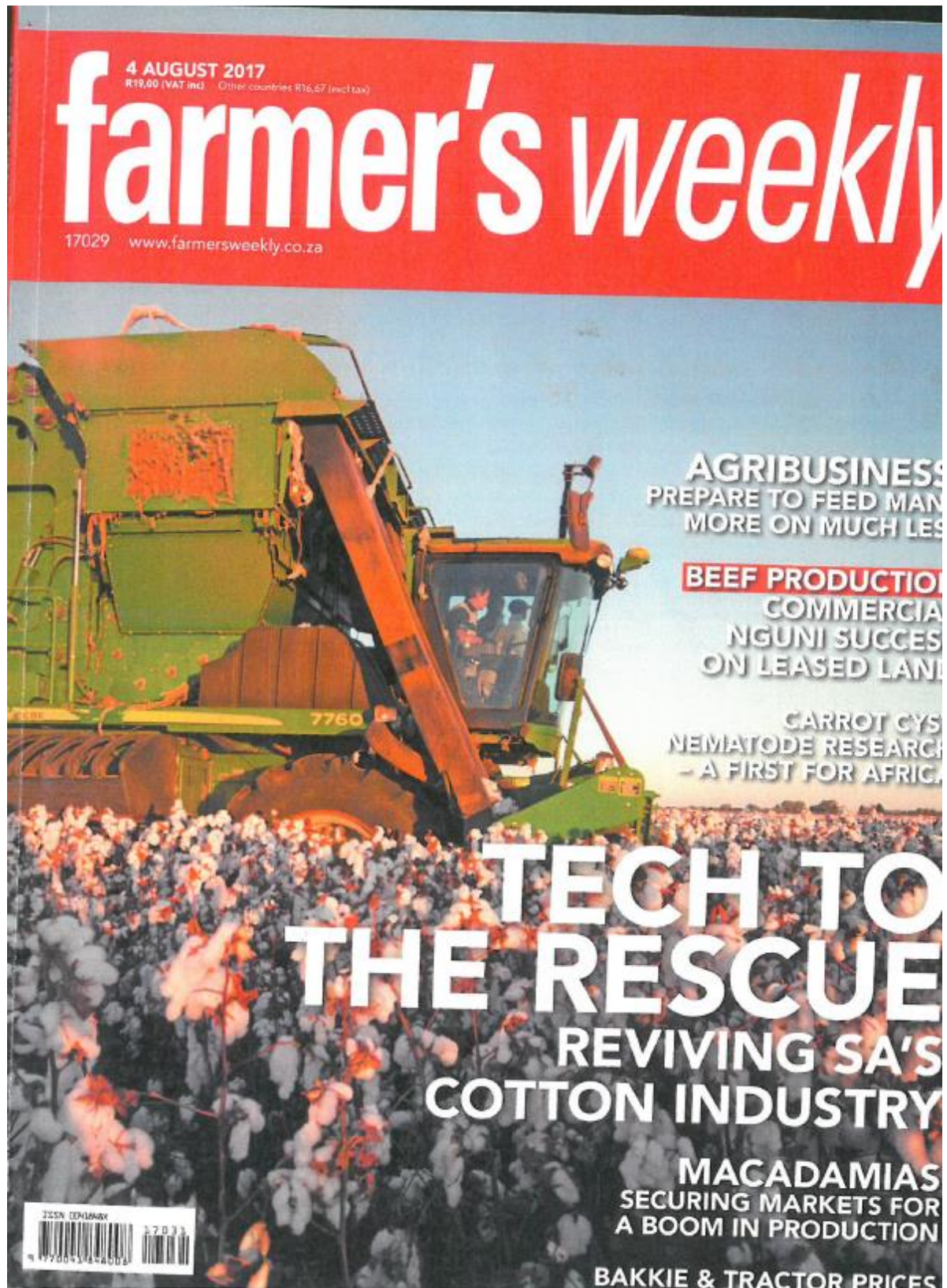


Figure 9. Measurements and length of the carrot taproots in Week 15 (Photo by A.R. Shubane).



Figure 10. Measurements and length of the carrot taproots in Week 18 (Photo by A.R. Shubane).

Appendix B: Published popular articles



Assessing the impact of carrot cyst nematode on SA production

The impact of carrot cyst nematodes on local production was studied by North-West University MSc student, **Adoration Shubane**. Her co-supervisor, **Dr Antoinette Swart** of the Agricultural Research Council's Plant Protection Research Institute, reports on the research findings.

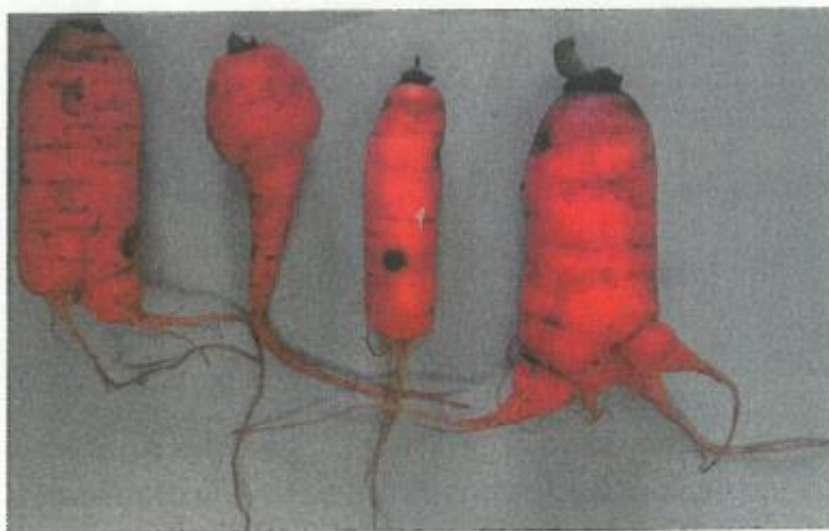
The carrot cyst nematode, *Heterodera carotae*, has never been studied under African conditions despite having an unquestionable impact on local carrot production.

The carrot (*Daucus carota* L.) is cultivated worldwide for the fresh market and processing industry. In South Africa, carrots are one of four major root and tuber vegetable varieties consumed.

Cyst-forming nematodes, or parasitic roundworms, are defined by their capacity to retain eggs inside the swollen female body, which is transformed into a resistant, tanned cyst that protects the embryonated eggs after completion of the female's life cycle. The infective juvenile (J2) emerges from the cyst when it detects root secretions from the growing host. It then enters the root tissue and settles in a permanent site to feed. Feeding is accompanied by swelling of the nematode's body during the third and fourth stages of juvenile development. In the final moult, the adult female remains swollen and sedentary.

The host range of the carrot cyst nematode is rather limited, and only cultivated and wild carrots are infected. The infective juveniles invade only carrot feeder roots, mainly at the tips. Although the nematode can develop in the early growth stage of the main root, no invasion or development occurs in the tap roots.

The plant reacts to the invasion of the juveniles by producing new rootlets, which, in turn, can be invaded by juveniles. The tap root of an infested



ABOVE: Nematode damage, resulting in deformities, was detected in the 15th week of the trial. PHOTOS AND GRAPH SUPPLIED

plant, therefore, often has a heavily bearded appearance.

According to observations in Europe, an infestation of a land usually manifests in patches of stunted plants on which the leaves become reddish before dying. Tap roots are greatly reduced in size and are unmarketable.

Complete failure of the crop may occur in heavily infested soil. In a 1980 Swiss study, an estimated tolerance limit of carrots to *H. carotae* of 40 cysts/250ml soil (16 cysts/100ml soil) was measured.

HISTORY

The carrot cyst nematode was first described in England in 1931, whereafter it was reported throughout the carrot-growing regions of Europe. The nematode has also been reported in the US and Canada. The only reports in Africa have come from South

Africa; it was first recorded near Phillipi in the Western Cape in 1996, and discovered here again in 2002. Thereafter, it was identified in carrots grown in Tarlton, Gauteng (2010 to present).

THE TRIALS

As part of her MSc in Environmental Sciences and Management at North-West University, Adoration Shubane initiated a study in November 2016 on a trial field infested with *H. carotae*, and sown with the Soprano carrot cultivar. Three types of fungicide (copper hydroxide, potassium phosphite and tebuconazole) were applied during the growing season.

The same cultivar was sown in an adjacent land on the same date, and a full, integrated pest management programme was followed here. This included administering two nematicides: oxamyl and Fenamiphos. The



LEFT:
Carrot cyst
nematode
infestation leads
to the stunting
or death of
carrot plants.

soil in the lands was sandy loam (sand: 76,2%; loam: 13,5%; clay: 10,3%) with a pH of 6,41. Soil and root samples were collected weekly from the trial field (from emergence to harvesting) for 18 weeks and nematode cysts extracted.

Shubane reached the following conclusions:

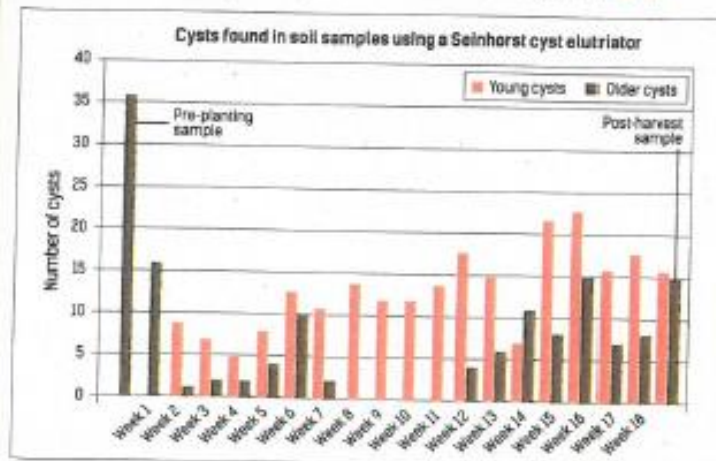
- A nematode's life cycle takes four weeks in the Tarlton area. About three life cycles can therefore be completed in summer, more than that recorded in Europe.
- The tolerance limit of carrots to the carrot cyst nematode in this trial was about 13 cysts/100ml of soil (see graph), which is lower than the European tolerance limit.
- Infective juveniles are present in the soil for the first six weeks after planting,

making this the optimal time to administer nematicides.

The total projected yield for the infected trial block was 36t/ha, of which 78% would be marketable, while the estimated yield for the nematicide-treated block was 57t/ha, with 73% of the crop being marketable.

The projected percentage of marketable produce did not differ significantly, which could be attributed to the fact that 52% of the marketable carrots in the trial block were deemed small (<16cm in length). This could indicate the effect of the carrot cyst nematodes on feeder roots, causing the carrots to be stunted or small. – Gerhard Uys

• Email Dr Antoinette Swart at Swart.A@arc.agric.za, or Adonation Shubane at Shubane.A@arc.agric.za. ■ FW



LEFT:
The graph shows
the number of
cysts found per
100ml of soil in
samples taken
from the trial site.

SAAISLENTER
'UITVINDERS'
KRY STRY

VOËLGRIEP
STAAT BETAAL
DÁN VIR VERLIESE

SPEENKALF-
PRYS GAAN
SY MAN STAAN

GRAANTOEKOMS
WIT MIELIES
VERLOOR VELD

BOEREKOS
LEKKER BOBO
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NUUT

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wat homself
oop- en toemaak!

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Boerehandelsmerk
gee deurslag

VELDBESTUUR

Hoe droër, hoe
langer die rustyd

JAN GREYLING

Hoekom die
beste tegnologie
in jou kop sit

DROOGTEFOKUS

- Besproei minder met dié tegnologie
- Só tel bekende kelder elke druppel
- Wes-Kaapse produksie lelik geknou

SPESIAAL
TLU SA VIER
120 JAAR



LNR: PLANTBESKERMING

DIÉ SIST-AALWURM EERSTE KEER IN AFRIKA BESTUDEER

Die geelwortelsist-aalwurm, *Heterodera carotae* Jones, kan tot merkbare afnames in oeste by geelwortels (*Daucus carota* L.) lei.

Die biologie van dié aalwurm word in 'n studie in die omgewing van Tlartton in Gauteng nagevors – die eerste studie in sy soort in Suid-Afrikaanse en Afrika-toestande.

Geelwortels is 'n belangrike groentesoort wat wêreldwyd vir die verspreidingsmark en die verwerkingsbedryf verbou word. Dit is ook een van die belangrikste wortel- en knolgroentes wat deur Suid-Afrikaners gebruik word.

Die aalwurm *H. carotae* is eerste in Engeland beskryf, waarna dit in al die wortelverbouende streke van Europa aangemeld is. Benewens Europa is dié aalwurm in Amerika, Kanada en Suid-Afrika aangemeld.

Plaaslik is dit die eerste keer in 1996 in Philippi in die Wes-Kaap aangemeld. Daarna is dit weer in 2002 in Philippi aangemeld en vanaf 2010 tot nou in die omgewing van Tlartton in Gauteng.

SIST BESKERM EIRS

Die gasheerreëks van die geelwortelsist-aalwurm is klein. Slegs veredelde (kommersiële) geelwortels en hul wilde eweknieë word daardeur besmet.

Die sistvormende aalwurms, of sist-aalwurms, word gekenmerk deur die vermoë van die wyfie om geëmbrioneerde eiers in haar geswolle liggaam te hou. Die liggaam van die wyfie verander dan in 'n weerstandbiedende sist wat die eiers beskerm.

Met die bespeuring van gasheer se wortelafskedings verlaat die besmetlike tweedestadiumlarwe die sist.

Die tweedestadiumlarwe dring dan die



Me. Adoration Shubane, hier met oestyd aan die werk op die proefperseel, is besig met haar M.Sc.-studie onder leiding van dr. Antoinette Swart (die Landbouuniversiteitsraad se Instituut vir Plantbeskerming, medestudente), dr. Rikus Knoetze (LNR-Infratec/Netwerkbij, medestudente) en prof. Dr. E. F. Fourie (Noordwes-Universiteit, studente).

wortelweefsel binne en begin met die sentere (sittende) voedingsfase, wat gekenmerk word deur die opswel van die derde- en vierdestadiumlarwes.

Ná die finale vervelling bly die wyfie opgeswel en sittend.

Die besmetlike larwe val die groeipunte van voedingswortels aan en nie die penwortel nie. Die plant se reaksie op dié aanval is om meer haarwortels te vorm, wat die penwortel 'n tipiese bebaarde voorkoms gee.

Die penwortel verdwerg en volgens Europese gegewens kan oeste met 'n hoë besmettingskoers heeltemal vernietig word.

PLAASLIKE STUDIE

In Switserland het R. Vallotton in 1980 'n drempelwaarde van 40 siste per 250 ml grond (16 siste per 100 ml grond) bereken. Aangesien hierdie waarde belangrik is wanneer sisttellings van 'n landery geëvalueer word, was dit een van die doelwitte van die huidige studie om 'n aanduiding van die drempelwaarde van geelwortels teenoor die geelwortelsist-aalwurm in Suid-Afrikaanse toestande te kry.

Die studie het in November verlede jaar begin, met die voorbereiding van 'n proefperseel wat met geelwortelsist-aalwurm besmet is.

Die geelwortelkultivar Soprano is gesaai en die perseel is deurlopend met swamdoders – koperhidroksied, kaliumfosfaat en tebukonasool (triasool) – behandel.

'n Soortgelyke kommersiële landery is op dieselfde datum met dieselfde kultivar gesaai,

maar die volledig geïntegreerde plaagbestrydingsprogram is in die groeityd toegepas. Dit het behandeling met die aalwurmdoders oksamil en fenamifos ingesluit.

Die grondklassifikasie van albei landerye is sanderige leem (sand: 76,2%; leem: 13,5% en klei: 10,3%) met 'n pH van 6,41.

Grond- en wortelmonsters is weekliks, vandat die plantjies opgekom het tot oestyd, vir 18 weke op die proefperseel geneem. Die Seinhorst-sist-onttrekker is gebruik om aalwurmsiste te onttrek.

MEER GESLAGTE

Die volgende afleidings is tot dusver uit die studie gemaak:

- Die lewensloop van *H. carotae* duur vier weke, wat beteken dat sowat drie geslagte in een somerseisoen voltooi kan word, meer as wat in Europa aangemeld is.
- Die drempelwaarde van geelwortels teenoor geelwortelsist-aalwurms is 13 siste/100 ml grond, teenoor die 16 siste/100 ml grond wat in Europa aangemeld is.
- Besmetlike larwes is vir die eerste ses weke ná plantyd in die grond teenwoordig (sien die **GRAFIK**), waarna hulle in die wortels inbeweeg. Dié ses weke is dus die beste tyd vir die toediening van aalwurmdoders.
- Die totale geskatte oesopbrengs in die proefperseel was 36 ton/ha, met 78% daarvan bemarkbaar. Op die kommersiële perseel met die volledig geïntegreerde plaagbestrydingsprogram, wat aalwurmdoders ingesluit het, was die geskatte oes 57 ton/ha, met 73% daarvan bemark-



Simptome van besmetting wat in die 15de week van die proef waargeneem is.



'n Groep verdwergde en doele plante tydens oestyd op die proefperseel. Die simptome is deur besmetting met die geelwortelsist-aalwurm veroorsaak.



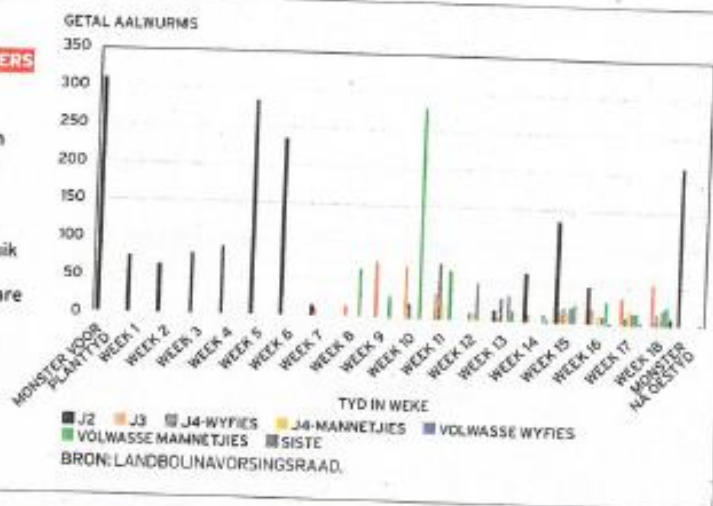
Geelblare van besmette plante met pestyd.

baar. 'n Opbrengsverskil van 21 ton/ha bestaan dus tussen die proef- en kommersiële perseel, hoewel die persentasie bemarkbare produk byna dieselfde was. Aangesien 52% van die bemarkbare wortels van die proefperseel as "klein" geklassifiseer is (< 16 cm lank), weerspieël dit die invloed van die geelwortelsist-aalwurm (sien simptome op die foto's) op geelwortels. – ADORATION SHUBANE, ANTOINETTE SWART, RINUS KNOETZE en DRIEKIE FOURIE

NAVRAE: Me. Adoration R. Shubane, tegnikus by die Landbounavorsingsraad se Navorsingsinstituut vir Plantbeskerming (NIPB), tel. 012 808 8243; e-pos: ShubaneA@arc.agric.za, of dr. Antoinette Swart, spesialisnavorsers by die NIPB, tel. 012 808 8279; e-pos: SwartA@arc.agric.za

AALWURM-STADIA IN GRONDMONSTERS GEVIND

Lewenstadia van aalwurms in grondmonsters wat gevind is deur die sif-suikerflottasie-tegniek te gebruik (getal/250 ml grond). Wortelhare met geswolle lewenstadia van die geelwortelsist-aalwurm is ook in die grond gevind.



Appendix C: Conference contributions.

A STUDY OF THE CARROT CYST NEMATODE, *HETERODERA CAROTAE*, IN SOUTH AFRICA

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Carrot (*Daucus carotae* L) is one of the four major root and tuber vegetables consumed in South Africa. The carrot crop therefore contributes much to the gross value of agricultural production in the country. In 2008 *Heterodera carotae* Jones, 1950 (carrot cyst nematode) was first reported from the Tarlton area, with concomitant crop damage. This nematode is generally considered a European species and no previous studies had been conducted on *H. carotae* under South African conditions. This presentation deals with a morphological, morphometrical and molecular study of populations of *H. carotae* in the Tarlton area, and results from this study are compared with those of European populations. To determine the number of generations that can be completed by *H. carotae* during a carrot-growing season in South Africa, the life cycle was investigated under field conditions. This involved weekly collections of root and soil samples, from seedling emergence to harvesting (about 22 weeks), extraction of all nematode life stages, and calculation of the abundance of each developmental stage. The soil temperature was recorded throughout the experiment. The time-periods and accumulated day-degrees above the developmental temperature (10°C) required by *H. carotae* to reach different life cycle stages are graphically presented.