Chapter 4
Techniques and Procedures

Mariette Marais, Antoinette Swart, Hendrika Fourie, Shaun D. Berry, Rinus Knoetze, and Antoinette P. Malan

4.1 Introduction

A prerequisite for sound and trustworthy nematology research and diagnostics is the accurate identification of nematode genera and species. A wide range of specialised techniques and procedures are available and have been published for use in Nematology. However, in this chapter only those techniques and procedures that are used on a routine basis in nematology laboratories in South Africa (SA) are described. An important aspect to keep in mind is safety prerequisites that have to be applied in a laboratory set-up (Box 4.1).

M. Marais (✉) • A. Swart
Nematology Unit, Biosystematics, Agricultural Research Council–Plant Protection Research Insitute, Private Bag X134, Queenswood 0121, South Africa
e-mail: maraism@arc.agric.za

H. Fourie
North-West University, Unit for Environmental Sciences and Management, Private Bag X6001, Potchefstroom 2520, South Africa

S.D. Berry
BASF Corporation, 26 Davis Drive, Research Triangle Park, Durham, NC 27709, USA

R. Knoetze
Department of Agriculture, Forestry and Fisheries, Plant Quarantine Station, Polkadraai Road, Stellenbosch 7600, South Africa

A.P. Malan
Department of Conservation Ecology and Entomology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, Stellenbosch, South Africa

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Box 4.1 Health and Safety in Research Environments
Health and safety in the laboratory, glasshouse, microplot and field is usually regulated by law. In South Africa, health and safety are regulated by the Occupational Health and Safety Act No. 85 of 1993 as amended by the Occupational Health and Safety Act No. 181 of 1993. Many of the chemicals, apparatus and devices used in nematology laboratories, glasshouses, microplots and fields are potentially dangerous or hazardous. Therefore, all devices and chemicals must be handled with due diligence, while standard procedures must be in place (e.g. the use of appropriate personnel protective equipment). These recommendations and regulations must be strictly adhered to by all research and support staff.

Detailed descriptions of specific methodologies used in nematology research and diagnostics are available in numerous chapters of books including Hooper et al. (2005), Been and Schomaker (2013), Bridge and Starr (2007), Khan (2008), Nguyen and Hunt (2007), Hunt and Handoo (2009), Manzilla-López (2012), Powers and Ramírez-Suárez (2012) and Subbotin et al. (2013). Summaries of basic methodologies used in laboratories in SA have been reported previously by Koen (1969), Keetch (1982), Kleynhans et al. (1996) and Van den Berg and Furstenberg (1982).

4.2 Nematode Sampling

One of the aims of nematode sampling is to determine the diversity and population densities of both plant-parasitic and non-parasitic nematodes for research, advisory or regulatory purposes. Since nematodes typically occur in patches (horizontally and vertically) in the soil (Fig. 4.1), sampling should be planned well in advance to enable the highest level of accuracy. This will minimise the inherent variability in the data obtained. To diagnose nematode problems accurately, the following information is crucial: (i) sampling techniques and apparatus used, (ii) sample size and area sampled, (iii) sampling depth, (iv) time of sampling and (v) sampling pattern. Also important is the handling, labelling, transport and storage of the samples.

4.2.1 Sampling Techniques

Several techniques are used to collect nematode samples from fields (Kleynhans et al. 1996; Kleynhans 1997; Hooper et al. 2005; Bridge and Starr 2007; Coyne et al. 2007; Khan 2008; Been and Schomaker 2013). Popular and widely used methods for sampling annual row crops or fallow fields (i.e. for pre-planting sampling) are the ‘zig-zag’, ‘W’ or ‘grid-pattern’ methods. Large fields should be divided in

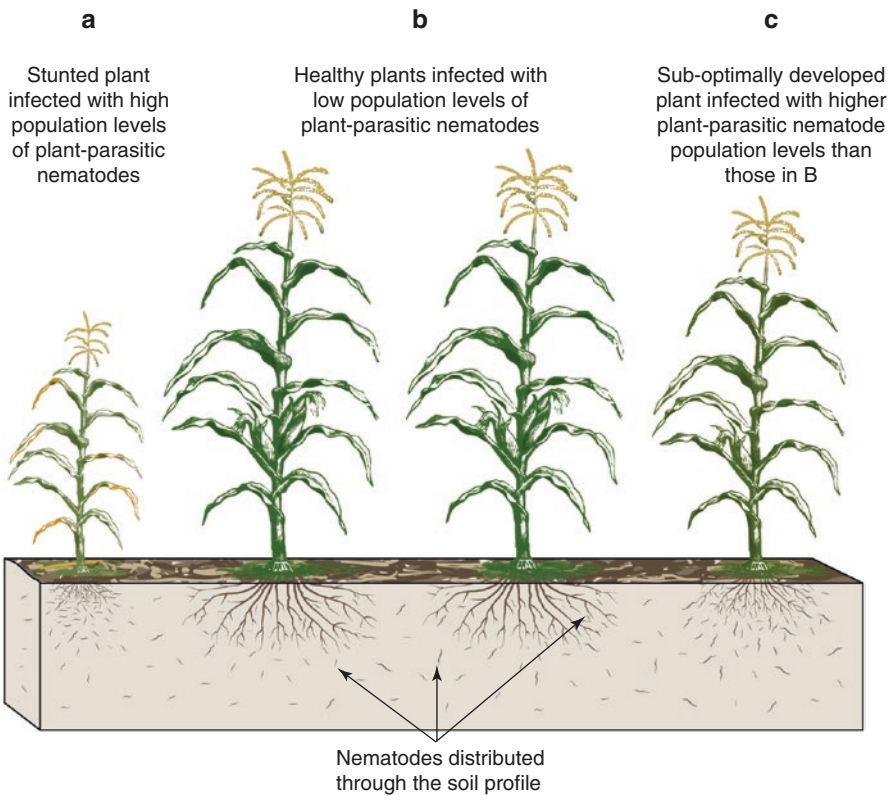


Fig. 4.1 Horizontal and vertical distribution of plant-parasitic nematodes in the soil in a maize field (Hannes Visagie, North-West University, Potchefstroom, South Africa)

smaller units of at least 1 hectare (ha) for sampling purposes. Soil samples should be collected systematically, not randomly, at equally-spaced points that cover the whole sampling unit, e.g. a 2×2 m or a 10×10 m pattern grid should be used. The smaller the distance between such points, the higher the sampling precision will be. Another sampling technique includes sampling a field at equally-spaced points along a diagonal line that runs across the field. Also, in experimental plots, annual and perennial row crops can be sampled within as well as across the crop rows. Root and soil samples from shrubs, trees and grapevine are usually taken underneath the canopy areas and on or near to the drip lines. Sampling is done on both sides of the stems and grapevine rootstocks to ensure that representative samples are obtained.

4.2.2 Sampling Equipment

Various apparatus are used for nematode sampling, including a garden spade, a trowel, a soil corer or an Edelman soil auger (Kleynhans et al. 1996; Kleynhans 1997).

4.2.3 Sampling Size or Area

The more samples (soil, roots and other plant parts) that are taken the greater the chance that they will be representative of a field or orchard. The number of samples depends mainly on the size of the field and the nematode extraction capacity available. Nematodes occur in uneven and aggregated patches in fields and hence sampling errors must be minimised by taking as many samples as practical possible. To increase the probability of detecting nematode species that may occur in low numbers, a large number of samples should be obtained according to a systematic pattern across a field (see Sect. 4.2.1). Nematode samples can be combined, thoroughly mixed and a single or a few subsamples taken and extracted. A typical subsample should preferably contain no less than 100 g of soil or 50 g of plant tissue (e.g. roots).

4.2.4 Sampling Depth

Soil and root samples of most annual crops should be collected to a depth of approximately 20–30 cm below the soil surface, where the majority of plant-parasitic nematode species occur and where their population densities are the highest (Fig. 4.1). However, the depth of sampling will also depend on where most of the roots occur, which can differ among crops. For example, rhizosphere soil from groundnut pods and roots will be collected at a shallower depth than soil around the roots of maize, soybean or sunflower. Samples should, however, also be collected at a depth of at least 30–45 cm in regions where hot, dry summers are experienced to ensure that also nematodes that migrated downwards in the soil profile are collected. Samples collected from perennial crops, trees and grapevine should be sampled in the soil layer were feeder roots are formed. For deep-rooted perennial crops, samples should be collected to a depth of approximately 20–30 cm as well as at lower depths up to 50–60 and even 100 cm. Feeder roots as well as roots that are present deeper in the soil profile are obtained this way.

4.2.5 Sampling Time

The timing of sampling depends on the purpose of the sampling. Taking samples before planting or during the early vegetative plant growth phase can provide evidence of a relationship between the nematode population densities in the soil or in the roots and the yield of annual crops. This information can be used to decide if treatment is necessary or not. Taking samples at regular intervals for the duration of a crop cycle can provide information on the population dynamics of the nematodes over time. For example, Riekert (1996) found that population densities of plant-parasitic nematodes peaked during the flowering of maize plants.

4.2.6 Handling, Labelling, Transport and Storage of Samples

Kleynhans et al. (1996) and Kleynhans (1997) published useful protocols on the handling, labelling, transport and storage of nematode samples. Soil and plant tissue samples should be placed in sturdy plastic bags, which can be sealed. The bags should be labelled with a permanent marker on the outside, preferably on masking tape or a sticky label, as ink of permanent markers can rub off. A bagged sample may be placed inside a second bag and a label inserted between the bags. Care should be taken that no labels are placed directly in contact with the soil or plant tissues. It is important to clearly label each sample. Information about the precise location, date of sampling, crop sampled, plant growth stage, name of farmer, Global Positioning System (GPS) coordinates where the samples were taken and crop history should be recorded in a field book (hard copy or electronic) or analysis form. This is an important aspect of the nematode sampling process.

It is preferable to place samples of rhizosphere soil and roots together in a bag. The plant tissues will be preserved for a longer time when stored this way. Other plant tissue samples, particularly aerial parts such as stems, leaves and seeds, should be wrapped in moist paper towels after sampling and placed in a separate bag. Samples should neither be dropped nor placed in direct sunlight or in a warm place. It is crucial that samples collected from fields containing dry soils not be moistened until they are processed in the laboratory. Processing of nematode samples should be done as quickly as possible after arrival at the laboratory. However, samples can be stored in a cold room or refrigerator at 5–10 °C, for as short a time as possible.

4.3 Visual Examination of Plant Material

It is advisable to examine both above- and below-ground plant parts for visual symptoms as a first measure to recognise nematode infections. Although symptoms are not always visible to the naked eye, small pieces of plant material can be inspected for nematode infection using a dissecting microscope.

The most typical symptom of nematode infection of plant tissues and an indication of potential damage are root galls induced by root-knot nematodes (*Meloidogyne* spp.) (Fig. 4.2a). Egg masses of root-knot nematode females may also be visible as small, white or brown mass protruding from the roots or visible just below the surface of infected roots/other plant parts. The egg mass can be stained pinkish-red with Phloxine B to facilitate observation and counting of egg-laying females (Fig. 4.2b).

On other plant parts, e.g. potato tubers, protuberances on the surface can also indicate the presence of root-knot nematodes (Fig. 4.3a). As in the roots of other plants, root-knot nematode females are generally visible as small, white, roundish organisms that produce white and brown egg masses below the skin of potato tubers (Fig. 4.3b).

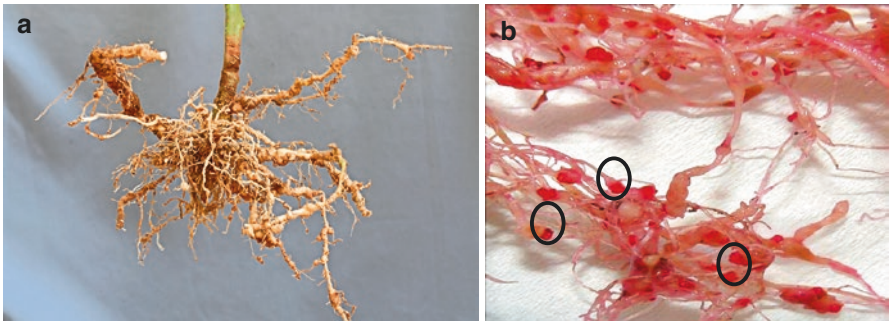


Fig. 4.2 Severe galling of tomato roots infected by root-knot nematodes (**a**) and pinkish-red stained egg masses (*black circles*) produced by females on tomato roots (**b**) (Kirk West, Port Elizabeth, South Africa)

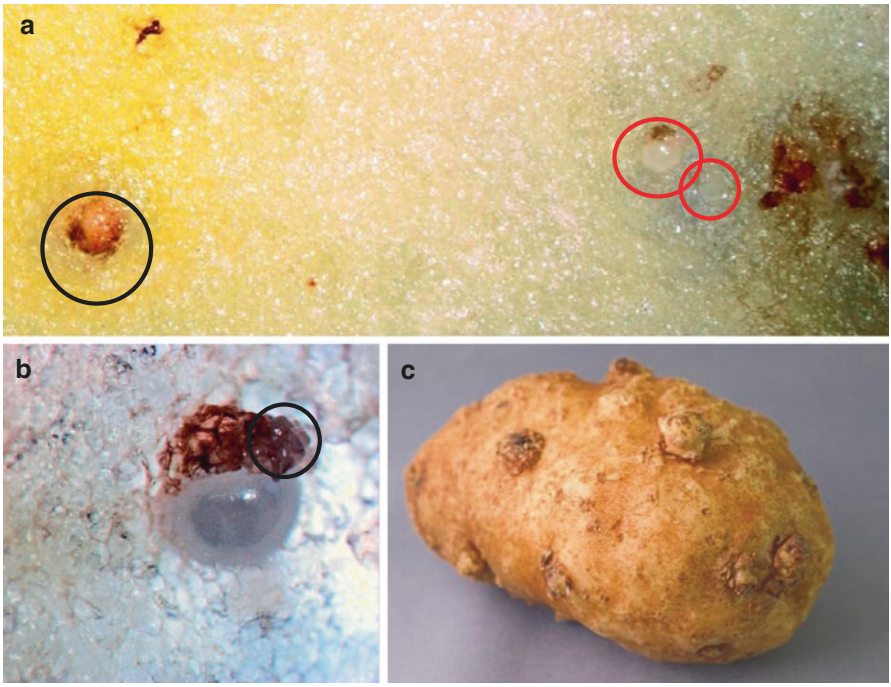


Fig. 4.3 White, roundish root-knot nematode females (*red circles*) and a brown egg mass (*black circle*) (**a**: 13× magnification), with a close-up view of a female and her brown egg mass (*black circle*) (**b**; 100× magnification) visible approximately 1 cm below the skin of an infected, galled tuber (**c**) (**a**: Kirk West, Port Elizabeth, South Africa; **b**, **c**: Gerhard du Preez, North-West University, Potchefstroom, South Africa)

Fig. 4.4 Yellowish (*yellow circle*) and brown (*red circle*) cysts of golden cyst nematodes on potato roots (Caroline Mouton, Department of Agriculture, Forestry and Fisheries, Stellenbosch, South Africa)

Cyst nematodes can also be observed on roots and tubers of infected crop plants. Towards mid-season, small, white to yellow, roundish young females (which may contain egg masses in some genera) may be visible on infected below-ground plant parts and at the end of the crop cycle as white to yellow and brown, roundish to oval-shaped cysts (Fig. 4.4).

Symptoms that result from infection by *Ditylenchus* spp. are also usually visible to the naked eye. Although the majority of species within this genus are mycetophagous, a few species are of great importance as parasites of higher plants. These include three species that are present in SA, namely the groundnut pod nematode *Ditylenchus africanus* Wendt, Swart, Vrain and Webster 1995 (see also Sect. 9.3.1, Chap. 9), *Ditylenchus dipsaci*, Kuhn, 1857 and *Ditylenchus destructor* Thorne, 1945 (Kleynhans et al. 1996). Groundnut pods and seeds infected with *D. africanus* show characteristic symptoms that are visible as corky, darkened brown-blackish tissue on the outside of the pods (Fig. 4.5), with brown and necrotic sections on the inside. Feeding of groundnut pod nematodes near or in the vascular bundles of the seed testa furthermore results in brown discolouration of the testa, with distinctly darkened veins being visible. Early germination of seeds is another distinctive symptom of *D. africanus* infection (Jones and De Waele 1990).

Symptoms caused by *D. dipsaci* infection generally are distorted and discoloured stems and leaves. Leaves may also develop small, yellowish galls or the lower parts of infected leaves may turn white ('white flagging') as is common in infected lucerne crops (Griffin 1998; Kleynhans et al. 1996). An *Aphelenchoides* sp. that can also cause damage to groundnut is the groundnut testa nematode *Aphelenchoides arachidis* Bos, 1977 (Lesufi et al. 2015). The symptoms caused by this nematode generally resemble those caused by the groundnut pod nematode and are illustrated in Chapter 9 (see Sect. 9.3.1.1, Fig. 9.8a, b).

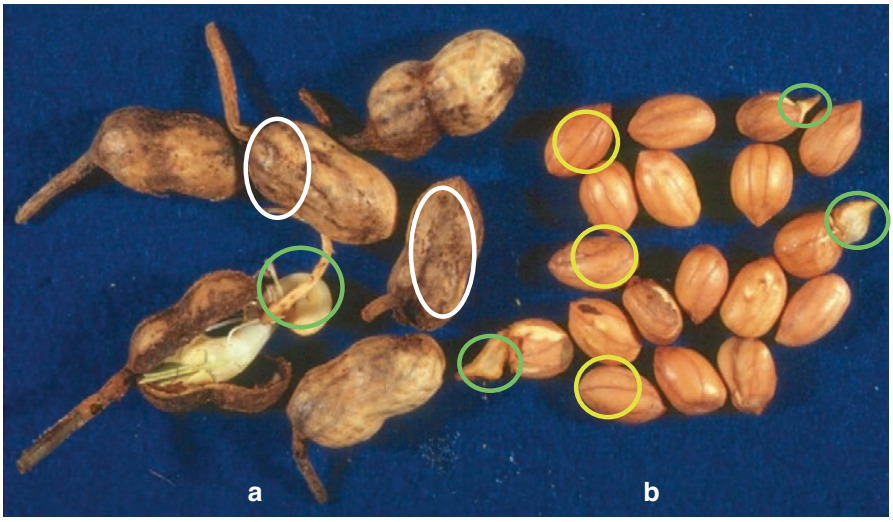


Fig. 4.5 (**a**) Below-ground symptoms caused by *Ditylenchus africanus* on infected groundnut pods, visible as brown/grey/black discolouration on pegs and hulls (*white circles*), and (**b**) darkened veins of the seed testa (*yellow circles*) and early germination of seeds (*green circles*) (Johan Els, Agricultural Research Council–Grain Crops Institute, Potchefstroom, South Africa)

Below-ground symptoms caused by root lesion nematodes (*Pratylenchus* spp.) are usually not easy to spot with the naked eye, but in some crops, such as banana, these symptoms can be observed as short to long, dark brown to black, lesions sometimes bordered by reddish cells as is the case for *Radopholus similis* Cobb, 1913 and some *Pratylenchus* species that parasitise banana. Dark, dry spots or pimples on the surface of potato tubers may also represent symptoms caused by lesion nematodes.

The examples mentioned above represent only a few of the symptoms caused by nematodes that can be detected through visual inspection of plant parts. Photographs of galled seeds as a result of infection by seed-gall nematodes (*Anguina* and *Subanguina* spp.) are included in Chap. 19. Photographs of the damage caused by some other nematode are included in some of the other chapters (see Chaps. 7–18).

4.4 Nematode Extraction

Extraction of nematodes from soil and plant tissue is a crucial step in determining the population densities of plant-parasitic nematode species or genera during the various growth stages of a crop. Although a wide variety of nematode extraction methods have been described, the most appropriate method should be used. The quantity of the (sub) sample, be it 100 g soil, 100 ml soil or 50 g roots, should

Table 4.1 A summary of the most common techniques and procedures used in laboratories in South Africa to extract nematodes from soil and plant tissue samples and the principles upon which they are based

Method	Principle	Soil	Plant tissue
Baermann funnel and tray	Active movement of nematodes	x	x
Decanting	Specific gravity of nematodes	x	
Sieving	Size of nematodes	x	
Flegg's method	Active movement of nematodes	x	
Insect-baiting technique (specifically for entomopathogenic nematodes)	Active movement of nematodes	x	
Maceration	Size of nematodes		x
Mistifier	Active movement of nematodes	x	x
Modified NaOCl method	Dissolving of egg masses by NaOCl		x
Oostenbrink's elutriation and sieving	Specific gravity and size of nematodes	x	
Seinhorst's cyst elutriator	Specific gravity and size of nematodes	x	
Sugar centrifugal flotation	Specific gravity of nematodes	x	x
Soaking	Active movement of nematodes		x

always be prepared in exactly the same way. Also, the processing of samples from the same experiment or study should be carried out by the same operator because even small differences in handling by different operators may result in differences in extraction efficiencies. The extraction method should also not be changed during an experiment or study. An extensive, useful and detailed document has been published by the European and Mediterranean Plant Protection Organization (EPPO) in which a wide range of methods used for extracting nematodes from soil and plant tissues are described (EPPO 2013). A few of these methods are also used in laboratories in SA on a daily basis. These methods can be divided into those used for the extraction of nematodes from soil (Sect. 4.4.1) and from plant material (Sect. 4.4.2). The most common methods used in SA as well as the principles upon which they are based are listed in Table 4.1.

4.4.1 Extraction of Nematodes from Soil

A number of methods used to extract nematodes from soil are based upon the specific gravity and the size of nematodes. During a specific period of flotation or elutriation, nematodes and soil particles with a similar specific gravity can be separated from heavier soil particles in water (or another substance) and then collected on one or more stacked sieves. Other methods are based on the active movement of nematodes. Often, different methods are combined.

4.4.1.1 Decanting and Sieving Method

This method is based on the specific gravity of nematodes (Jenkins 1964). The specific gravity of terrestrial nematodes is approximately 1.08 g cm^{-3} and that of marine nematodes about 1.13 g cm^{-3}. In a water layer, terrestrial nematodes will slowly sink to the bottom but not as fast as soil particles with a higher specific gravity such as sand particles. This difference allows the separation of terrestrial nematodes from a large part of the particles present in a soil. The method described below is a modified method based on descriptions by inter alia Jenkins (1964), Kleynhans (1997) and Hooper et al. (2005). It allows the extraction of both active and inactive nematodes.

A soil sample is soaked in tap water and washed through a coarse-meshed 2-mm-aperture sieve into a plastic bucket. The residue on the sieve (that may include plant tissues) is discarded and the bucket filled up to 5 l with tap water. The soil within the bucket is then thoroughly mixed with the tap water and the mixture allowed to stand for 30 s. The sediment that settles at the bottom of the bucket is left behind when only the supernatant is decanted through stacked 45- and 25-µm-aperture sieves. The nematodes that are suspended in the supernatant are retained on the sieves and washed into a beaker with distilled water. The entire procedure is repeated twice but each time with a shorter settling time (20 and 10 s). Each time, the residue on the sieves is washed into the beaker. The content of the beaker is then mixed thoroughly and the suspension divided over 100-ml centrifuge tubes to be centrifuged.

4.4.1.2 Sugar Centrifugal Flotation Method

Different flotation methods are commonly used for the extraction of nematodes. These methods are also based on the specific gravity of nematodes. They involve the use of solutions with different densities to separate unwanted materials from nematodes by either flotation or precipitation. While water typically represents the low specific gravity (1 g cm^{-3}) solution, sugar or NaCl can be used to create the high specific gravity (1.15–1.3 g cm^{-3}) solution. After the centrifugation process in water, only organic material with a specific gravity <1 g cm^{-3} will remain in suspension and can be discarded. When centrifuged in a sugar solution with a specific gravity of 1.15 g cm^{-3}, the nematodes will remain in suspension and can be separated from soil particles with a higher specific gravity. This method also allows the extraction of both active and inactive nematodes. It is a rapid technique which enables the processing of a large number of samples within a relative short time. A limitation of this method is that the required equipment is expensive. Exposure of the nematodes to the sugar solution also represents a risk since this may cause plasmolysis if the exposure time is too long. The use of Ludox offers an alternative in this respect (see Sect. 4.4.1.9).

First, 5 ml kaolin (see Sect. 4.4.2.3) is added to each centrifuge tube, mixed with the nematodes suspended in distilled water, and the suspension centrifuged for 4 min at 1,800 *g*. The supernatant is decanted and discarded. From this step

onwards, the nematodes are subjected to sugar centrifugal flotation. A sucrose solution with a specific gravity of 1.15 g cm^{-3} is added to each of the centrifuge tubes. This solution is prepared by adding 624 g of sugar to 1,000 ml of tap water. The sugar solution and the sediment, containing the nematodes, are thoroughly stirred with a spatula and re-centrifuged for 3 min at 1,800 g. The spatula is rinsed every time after it has been used to prevent cross contamination among the centrifuge tubes. After the centrifugal flotation has been completed, the supernatant that contains the nematodes is decanted through a 25-µm-aperture sieve and the nematodes washed into a beaker with distilled water for counting and identification. The time that the nematodes are exposed to the sugar solution should be as limited as possible to prevent plasmolysis of the nematodes and minimise osmotic stress.

4.4.1.3 Flegg's Method

The method described by Flegg (1967) is based on the active migration of nematodes and especially suited to extract larger nematodes (e.g. *Xiphinema, Longidorus* and *Paralongidorus* spp.) from soil samples. This method is, however, not suited to extract cysts, sluggish or inactive and small nematodes.

A known soil volume, e.g. 500 ml, is soaked in water for 1 h (for high clay content soils) while it is stirred intermittently. The resulting suspension is washed through a 4-mm-aperture sieve into a 5 l plastic bucket to separate coarse soil particles from the nematodes. The water in the bucket is stirred vigorously by hand to suspend all soil particles and nematodes, and then allowed to stand for 30 s. The supernatant is decanted through three stacked 150-µm-aperture sieves. The residue on the sieves is thoroughly rinsed with a gentle stream of tap water before it is washed with distilled water into a beaker. Water is again added to the 5 l bucket and the process repeated once more. The residue collected in the beaker is gently stirred before decanting through a 90-µm-aperture polyethylene-supported nylon sieve. This sieve is placed in a Petri dish and distilled water added in such a way that it just touches the residue. After 24 h the nematodes will have moved through the sieve in to the Petri dish and are ready for counting and identification.

4.4.1.4 Baermann Funnel Method

In contrast to Flegg's method, the Baermann funnel and tray methods are not suited to extract large nematodes from soil. A known volume of soil, e.g. 50 ml, is evenly spread on paper tissue which is placed on a 710-µm-aperture sieve. The sieve is rested onto the wide top of a funnel which is supported in a ring stand or rack. A pinch clamp is attached to a rubber or plastic tube clipping the funnel stem to seal it off during the extraction process. The funnel is filled with distilled water until the soil on the filter is slightly submerged. Nematodes will migrate from the soil through the paper tissue and the meshes of the sieve into the water in the funnel and

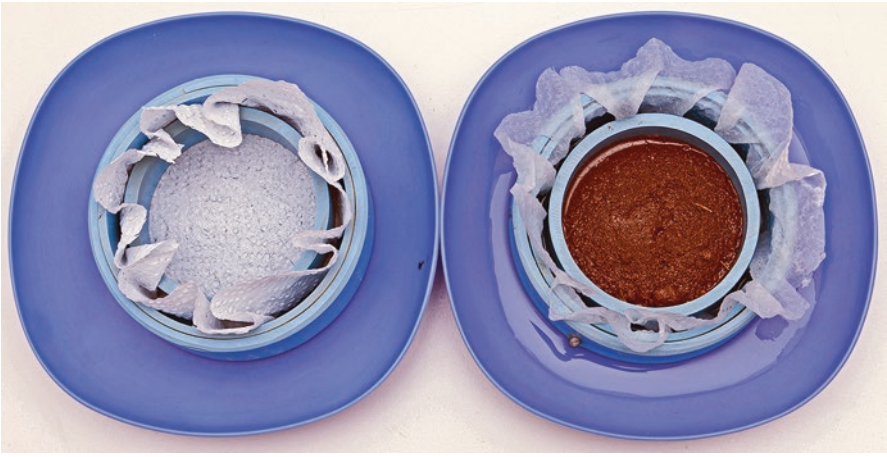


Fig. 4.6 Modified Baermann trays used for the extraction of nematodes from soil, consisting of 1-mm-aperture sieves lined with paper tissue (Kirk West, Port Elizabeth, South Africa)

sink to the funnel stem. After 24 h at room temperature, the nematodes can be collected by opening the pinch clamp. The water in the funnel can then be filled up to the original mark to replace the water that had evaporated and after another 48 h the nematodes can be collected again in a beaker. This method allows extraction of nematodes without using any substance that can be harmful to them. This method is, however, also only suited to allow extraction of only actively moving nematodes.

4.4.1.5 Baermann Tray Method

The Baermann tray method is a modification of the Baermann funnel method (Hooper and Evans 1993). The funnel is replaced by a shallow tray in which a 1-mm-aperture sieve or a kitchen sieve is placed and lined with paper tissue (Fig. 4.6). A known volume of soil is evenly spread on the paper tissue. Nematodes that migrated from the soil into the water can be collected after 24–72 h (Kleynhans 1997).

4.4.1.6 Oostenbrink's Elutriation and Sieving Method

Vermiform nematodes can be extracted from both wet and dry soil samples by keeping them in suspension in water using a controlled upward current of water in a so-called elutriator. Oostenbrink's elutriation and sieving method combines this principle with sieving (Oostenbrink 1960) (Fig. 4.7). This method is only suited for the extraction of active nematodes and is not suited to extract cysts, sluggish or inactive and large nematodes.

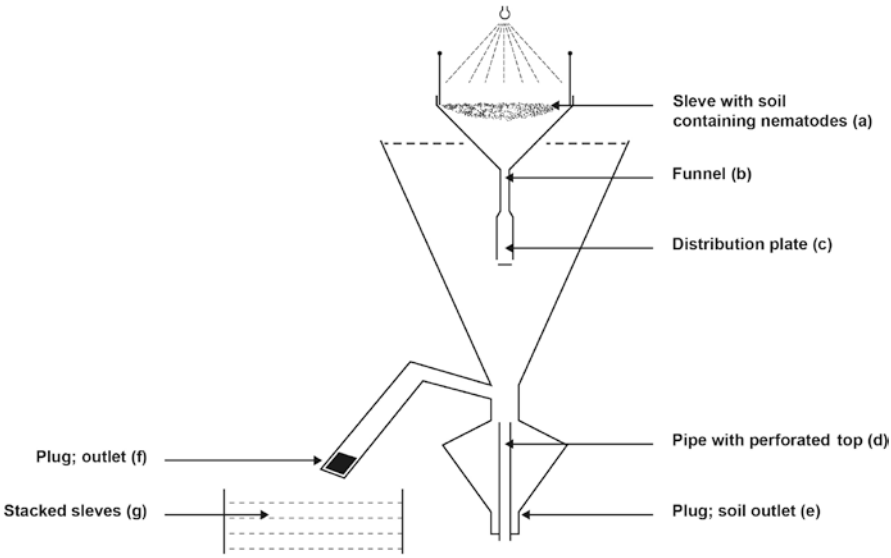


Fig. 4.7 Oostenbrink's elutriation apparatus used for the extraction of nematodes from soil; sieve with soil containing nematodes (**a**), funnel (**b**), distribution plate (**c**), pipe with perforated top (**d**), plug to control soil outlet (**e**), plug to control water outflow in which nematodes are suspended (**f**), stacked sieves on which nematodes are collected (**g**) (Redrawn by Hannes Visagie, North-West University, Potchefstroom, South Africa)

Water enters the bottom of the apparatus at about 1,000 ml min^{-1} through a pipe with a perforated top (d). When the water reaches the small plate, a 250 ml soil sample is placed on a 4-mm-aperture sieve (a) and washed into the flotation apparatus via the funnel (b). The upward water current prevents nematodes and fine soil particles from entering the neck of the apparatus. Heavy soil particles settle at the bottom of the apparatus. When the apparatus is almost full, the outlet plug (f) is removed and the water containing the nematodes is drawn off and transferred to four stacked 20-cm-diameter sieves (one 90-µm-, two 53-µm- and one 45-µm-aperture sieve) (g). The outlet pipe (f) is regulated with a clamp to prevent excessive water flow clogging the sieves. The nematodes and the fine soil particles are immediately washed with distilled water from the sieves into beakers and then transferred to paper tissue on a sieve placed in a Petri dish and filled with distilled water. After 24–48 h, the nematodes in the Petri dish can be counted and identified. Alternatively, the suspension of nematodes and small soil particles may be subjected to the centrifugal flotation method.

4.4.1.7 Seinhorst's Cyst Elutriator Method

Cysts of the Heteroderidae can also be extracted from both wet and dry soil samples using an elutriator (Seinhorst 1964). This apparatus consists of a cylinder with a bowl at the top (Fig. 4.8). Below the rim of the bowl is a sloping collar with an

upright rim (a). The collar tapers towards an outlet (b), and the cylinder is closed with a rubber plug at the bottom (e). A water inlet (d), which is supplied from a constant-flowing current from a header tank, is present at the bottom of the cylinder. A lower outlet is situated in the lower half of the cylinder (c). After the cylinder and lower outlet are closed with the rubber plug, the stopcock of the header tank is opened to the desired setting (3.5 l min^{-1}). As water rises in the cylinder, a 500 ml soil sample is washed through a 2-mm-aperture sieve into the bowl. Water that spills over the collar is collected in two stacked sieves (840- and 250-µm-aperture). After approximately 30 s, the lower outlet is opened and the residue collected on the bucket sieves. The bowl and cylinder are then rinsed until the water in the cylinder is clear. The content of the 840-µm-aperture sieve is washed gently, but thoroughly, onto the 250-µm-aperture sieve. The residue on the latter sieve is then transferred to filter paper that lines a funnel and is left to dry. The dried debris containing the cysts is ready to be examined and the cysts counted using a dissecting microscope.

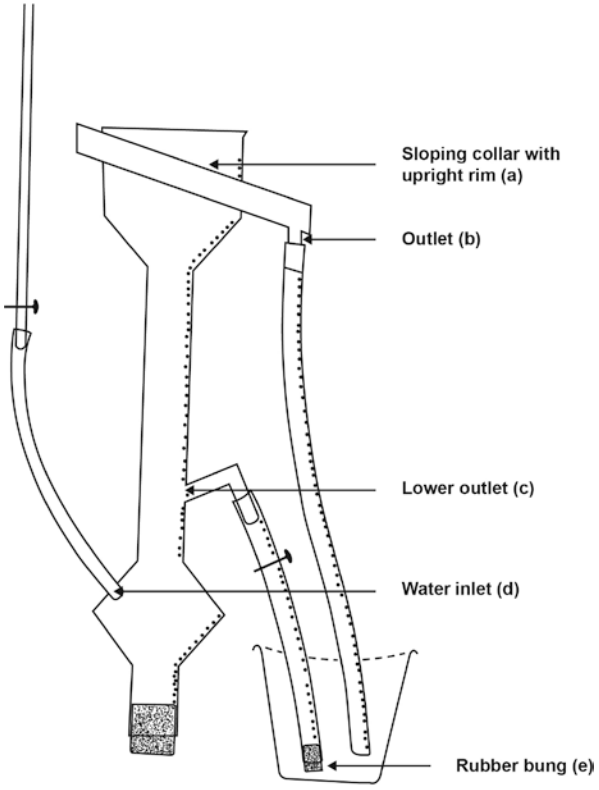


Fig. 4.8 Seinhorst's elutriator apparatus used for the extraction of cysts from soil; sloping collar with upright rim (**a**), outlet (**b**), lower outlet (**c**), water inlet (**d**) and rubber bung (**e**) (Redrawn by Hannes Visagie, North-West University, Potchefstroom, South Africa)

4.4.1.8 Alternative to Seinhorst's Cyst Elutriator Method

With this alternative method cysts of the Heteroderidae can be extracted from both wet and dry soil, but better results are obtained with dry soil (Seinhorst 1964).

A large glass trough, about 19-cm-diameter, is lined with a 7×60 cm strip of blotting paper. Tap water is added to the trough until the lower half of the paper is submerged. A known volume or mass of air-dried soil, e.g. 50 ml or 50 g, is crumbled and submerged in the water. The heavier soil particles will settle at the bottom of the trough whereas some light debris and the cysts will float. After stirring the suspension, the cysts adhere to the blotting paper. To separate the cysts from the suspended particles, the level of the suspension has to be raised momentarily. To achieve this, a large conical flask, half-filled with water, is slowly pushed down into the container and after about 15 s pulled out of the water. Adhering particles are washed into the container with a wash bottle. The water in the trough is siphoned off, and the paper strip with adhering cysts and debris is removed and laid flat on a strip of Perspex. The cysts are then separated from the debris with a needle using a dissecting microscope. The cysts are collected in a dish with a wet camel-hair brush. This method is illustrated in Kleynhans (1997).

4.4.1.9 LUDOX® Centrifugal Flotation Method for Extracting Nematodes from Sediment

As mentioned above (see Sect. 4.4.1.2), a sugar solution is very viscous and a NaCl solution causes osmotic stress which can severely damage the nematodes. As an alternative LUDOX®, a colloidal silica solution with low viscosity and osmolarity, can be used to create the required specific gravity solution. For a single extraction an efficacy rate of 70 % in terms of nematode recovery can be achieved. However, if the procedure is repeated three or more times, between 95 and 100 % of the nematodes can be extracted from a sample (Hodda and Eyualem-Abebe 2006).

A commonly used flotation method for the extraction of freshwater nematodes from sediment, which can also be used to extract nematodes from soil, is as follows: The sample is washed with distilled water into centrifuge tubes up to one-third of the total volume. Each tube is then filled with an equal amount of water to balance the centrifuge. If necessary, kaolin (see Sect. 4.4.2.3) is added to separate and settle the sediment from the organic material fraction. The tube is then shaken manually or using a mechanical device and centrifuged at 400 *g* for 5 min. After discarding the supernatant, the tube is filled with a LUDOX® solution (with a specific gravity of 1.15 g cm^{-3}) and the content resuspended. After centrifugation at 300 *g* for 5 min, the supernatant (now containing the nematodes) is decanted through a 38-µm-aperture sieve and rinsed well with distilled water. The content of the sieve is finally washed with distilled water into a beaker.

4.4.1.10 Isolation of Entomopathogenic Nematodes from Soil Samples: Insect-Baiting Technique

The same principles for the sampling and handling of plant-parasitic nematodes are used for the sampling of entomopathogenic nematodes (EPN) belonging to the Steinernematidae and Heterorhabditidae families. Individuals are recovered from soil samples by using an insect-baiting technique (Bedding and Akhurst 1975). The soil sample is placed in 250 ml plastic containers. Any suitable insect can be used as a trapping host, but in general 5 larvae of either the greater wax moth (*Galleria mellonella*) or the mealworm (*Tenebrio molitor*) are placed on the soil surface of each container, which is then closed (Fig. 4.9a). The two trapping hosts are added together or the one followed by the other, depending on their availability. During a 7-day period, for wax moth, and a 14-day period, for mealworm, the samples are periodically checked for the presence of dead insects. Dead, non-putrefied insects are washed with water to remove all surface scavenger nematodes and especially mites.

Insect cadavers that may be infected with EPN are washed and placed on a modified White trap (Figs 4.9b, c) (Kaya and Stock 1997). Nematodes are harvested within the first week of emergence. Nematode isolates are maintained in 150 ml of filtered tap water in vented culture flasks, which are kept horizontal at 14 °C and shaken weekly. Infective juveniles (IJ) are maintained by recycling through wax moth larvae within 3 months for heterorhabditids and within 6 months for steinernematids (Nguyen and Hunt 2007).

4.4.2 Extraction of Nematodes from Roots

4.4.2.1 Modified Baermann Funnel and Baermann Tray Methods

These methods are the same as used for the extraction of nematodes from soil samples (see Sect. 4.4.1.5) (Hooper and Evans 1993). Instead of soil, a small sample of plant tissues, e.g. 5 g roots, is placed on a paper tissue that is placed on a 2-mm-aperture sieve. The same procedures as described in Sects. 4.4.1.4 and 4.4.1.5 are followed from this step onwards. Plant material can also be first macerated in 100 ml

Fig. 4.9 Insect larvae of *Tenebrio molitor* used as bait to recover entomopathogenic nematode infective-stage juveniles (IJ) from soil (**a**), a modified White trap consisting of a small Petri dish covered with a filter paper and placed in a larger Petri dish partly filled with water (**b**) and IJ emerging from a wax moth larva on the moistened filter paper (**c**) (Kirk West, Port Elizabeth, South Africa)

of tap water in a kitchen blender for 30 s. The suspension containing the nematodes is then gently poured onto the paper tissue.

4.4.2.2 Modified Mistifier Method

This is a popular method to extract active nematodes from plant tissues. It was originally described by Seinhorst (1950). In principle it is another version of the Baermann funnel method, but in this method the funnels are placed in a chamber wherein a fine mist of water is sprayed over the plant tissue. The fine mist is produced by nozzles similar to those used in the irrigation systems of glasshouses. Debris (soil and plant material) is washed off the plant tissue and allows the extraction of nematodes in clean water. The water also remains well oxygenated which is a major advantage over the Baermann funnel method if used for 48 h or longer. The incubation time can be chosen, e.g. should 24-h-old second-stage juveniles (J2) of root-knot nematodes be needed for a specific experiment, they can be collected at the appropriate time. The advantages of this method outweigh the disadvantages. For example, extraction of nematodes can be done over a prolonged period which allows *Meloidogyne* J2 to hatch from the eggs, a high extraction efficiency is obtained and nematodes are usually in a better condition compared to those extracted using other methods. However, this method is time consuming and uses high volumes of water, although this can be reduced by regulating spray-time intervals. The use of a water softener is advised in areas where the water has a high mineral content. A version of the mistifier apparatus that is used at the Nematology Laboratory of North-West University is shown in Fig. 4.10.

4.4.2.3 Centrifugation

Nematodes can also be extracted from plant tissues using an adapted sugar flotation method (Coolen and D'Herde 1972; Hooper et al. 2005).

The Importance of Kaolin

Kaolin is a clay mineral with a specific gravity of 2.6 g cm^{-3} and consists of particles that range from 2–3 μm in size (Coolen and D'Herde 1972). Although the specific gravity of kaolin is higher than that of nematodes, kaolin particles are small and flat and therefore sink to the bottom of a centrifuge tube more slowly than nematodes. This way kaolin spreads out to form a layer over the loose sediment on the bottom of the centrifuge tubes and seals it off when the supernatant is decanted. When the sucrose solution is added to the sediment the mixture must be stirred again thoroughly to break the kaolin layer. Another advantage of kaolin is that it also precipitates during the sugar centrifugation, thus preventing the remixing of the sedimented debris when the sugar solution with the nematodes in suspension is decanted. Thus, a more clear suspension of nematodes can be obtained.

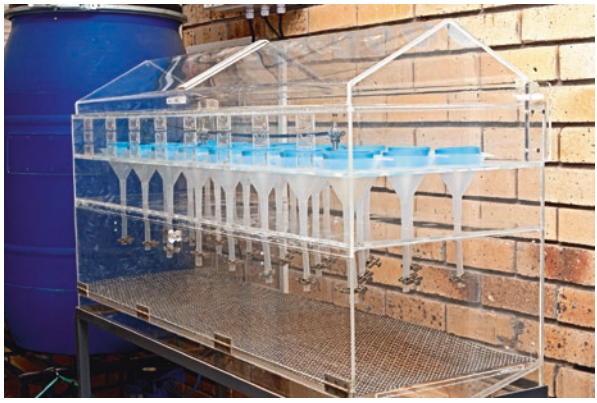


Fig. 4.10 Mistifier apparatus used to extract nematodes from soil and plant tissues (Kirk West, Port Elizabeth, South Africa)

An Adapted Sugar Centrifugal Flotation Method

This method has been adapted from those described by De Waele et al. (1987) and Kleynhans (1997). A plant tissue sample, not less than 5 g, is cut into 2–5-mm pieces. It is then macerated in 250 ml tap water at medium speed in a kitchen blender for 30–45 s to release the nematodes from the tissue. The water suspension, containing the nematodes and root fragments, is decanted through a 1-mm-aperture sieve that is stacked on 150-, 45-, 38- and 25-µm-aperture sieves. The use of several sieves prevents clogging of the 25-µm-aperture sieve. The root pieces on the 1-mm-aperture sieve are washed thoroughly with tap water and discarded. The residue on the stacked sieves is collected and decanted into centrifuge tubes. Kaolin is added to each of the tubes, the content of the tubes stirred well and the tubes centrifuged at 3,484 g for 7 min. The supernatant that contains mainly debris is decanted and a sucrose solution with a specific gravity of 1.15 g cm^{-3} added to each tube. The content of the tubes, containing the nematodes, is stirred well and again centrifuged at 3,484 g for 3 min. The supernatant, containing the nematodes, is decanted through a 25-µm-aperture sieve and rinsed well with distilled water to remove the sucrose solution. The residue on the sieve, containing the nematodes, is washed with distilled water in a beaker for counting and identification.

4.4.2.4 Extraction of Eggs and Second-Stage Juveniles of Sedentary Endoparasitic Nematodes from Plant Tissue

Eggs and J2 from nematodes that produce gelatinous egg masses (e.g. *Meloidogyne* spp., *Rotylenchulus* spp., *Tylenchulus semipenetrans* Cobb, 1913) can be extracted from roots of infected plants using the NaOCl method. This method was originally described by McClure et al. (1973), is used routinely in nematology laboratories across the globe and has been adapted by Riekert (1995). It is

important to note that there is no known method, except molecular analyses (Bekker et al. 2016), to distinguish between the eggs of different plant-parasitic nematode genera or species.

Riekert's Adapted NaOCl Method

A known mass of roots, e.g. 50 g, is taken from the root system of an infected plant, cut into 10-mm pieces and mixed thoroughly. The root sample is shaken thoroughly for 4 min in 400 ml of a 1 % NaOCl solution. The bleach solution breaks down the gelatinous matrix surrounding the eggs. The solution, containing the eggs and J2, is decanted through a range of stacked sieves, from top to bottom: a 720-, 75-, 25- and 20-µm-aperture sieve. This procedure ensures less clogging on the bottom of the 20-µm-aperture sieve. A vacuum pump is connected to the 20-µm-aperture sieve and suction is applied. This enhances the passing of the suspension containing the eggs and J2 through the sieves. Root fragments are washed thoroughly through the stacked sieves for about 4 min with tap water before the eggs and J2 are collected on the 20-µm-aperture sieve. Eggs and J2 are finally washed with distilled water in a beaker for identification and counting. The set-up of the sieves and vacuum pump are illustrated in Fig. 4.11.

4.4.2.5 Extraction of Nematodes from Groundnut Pods

Bolton et al. (1990) published the following protocol for the extraction of *D. africanus* of all developmental stages from groundnut hulls and seeds. A 5 g subsample of the hulls and the seeds are broken by hand or cut with scissors into smaller pieces of about 3-mm-in-diameter. The hull and seeds tissues are then soaked for 24 h at room temperature in a Petri dish containing 20 ml of distilled water. This allows ample time for the nematodes to move out of the hull and seed tissues into the water. The content of the Petri dish is then washed through a 750-µm-aperture sieve, which is stacked on a 25-µm-aperture sieve. The nematodes retained on the 25-µm-aperture sieve are washed with distilled water in a beaker for counting. The same protocol is also followed for the extraction of nematodes from grass seeds and other aerial plant parts (Bekker 2009).

4.4.2.6 Extracting Nematodes from Onion Seed

An adapted method to extract nematodes from onion seeds was described by Southey (1965). Onion seeds are mixed, weighed and a subsample of 14 g soaked in distilled water for 48 h at room temperature in a Baermann tray (see Sect. 4.4.2.1). The water in the tray is then poured off in a beaker and allowed to stand for at least

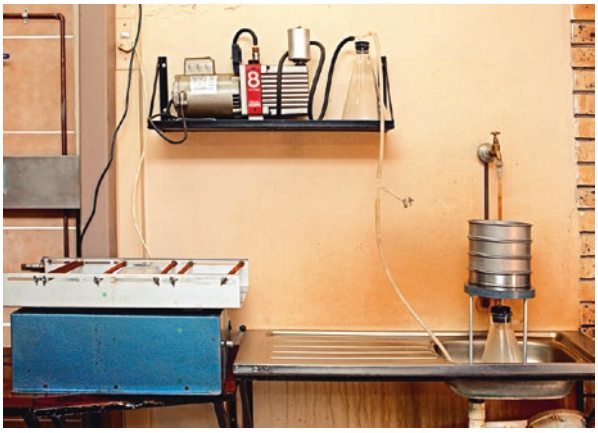


Fig. 4.11 Stacked sieves, vacuum pump and shaker set-up for the extraction of eggs and second-stage juveniles (J2) of sedentary endoparasitic nematodes that deposit their eggs in gelatinous egg mass (Kirk West, Port Elizabeth, South Africa)

1 h, thereby allowing the nematodes to sink to the bottom of the beaker. The bottom 10 ml suspension, containing the nematodes, of each beaker is removed using a pipette and examined for the presence of nematodes.

4.4.2.7 Extraction of Nematodes from Carrot Tissue

This method was developed by the Nematology Unit, ARC-PPR (Marais and Shubane 2012). Carrot roots are washed clean of soil and peeled to a depth of about 2 mm using a vegetable peeler. The peeled strips, and if present the secondary roots, are cut into 2-mm pieces, thoroughly mixed and a 100 g subsample then macerated in water for 40 s in a kitchen blender. The macerated carrot tissues and the nematodes are washed into a 250 ml bottle and 10 ml of a 1 % NaOCl solution is added before the bottle is closed with a lid through which an aquarium tube is threaded. The suspension is aerated for 72 h and then washed through a stack of 1,000-, 150-, 45- and 38-µm-aperture sieves. The residue on the top sieve is discarded, and the residues on the other sieves are washed with distilled water into a centrifuge tube and processed by the sugar centrifugal flotation method (see Sect. 4.4.1.2). This technique is also used to extract nematodes from beetroot, groundnut and potato tissues.

4.4.2.8 Extraction of Nematodes from Wood

Swart (1997) developed a method whereby standing trees may be sampled by a forestry pressler borer or, in the case of dead or dying trees, by felling and taking stem discs at various levels. Imported coniferous wood should be randomly sampled using a low-speed drill, borer or saw. However, preference must be given to select

wood pieces with grub holes or fungal growth, especially blue stained wood, which is a typical symptom of *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle, 1970 infection. The presence of flat-headed larval stages of the *Monochamus* beetles or pupae in galleries with an oval diameter and galleries sometimes blocked with wood particles may also indicate the presence of *B. xylophilus*. The emergence holes of the *Monochamus* beetles are round. Live nematodes can be extracted from infected wood using a Baermann tray (see Sect. 4.4.2.1).

4.4.2.9 Assessing Numbers of Citrus Nematode Females in Roots

This method was adapted from the method used by the Diagnostic Centre of Citrus Research International in Mbombela, Mpumalanga Province (Laura Huisman 2010, Mbombela, personal communication). Citrus roots shaken free from soil are cut into approximately 1-cm pieces, suspended in 150–250 ml of water and macerated at a low speed for 20 s using a kitchen blender. The macerated suspension is then decanted through stacked sieves with apertures of 1,000, 150 and 38 µm. The residue on the top sieve is washed using a gentle stream of water. *Tylenchulus semipenetrans* Cobb, 1913 females are collected on the 38-µm-aperture sieve and washed with distilled water into a beaker for identification and counting.

Alternatively, a staining solution containing 10 ml of a 1 % acid fuchsin solution, 10 ml of a 1 % orcein solution, 200 ml distilled water, 188 ml phenol, 165 ml lactic acid and 318 ml glycerol is prepared. The nematodes collected on the 38-µm-aperture sieve (as described above) are washed through another 38-µm-aperture sieve, using a 2 % acetic acid solution. The sieve is then submerged into the staining solution for 60 min. Thereafter, the root tissues are rinsed with tap water and washed into a beaker to which a few drops of 5 % acetic acid are added. The stained citrus nematode females are visible and can be counted using a dissecting microscope.

4.5 Counting of Nematodes

After nematodes have been extracted from soil or plant tissues, they can either be counted and identified to genus level using a counting dish or counting slide or fixed prior to mounting on microscope slides for species identification. Counting and identification of live nematodes is usually more effective in terms of time saved by not fixing and mounting the specimens. Also, the characteristic movement of certain nematode species may aid in their identification. Substantial practice and experience are required to identify nematodes to genus level with a dissecting microscope. The task of counting a large number of nematodes present in one sample can be eased by counting several subsamples (Kleynhans 1997).

De Grisse (1963) designed a counting dish that is used when counting the number of nematodes extracted from soil or plant material. The process generally followed to count nematodes is as follows: a 10 ml subsample taken from a 100 ml

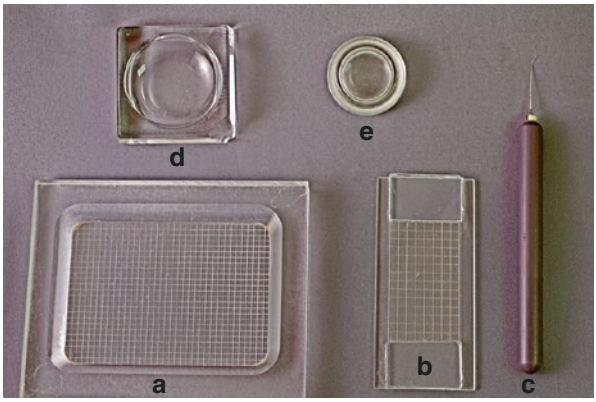


Fig. 4.12 A modified version of a De Grisse counting dish (**a**) and a Peters counting slide (**b**), fishing needle (**c**), block glass dish (**d**) and Syracuse dish (**e**) used for handling, counting, fixing or staining nematodes for identification purposes (Kirk West, Port Elizabeth, South Africa)

nematode suspension is transferred to a De Grisse counting dish (Fig. 4.12a) using a pipette. A dissecting microscope, fitted with a transmitted light source, is used to identify and count the nematodes to genus level. The subsample is returned to the nematode suspension, the suspension stirred well and another subsample taken to repeat the counting procedure The same process described above are also used to count nematodes with a Peter's slide (Fig. 4.12b), although only a 1ml subsample is used for such purposes.

4.6 Handling of Nematodes

Because of their small size, nematodes are handled in a fluid medium using a dissecting microscope. For individual specimens a handling tool is used such as a sharpened bamboo splinter, a mounted eyebrow hair, a No. 0 insect mounting pin with a re-curved tip, the fine tip of a 10 µl glass pipette or a tailor-made fishing needle (Fig. 4.12c), etc. Once picked up with the handling tool, the individual specimen is transferred to a drop of distilled water on a glass slide or in a small Syracuse dish (Fig. 4.12d) or watch glass (Fig. 4.12e) containing distilled water.

4.7 Killing, Fixing and Mounting of Nematodes

Fixation kills and hardens animal tissues and preserves the cellular structure of organisms. Nematode specimens should be fixed immediately after killing, or killed and fixed simultaneously (Kleynhans 1997). Proper methods of killing, fixing and mounting nematodes are essential to ensure that specimens remain in good condition for many years.

4.7.1 Preparation of Vermiform Nematodes for Temporary Mounting

During routine identification of vermiform nematodes, it is often necessary to make temporary slides. After the nematodes have been picked up from the liquid in which they were suspended for inspection and counting, they are transferred to a glass microscope slide with a drop of liquid and mounted by applying a coverslip onto the drop. The specimens can then be examined for their finer morphological features using a compound microscope.

4.7.2 Preparation of Vermiform Nematodes for Permanent Mounting

4.7.2.1 Killing and Fixing

One method to kill nematodes is to heat them in water in a small Syracuse dish over a spirit flame. Care must be taken not to boil the water. Specimens should be checked frequently and heating stopped as soon as the specimens stop moving. Most of the water in the dish is then pipetted off cautiously and the specimens are mounted on glass slides for identification. Alternatively, nematodes can be killed and fixed simultaneously by adding an equal volume of a boiling (at approximately 90 °C) 8 % formaldehyde solution. Because of the dilution, the final concentration of the formaldehyde will be 4 %. Another fixative, containing 10 ml of a 40 % formaldehyde solution, 1 ml glacial acetic acid and 2 ml glycerol, made up to 100 ml with distilled water, can be heated at 90–100 °C and used to kill and fix nematodes simultaneously.

To study their finer morphological structures (Box 4.2) individual nematodes can be mounted in a number of media. Glycerol is a medium with a refractive index that is nearly the same as that of glass. However, if live or fixed nematodes are suddenly placed in pure glycerol, they will plasmolyse. Therefore, the transfer of nematodes to glycerol must be done gradually. The Syracuse dish with the nematodes suspended in water should be partly covered and kept for 2–3 weeks at room temperature to allow the fixative (that contains a small amount of glycerol) to slowly evaporate, leaving the nematodes in pure glycerol. The Syracuse dish with the nematodes is then stored in a desiccator, with Calcium chloride as the desiccant, until the specimens can be mounted (Kleynhans 1997).

Another method, an adaptation of the method originally described by De Grisse (1965), can also be used to kill and fix vermiform nematodes. The nematodes are placed in distilled water in a small glass dish with a volume of 5 ml. Most of the

Box 4.2 Morphometric and Morphological Characters Commonly Used in Nematode Systematics

Symbols and Abbreviations:	Habitus	Width of annules at excretory pore and at mid-body
L = Total body length	Width of amphid aperture	Width of lateral field at mid-body and number of lines in lateral field
a = Body length divided (/) by body width	Lip height and lip width	Lateral field width/body width × 100
b = Body length/ oesophageal length	Basal ring width	Position of phasmid/scutellum
b' = Body length/ anterior end to posterior end of oesophageal gland	Stylet/ondontostyle/onchiostyle length	Diameter of phasmids/ scutellum
c = Body length/tail length	Length of odontophore	Tail length
c' = Tail length/body width at anus or cloaca	Telenchium and metenchium length in Tylenchida	Length of peg on tail terminus
o = Distance of dorsal oesophageal gland opening from stylet knobs/stylet length × 100	Stylet knob height and width	Number of ventral annules on tail
m = Length of conus/ stylet length × 100	Position of dorsal gland opening behind stylet knobs	Position of vulva
V = Distance of vulva from anterior end/ length of the body × 100	Position of guiding ring	Length of vagina
OV_1 = Length of anterior gonad/body length × 100	Position of median bulb	Length of anterior and posterior genital branches in females
OV_2 = Length of posterior gonad/body length × 100	Length and width of median bulb	Position and form of spermatheca
T = Length of testis/ body length × 100	Length and width of median bulb valve	Diameter of sperm in spermatheca
h = Hyaline length on tail	Length of oesophagus	Length of testis
	Position of excretory pore	Spicule length
	Position of hemizonid and hemizonion	Gubernaculum length
	Position of dorsal gland nuclei	
	Body width at excretory pore, mid-body, vulva and anus or cloaca	

water in which the nematodes are suspended is carefully removed with a pipette. A solution containing 100 ml of 40 % formaldehyde, 10 ml propionic acid, 890 ml distilled water and a pinch of picric acid (to stain the solution citrus yellow) (FPG) is prepared and heated in a water bath to 60–70 °C. The hot FPG fixative is added to the glass dish containing the nematodes and placed in a Petri dish with a closed lid, which is then placed in a desiccator with an atmosphere saturated with the FPG solution. The latter is obtained by adding FPG to the bottom of the desiccator. The desiccator is placed in an oven at 38–40 °C for at least 3 days, but this period could be extended to 3 months. After this time, the lid of the Petri dish is removed and half of the FPG is carefully withdrawn with a pipette from the glass dish in which the nematodes are suspended. A small amount of a Solution 1 that contains 200 ml of a 95 % ethanol solution, 10 ml glycerol and 790 ml distilled water is then added to the glass dish. Without replacing the cover lid, the Petri dish with the nematodes is returned to the desiccator for 12 h. A 95 % ethanol solution is then added to the bottom of the desiccator. The desiccator is returned to the oven during this step for 12 h after which half of Solution 1 is carefully removed from the glass dish and replaced with Solution 2. The latter solution contains 950 ml of a 95 % ethanol solution and 50 ml glycerol. After this step, the Petri dish is partially covered with the lid to ensure that evaporation occurs slowly. The desiccator is returned to an incubator at 38–40 °C for 2–3 days or until all the ethanol had evaporated. The Petri dish containing the glass dish is then removed from the incubator and the glass dish with the nematodes placed in a desiccator with silica on the bottom for another 2 days. After that time, the nematodes are ready to be mounted on microscope slides.

A method to prepare nematodes for permanent mounting involves the transfer of individual specimens from a fixative solution to a small glass dish containing 0.5 ml of Seinhorst's first solution (20 parts of 96 % ethanol, one part glycerol and 79 parts of distilled water) (Seinhorst 1959). The glass dish with the nematodes and the fixative is then placed in a closed glass vessel containing an excess (e.g. 1/10 volume of the vessel) of a 96 % ethanol solution and left in this saturated atmosphere for at least 12 h at 35–40 °C. The evaporation of the water results in the nematodes becoming suspended in a mixture of glycerol and ethanol. The glycerol-ethanol solution is then carefully removed using a pipette. The container is filled with Seinhorst's second solution, which contains five parts glycerol and 95 parts of 96 % ethanol, and placed in a partially covered Petri dish in an oven at 40 °C until the ethanol has evaporated. The duration for the latter step is at least 3 h. After this time the nematodes are suspended in pure glycerol and are ready to be mounted on slides. Using this method, nematodes can be preserved and mounted permanently. If shrinkage of the nematode specimens occurs after the first incubation, more water should be added and allowed to evaporate slowly until a 98 % ethanol solution remains. If shrinkage of nematodes occurs after the second incubation, the process should be repeated from the very beginning.

4.7.2.2 Mounting of Vermiform Nematodes

Melted paraffin wax is mixed with liquid paraffin to prevent the solid wax from becoming brittle. The end of a 7-cm-long copper tube with an internal diameter of 10 mm and wall thickness of 1 mm is heated for a few seconds in the flame of a spirit lamp and then pressed into the solid wax to a depth of approximately 2 mm. The wax will melt and cling to the tip of the copper pipe which is then pressed onto a glass slide to produce a wax ring which will cool down and solidify rapidly. A small drop of distilled water for temporary slides, 5 % formaldehyde for semi-permanent slides or glycerol for permanent slides is added in the middle of the ring and the fixed nematodes placed therein using a fine handling tool such as a fishing needle. A circular or rectangular cover slip is placed on the wax ring and the glass slide is then heated from below on a warm plate at 50 °C or by using a Bunsen burner flame to melt the wax. Alternatively a small drop of glycerol can be added to the centre of a microscope slide and several small fibreglass rods arranged around the edges of the glycerol drop. The rods must match the nematodes in thickness. Several fixed nematode specimens are then placed in the glycerol. A cover slip is heated over a spirit flame and is gently lowered onto the glycerol drop and fixed at three points with glyceel or clear nail varnish. The coverslip is finally sealed with glyceel or clear nail varnish and the specimens are ready for identification (Kleynhans 1997).

4.7.3 Preparation of Swollen Nematodes

4.7.3.1 Temporary Mounting of Nematode Cysts

The method described by Turner (1998) can be used for the temporary mounting of nematode cysts. The cysts are soaked in tap water for 24 h at room temperature. Each cyst is then transferred to a drop of water mixed with pure glycerol. The posterior end of the cyst is cut off with a scalpel and the body contents removed with a dissecting needle without damaging the structures in the vicinity of the vulva. The cuticle around the vulval area is also trimmed away using a scalpel. The cuticle piece, containing the vulval cone is then transferred to a drop of pure glycerol that is placed on a glass slide with the outer surface facing upwards. The cuticle piece is gently pressed down onto the surface of the slide and a cover slip lowered onto the drop of glycerol, which is heated gently over a spirit flame. Glass rods are used as cover slip supports to prevent the vulval cone being flattened and damaged.

4.7.3.2 Permanent Mounting of Perineal Patterns and Anterior Ends of Root-Knot Nematode Females

Methods for the preparation and mounting of perineal patterns and anterior ends of mature root-knot nematode females for identification have been described by various authors including Kleynhans (1991, 1997). Plant material infected with

root-knot nematodes is washed gently with tap water to remove adhering soil and debris. The plant material is then placed for approximately 3 min in a fuming (not boiling) lactophenol solution that contains 20 ml liquid phenol, 20 ml lactic acid, 40 ml glycerol and 20 ml distilled water. Inhalation of phenol fumes is dangerous and should be avoided by conducting this activity in a fume hood. Otherwise, the phenol can be replaced with glycerol. After removing the plant material from the fuming lactophenol solution, it is transferred to a cold, clear solution of lactophenol (or glycerol). Mature root-knot nematode females are removed from the plant material using a scalpel (Fig. 4.13a) and stored in clear lactophenol or glycerol in labelled vials until mounting.

To mount the females they are first incubated with lactophenol in an oven at 40 °C for 4 days. One by one the root-knot nematode females are then transferred to a small drop of 100 % lactic acid in a plastic Petri dish. The body is punctured using a sharp dissecting or insect needle (Fig. 4.13b) and cut in half just above mid-body using a ocular scalpel or insect needle. The anterior half of the body is transferred to a small drop of glycerol on a glass microscope slide. The body contents spilling out from the posterior half of the female body are removed with an insect or dissecting needle. The anterior half of the body is transferred to a small drop of glycerol on a glass microscope slide, and the cuticle around the perineal pattern is trimmed to a neat square using an ocular scalpel or insect needle. This cuticle piece, containing the perineal pattern, is transferred to the drop of glycerol in which the anterior part

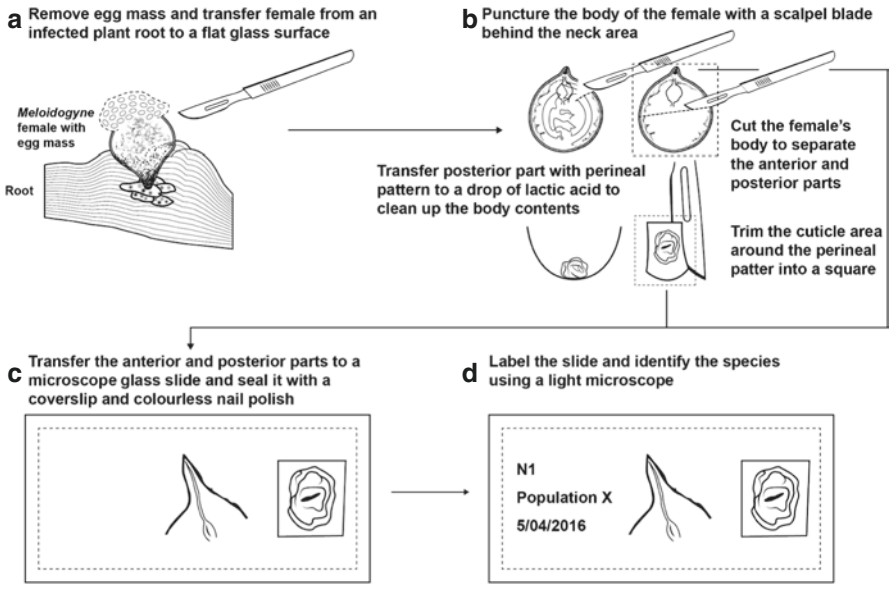


Fig. 4.13 Procedure used for mounting the perineal and anterior ends of a mature root-knot nematode females: the female is removed from a root fragment (**a**), the body is punctured, cut in half and the body content gently removed (**b**), the cuticle around the perineal pattern is trimmed (**c**) and the oesophageal and perineal pattern of the females are mounted in glycerol on glass microscope slides (**d**) (Hannes Visagie, North-West University, Potchefstroom, South Africa)

of the body has been placed (Fig. 4.13c). The cuticle piece is carefully pressed down to ensure that the outer surface of the cuticle is facing upwards. The anterior and posterior parts of several individuals are mounted on the same slide, covered with a cover slip and sealed with either glyceel or clear nail varnish (Fig. 4.13c-d). Care must be taken when using lactic acid as it is corrosive.

4.7.3.3 Permanent Mounting of *Globodera* Cysts

The procedure described below has been adapted from Kleynhans (1997). Dried cysts are soaked in distilled water for 24 h, transferred to a fuming (not boiling) lactophenol solution and then to 100 % lactic acid in a cavity glass slide. While suspended in the lactic acid, the posterior end of the cyst is cut off with a scalpel and the body contents removed. These handlings should be carried out without damaging the structures that are situated in the vicinity of the vulva and anus (the so-called terminal pattern) that are crucial for species identification. The cuticle around the terminal pattern is trimmed away and the piece of cuticle that contains the terminal pattern is first transferred to a drop of distilled water, then to a drop of a 96 % ethanol and finally to a drop of xylene for clearing. From the xylene, the cuticle piece is finally transferred to a drop of gently heated glycerol jelly that is placed in the middle of a glass slide. The cuticle piece is positioned while the jelly is still liquefied in such a way that the outer surface faces upwards. As soon as the glycerol jelly has solidified, a heated cover slip is applied and sealed with glyceel or clear nail varnish.

4.7.3.4 Permanent Mounting of *Heterodera* Cysts

The preparation of the vulval cone (with the vulva, fenestra and associated structures) for mounting in glycerol jelly is the same as for *Globodera* cysts (see Sect. 4.7.3.3), except that the cover slip needs to be supported (e.g. using glass slivers from a broken cover slip) so that the vulval cone is not crushed. As soon as the glycerol jelly has solidified, a heated cover slip is placed over the vulval cone and sealed with glyceel or clear nail varnish. Various morphometric and morphological characters are then measured and observed to identify the *Heterodera* species. Characters that are commonly used in nematode systematics are listed in Box 4.2.

4.8 Preparation of Nematodes for Histological Studies

4.8.1 Transmission Electron Microscopy

The method described below for preparing nematodes for transmission electron microscopy (TEM) studies has been described by Fourie et al. (2013) and has been modified for the preparation of root-knot nematode-infected soybean roots. Infected

plant tissues are first inspected using a dissecting microscope to identify swollen areas on the roots where various developing stages of root-knot nematodes (all juvenile stages and females) are situated. Infected areas are then cut into approximately 5-mm-sections and fixed in Todd's fixative (pH 7.5) in 20 ml glass flasks at room temperature for 12 h (Todd 1986). The flasks are sealed with perforated plastic plugs and placed in a vacuum for approximately 2 min to remove all air trapped inside the root tissues. From this point onwards the fixation and infiltration methods of Murphy et al. (1974) and Spurr (1969) are used. The nematode-infected sections are washed three times for 15 min in a 0.05 M sodium cacodylate buffer (pH 7.4), post-fixed in 1 % osmium tetroxide for 1 h and washed three times in distilled water. The root pieces are subsequently dehydrated in an acetone series of 50, 70, 90, 100 and again 100 % acetone, each for a 15 min period. The sections are then infiltrated with a 1:1 mixture of acetone and resin and left for 3 h during which the root sections sink to the bottom of the flasks. The 1:1 acetone-resin mixture is replaced with a freshly mixed resin solution (100 %) and the root sections transferred to it for 5–12 h, again allowing the sections to sink to the bottom of the flasks. The root sections are subsequently placed in the acetone:resin mixture for 2 h, followed by embedding of each root section in resin (100 %) in a flat mould. Moulds containing the root sections are then cured in an oven at 65 °C for 8–12 h to allow the resin to harden. Embedded resin blocks containing the root-knot nematode-infected root sections are inspected using a dissecting microscope and excessive resin removed with sandpaper to position the sections containing the root-knot nematodes for optimal sectioning. The root sections embedded in resin are sectioned transversely using an Ultracut Microtome (±100 nm) and fixed on glass slides. Fixed sections are stained with toluidine blue (Reynolds 1963) for light microscope observations, and with 2 % uranyl acetate and lead citrate (pH 12) for TEM observations. Slides containing the fixed root sections are examined and photographed using a light microscope at 80, 100, 200, 400 and 1000× magnifications. Root sections are examined with TEM using a Philips CM 10 at 80 kv at a range of 2,200–21,000× magnification. The same procedure as described above can be used for preparing individual nematodes, not contained within plant tissues, for examining changes at the ultrastructural level. A detailed account of the use of TEM to study nematodes is given by Carta (1991).

4.8.2 Scanning Electron Microscopy

Live individual nematodes to be prepared for scanning electron microscopy (SEM) are transferred into tap water in a small Syracuse dish. The dish containing the nematodes is heated over a spirit flame until all specimens stop moving. The dish is left to cool down completely after which most of the water is drawn off with a syringe. TAF fixative is poured over the specimens and they are left at room temperature for a week. The specimens are then transferred to a range of ethanol solutions viz. 70, 80, 90 and 96 % at 3 h intervals. Rinsing in 96 % ethanol is repeated three times. The specimens are

then critical-point dried using liquid carbon dioxide and stuck up against a short hair stuck on copper foil conductive tape on a SEM viewing stub. The stub containing the nematodes is then sputter-coated with gold (66%) palladium (33%) pieces ranging 21–25 nm in size (Minagawa 1986). Mounted nematode specimens can also be prepared for SEM investigations. This is done by removing the specimens from the slides, rehydration of the specimens in a graded series of glycerol-thinning media and finally suspension in distilled water. The thinning medium consists of 30% absolute ethanol mixed with distilled water. The percentage glycerol in the thinning medium in the graded series is 85, 65, 45, 25, 5 and 0%. The various thinning medium concentrations are replaced at 15–30 min intervals. Rinsing of the nematode specimens during the last step of this series with a 70% ethanol solution is repeated three times. The nematodes are then hydrated by placing the specimens for 15–30 min in a 15% ethanol solution, followed by a 5% ethanol solution and then distilled water. Rinsing of the nematodes in distilled water is repeated three times. The specimens are then transferred to a capsule containing a 30% ethanol solution and transferred to a 50, 75, 95 and 100% ethanol solution for 20 min each. The last step is repeated twice. The specimens are then critical-point dried and coated with gold palladium as described above.

The external (e.g. cuticular pattern of body and vulval regions) and internal (e.g. bullae and underbridge) structures of *Globodera* and *Heterodera* cysts can also be studied with SEM (Lax and Doucet 2002). Other procedures for the preparation of nematodes for SEM studies have been described by Eisenback (1985) and Hooper (1998).

4.8.3 Interference Microscopy

Roots, pegs, hulls and seeds of groundnut plants infected with *D. africanus* have been studied by Jones and De Waele (1990) to establish the time and mode of entry of these nematodes. Infected plant tissue sections are fixed in FAA (20 ml 96% ethanol, 6 ml formaldehyde, 1 ml glacial acetic acid and 40 ml distilled water) and dehydrated in a series of alcohol solutions. The sections are transferred to propanol and butanol and embedded in paraplast. Transverse and longitudinal sections (12-µm-thick) are cut with a microtome, stained with Mallory triple stain (Basson et al. 1991) and mounted in DPX (BDH Ltd, Broom Rd, Poole, Dorset BH12 4 NN, England). These sections can then be examined using interference microscopy using the protocol described by Lille and Fullmer (1976). Other accounts of the use of interference microscopy to study nematodes are given by Shaham (2006) and Seacor et al. (2015).

4.9 Nematode Identification using Molecular Techniques

The use of molecular techniques to complement the morphological and morphometric identification of nematode species has increased considerably during the last two decades. Protein electrophoresis was the first molecular-based technique applied in

Nematology (Esbenshade and Triantaphyllou 1985a, b, 1987; Karssen et al. 1995). Since, new techniques were developed and further optimised for the efficient and accurate identification of nematodes including the polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), multiplex PCR, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), reverse dot blot hybridisation, sequencing of DNA, DNA bar-coding and real-time PCR (Carneiro et al. 2000; Zijlstra 2000; Zijlstra et al. 2000; Hernandez et al. 2004; Adams et al. 2009; Blok and Powers 2009; Subbotin et al. 2013).

Currently, DNA-based methods are used on a regular basis as an efficient, diagnostic tool to confirm and supplement the identification of a wide range of nematode species, both parasitic and non-parasitic. In SA, the first molecular characterisation of a new plant-parasitic nematode was the description of *D. africanus* by Wendt et al. (1995). During the beginning of the 21st century, Fourie et al. (2001) differentiated six root-knot nematode species using the SCAR-technique. Numerous papers using molecular techniques to characterise nematodes from SA have since been published (Table 4.2).

For DNA extraction from nematode individuals, various protocols are used by different researchers (see, for instance, Zijlstra et al. 2000; Hooper et al. 2005; Subbotin et al. 2013). For entomopathogenic nematode species, DNA is generally extracted using the method described by Nguyen (2007). For example, one first generation female for steinernematids or one hermaphrodite for heterorhabditids is placed in 30 ml lysis buffer (16 mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1 %

Table 4.2 Nematode genera occurring in South Africa of which morphological and molecular characterisation have been done

Nematode genera	Reference
Anguina spp.	Swart et al. (2004)
Aphelenchoides spp.	Lesufi (2007); Lesufi et al. (2015)
Criconemoides spp.	Van den Berg et al. (2012)
Ditylenchus spp.	Wendt et al. (1995)
Globodera spp.	Knoetze et al. (2006, 2013)
Helicotylenchus spp.	Subbotin et al. (2011)
Hemicycliophora spp.	Van den Berg et al. (2010); Subbotin et al. (2014)
Heterorhabditis spp.	Malan et al. (2008, 2011, 2014)
Hirschmanniella spp.	Van den Berg et al. (2009)
Meloidogyne spp.	Fourie (1998); Fourie et al. (2001); Berry et al. (2008); Ntidi et al. (2012); Onkendi and Moleleki (2013a, b)
Paratylenchus spp.	Van den Berg et al. (2014)
Pratylenchus spp.	Berry et al. (2008)
Rotylenchulus spp.	Van den Berg et al. (2016); Bekker et al. (2016)
Scutellonema spp.	Van den Berg et al. (2013)
Steinernema spp.	Nguyen et al. (2006); Çimen et al. (2014a, b); Malan et al. (2011, 2015); Nthenga et al. (2014); Stokwe et al. (2011); Malan et al. (2016)
Subanguina spp.	Bekker (2009)
Xiphinema spp.	Knoetze et al. (2000); Berry et al. (2008)

Tween 20) containing 60 µg ml^{-1} proteinase K on the side of a 0.5 ml Eppendorf tube. The nematode is cut into pieces with the sharp side of a syringe needle and immediately put on ice and frozen overnight at –80 °C. The Eppendorf tubes are transferred to a thermocycler at 65 °C for 1 h followed by 95 °C for 10 min and then centrifuged for 2 min at 11,600 *g*. The top 20 µl of the solution is transferred to a clean Eppendorf tube and kept at 20 °C. The 18S and 26S primers suggested by Vrain et al. (1992) are used for amplification of the ITS region. If a good sequence is not obtained, the primers TW81 and AB28 (Hominick et al. 1997) are used. The technique of Nguyen (2007) for PCR amplification is then also followed, with purified DNA being sequenced at the Analytical Centre of the Department of Genetics at Stellenbosch University using the BigDye 3.1 chemistry (PE Applied Biosystems). The base-pair calls of the sequences are verified and edited, using the software CLC DNA Workbench, Version 6. To indicate the phylogenetic position of the nematode isolates, sequences of *Heterorhabditis* and *Steinernema* isolates are compared with sequences obtained from Genbank. Phylogenetic and molecular analyses are conducted and based on maximum parsimony of the ITS region using the software ClustalX ver. 1.83 (Thompson et al. 1997) and PAUP Version 4.08b (Swofford 2002) or maximum likelihood using Mega5 (Tamura et al. 2013).

4.10 Preparation of Nematode Inoculum and Inoculation of Plants

The basic procedure used for preparing inoculum of the groundnut pod nematode (*D. africanus*) for experimental purposes has been described in detail by Mc Donald (1998) but can be used for other nematode species too. Mc Donald used *D. africanus* individuals of all developmental stages obtained from in vitro cultures (see Sect. 4.11.2). The nematode eggs, juveniles and mature adult stages were placed in a calibrated glass flask and kept in suspension by means of a magnetic stirrer throughout the inoculation process. Air could be bubbled through the nematode suspension with an aquarium pump should a magnetic stirrer not be available.

At least 10 aliquots of 10 ml each of the nematode suspension are collected with a 10 ml pipette, poured into a counting dish and counted to check for at least 95 % accuracy.

Roots or other below-ground plant parts to be inoculated are exposed by removing the soil. The prepared nematode inoculum is pipetted directly onto the plant part(s). The inoculation flask is rinsed and the process repeated. The soil that was removed before inoculation is then replaced. Excessive watering of plants should be avoided for at least 48–96 h after nematode inoculation was done to ensure optimal penetration of nematodes into the plant tissue. By preference, watering should be added to the saucers in which the pots are placed. This inoculation procedure is suitable for potted plants, microplots and small field trials.

4.11 Mass Rearing of Nematodes

4.11.1 In Vivo *Mass Rearing of Root-Knot Nematodes*

The protocol described here is based on Fourie et al. (2012). Root-knot nematode populations are obtained from various localities. The GPS coordinates as well as relevant information, including crop sampled and field history, should be listed in a field book. This information is necessary to ensure that the origin of the root-knot nematode population is precisely known. Populations of single species of *Meloidogyne* are established by collecting individual egg masses from infected plant material. Each egg mass is placed near the roots of a susceptible tomato seedling (for instance, the cultivars (cvs) Moneymaker, Rodade, Floradade or UC82B) or susceptible cvs of other crops that are easy to maintain in a steam-sterilised or fumigated soil (>90 % sand) in 25 l pots in a glasshouse. By rotating the host plant on a routine basis with susceptible cvs of different crops (e.g. soybean cv. LS6248R, maize cv. DKC77-77BR, *Amaranthus cruentus* accession Arusha), the virulence of the different single species populations can be maintained.

Nutrients are added according to soil nutrient analyses. The root-knot nematode population should be well established within 56–90 days after inoculation with a single egg mass. At this stage, root-knot nematode-infected roots can be removed, and the eggs and J2 extracted. As a precaution, different *Meloidogyne* spp. populations should be kept well separated, preferably in different glasshouses. An ambient temperature range of 18–26 °C and a 14:10 (light:dark) photoperiod should be maintained in the glasshouse throughout the culturing period of termophylic species. Single species populations or a mixture of more than one species can be used, depending on the objective of the research. According to Hussey and Jansen (2002), a combined inoculum is a precautionary measure to ensure that germplasm is screened against a spectrum of nematode populations. This reduces the risk of finding resistance that is not durable and can be overcome by the concommittant occurrence of more than one nematode species in the field.

4.11.2 In Vitro Mass Rearing of the Groundnut Pod Nematode

Ditylenchus africanus can be cultured on groundnut callus tissue, derived from groundnut leaves that have been placed on an agar medium containing growth promotors (usually 2,4-D) (Van der Walt and De Waele 1989). The procedure is conducted in a sterile environment (laminar flow cabinet). Callus tissue is initiated from 4-week-old groundnut (cv. Sellie) leaves. The leaves are sterilised in 70 % ethanol for 30 s and then suspended in a 0.5 % NaOCl solution for 15 min. The leaves are rinsed four times in sterile, distilled water and cut into 1-cm-pieces. They are then transferred to 9-cm-diameter Petri dishes containing 25 ml of a nutrient medium. The pH of the medium is adjusted to 5.7 and the nutrient autoclaved for

15 min at 121 °C. The callus tissue cultures are incubated in the dark in a growth chamber at 25 °C for 4 weeks. Callus tissue that increased in volume after this period is transferred to Petri dishes containing freshly-prepared medium and inoculated with surface-sterilised *D. africanus* individuals. The Petri dishes are sealed with parafilm and incubated in a growth chamber at 25 °C for 1–5 weeks. Nematodes are extracted by transferring each infected callus tissue to a Petri dish containing 25 ml water and cutting the tissue into smaller pieces. The Petri dishes are left at room temperature to allow *D. africanus* individuals to emerge from the infected callus tissue and migrate into the water. The nematode suspension from each Petri dish is decanted through a 25-μm-aperture sieve and the nematodes collected in a beaker.

4.11.3 In Vitro Mass Rearing of Lesion and Burrowing Nematodes

The monoculture of the lesion nematode species *Pratylenchus brachyurus* (Godfrey 1929) Filipjev & Schuurmans Stekhoven, 1941 on carrot discs reported by Fourie et al. (2003) is described below. This method has been adapted from the method used to rear the burrowing nematode *R. similis* on carrot discs (Stoffelen et al. 1999). Specimens of *P. brachyurus* are extracted from infected material, surface-sterilised in 2 % streptomycin sulphate and rinsed twice in sterile water prior to inoculation. Carrots are sterilised with 99.9 % ethanol, peeled, flamed, cut in discs (approximately 20-mm-diameter and 5-mm-thick) and placed in Petri dishes. An inoculation density of 50 *P. brachyurus* juveniles and adults carrot disc^{-1} is used. Discs that were kept in sealed Petri dishes and incubated at 30 °C yielded a large number of this species, viz. 3,580 individuals disc^{-1} in 60 days and 48,928 individuals disc^{-1} in 80 days. At the end of the incubation period, *P. brachyurus* individuals were extracted by macerating individual carrot discs for 15 s in a kitchen blender and passing the suspension through a range of stacked sieves, viz. 710-, 250-, 75-, 25- and 20-μm-aperture sieves. The nematodes were collected on the 25- and 20-μm-aperture sieves.

4.11.4 Mass Rearing of Entomopathogenic Nematodes

Large numbers of EPN can be cultured using either in vivo or in vitro culture techniques. In vivo production is a low-technology and labour-intensive process. However, it is easily implemented in research laboratories and can be used for small-scale nematode production for field trials. Compared to in vivo production, the in vitro production of EPN demands a high level of technology input and capital investment, thus making it more suitable for commercial mass production. Recent reviews of the methods used for the culturing of EPN have been reported by Ferreira and Malan (2014), Shapiro-Ilan et al. (2014) and Van Zyl and Malan (2014a).

4.11.4.1 In Vivo Production of Entomopathogenic Nematodes

In vivo production of IJ of EPN is based on the White trap method (White 1927) and on modifications of the method (see Sect. 4.4.1.10). This procedure begins with the inoculation of a susceptible insect host and ends with the harvesting and concentration of the IJ. This method makes use of the natural migration of the IJ away from the infected cadaver at the end of the life cycle. Nematode-infected insects are placed on moist filter paper in a 9-mm-diameter Petri dish, which, in turn, is placed in a 15-cm-diameter Petri dish, the bottom of which is filled with just enough water so that the smaller dish still rests on the bottom, without causing it to float. The lids of both dishes are closed to maintain a moist atmosphere during the development of the nematodes. The length of the cycle, which depends on the size of the host, tends to last from 7 to 14 days. During this period, small drops of water should periodically be added to the filter paper to keep it moist.

When the food source in the insect cadaver is depleted, the IJ move over the side of the Petri dish in search of a new host, whereupon they are trapped in the surrounding water contained in the bigger Petri dish. Just before emergence, the lid of the smaller Petri dish should be removed and the nematodes that will have by this time aggregated on the inside of the lid should be washed off into the larger Petri dish. High-quality IJ can be obtained using this technique. The most widely used insect hosts are wax moth larvae and mealworms. Wax moth larvae, highly susceptible to EPN, can easily be cultured in large numbers, but unfortunately are not easily stored. Mealworms are more easily cultured and stored, but they tend to be less susceptible to infection and fewer IJ are produced per host (Van Zyl and Malan 2014a, b).

4.11.4.2 In Vitro Production of Entomopathogenic Nematodes

The in vitro production of nematodes is essential for the commercial use of EPN. In vitro cultures can be reared on a solid medium or in a liquid. For in vitro production of EPN, a monoxenic culture is a prerequisite. This requires that the symbiotic bacteria are isolated and that the nematodes are produced free of bacteria. The procedure for culturing symbiotic bacteria is described by Kaya and Stock (1997) and Ferreira et al. (2014, 2016). The establishment of nematodes without their symbiotic bacteria can be achieved by harvesting eggs from gravid females (Han and Ehlers 1998; Ferreira et al. 2014, 2016).

In vitro techniques for culturing EPN on a solid medium involve the preparation of the medium, inoculation of the bacteria and then the nematodes and, finally, harvesting of the progeny. A culture on a solid medium can be accomplished on various agar media in the same Petri dish or in a so-called three-dimensional rearing system consisting of a nematode culture that is reared on crumbled polyether polyurethane foam coated with a diet (Bedding 1981).

In a liquid culture, the bacteria are generally introduced first followed by the nematodes. Nematodes can be produced in Erlenmeyer flasks on orbital shakers or in bioreactors with a capacity of up to 80,000 l under sterile conditions. As the process time (meaning the completion of the life cycle to the IJ stage) can last up to 3

weeks, the maintenance of sterile conditions throughout the culturing poses a challenge (Ehlers and Shapiro-Ilan 2005).

4.11.5 Surface Sterilisation of Nematodes

Nematodes are routinely sterilised before they are used to establish in vitro populations (Van der Walt and De Waele 1989). Nematode individuals used for this purpose should preferably be extracted using a method such as the Baermann funnel or Baermann tray methods to ensure that they are not damaged in any way. The nematodes are then transferred to a Syracuse dish containing sterile water and then into sterilised centrifuge tubes containing 2 ml of a 0.1 % $HgCl_2$ solution and a piece of milk filter at the bottom. The tubes containing the nematodes are closed with aluminium covers and centrifuged for 2 min at 1,750 *g*. The $HgCl_2$ solution is decanted and the nematodes are trapped on the milk filter. The milk filter is rinsed three times in 2 ml of sterile water. The nematodes are now surface-sterilised and ready for use.

4.12 Reproductive Potential of Root-Knot Nematodes

Root-knot nematode egg masses are stained to facilitate counting and thus establish the reproductive potential of a population on a specific crop cv. The method described here is adapted from Hussey and Boerma (1981).

Plants of the crop to be examined are inoculated with a given number of eggs and J2 or only J2 (e.g. 5,000 per seedling). Fifty-six days after inoculation, the root systems are lifted and excised, rinsed free of adhering soil and debris with running tap water. The root systems are blotted dry with paper towel and weighed, and the number of egg masses per root system counted. Egg masses are stained by immersion of the roots for 20 min in a 0.1 % Phloxine B solution (Hussey and Boerma 1981). Each root system is cut into approximately 10-mm pieces and transferred to a container filled with approximately 200 ml water. The root pieces are individually inspected and the red-stained egg masses counted. Egg masses coming loose from the root pieces during the staining and counting process are collected using a Pasteur pipette and are also counted. The egg-laying-female index for each root system is determined according to the method of Hussey and Boerma (1981). This index is based on a 0–5 scale where 0 = no egg masses; 1 = 1–2 egg masses; 2 = 3–10 egg masses; 3 = 11–30 egg masses; 4 = 31–100 egg masses and 5 => 100 egg masses system^{-1}. Subsequently, the eggs are extracted from the stained egg masses using the modified NaOCl method (Riekert 1995) and counted using a dissecting microscope. The reproduction potential of the nematodes is determined using Oostenbrink's Rf value as published in Windham and Williams (1987): Rf = Pi (initial number of nematodes inoculated per root system/Pf (final number of nematodes per root system). The number of eggs per g of fresh roots is determined for each plant.

4.12.1 *Estimation of Juvenile Numbers and Viability of Cyst Nematodes*

Depending on the age and state of heteroderid cysts, their egg and J2 content can vary considerably. The viability of cysts can be determined by examining the biological status of the eggs and J2 they contain. A technique to do this is to place cysts, that had been soaked for 12 h in water, into a 0,05 % aqueous solution of Meldola's blue (Shepherd 1962) for 1 week. The cysts are then crushed in a microcentrifuge tube with a homogeniser, and the eggs and J2 are released by vortexing. The eggs are separated from the cyst fragments by washing them through a 90-µm-aperture sieve and collecting them on a 25-µm-aperture sieve. Eggs and J2 are left in a water suspension for up to 12 h to remove excess stain. Two aliquots are then pipetted onto a counting chamber and observed using a light microscope. The viability of the eggs are estimated by counting stained (non-viable) and non-stained (viable) eggs. Spontaneous hatching of J2 are estimated by counting empty eggs. Turner (1998) and Le Roux (2000) also described useful methods to estimate the viability of cysts.

4.13 Staining of Roots to Establish Nematode Penetration and Development

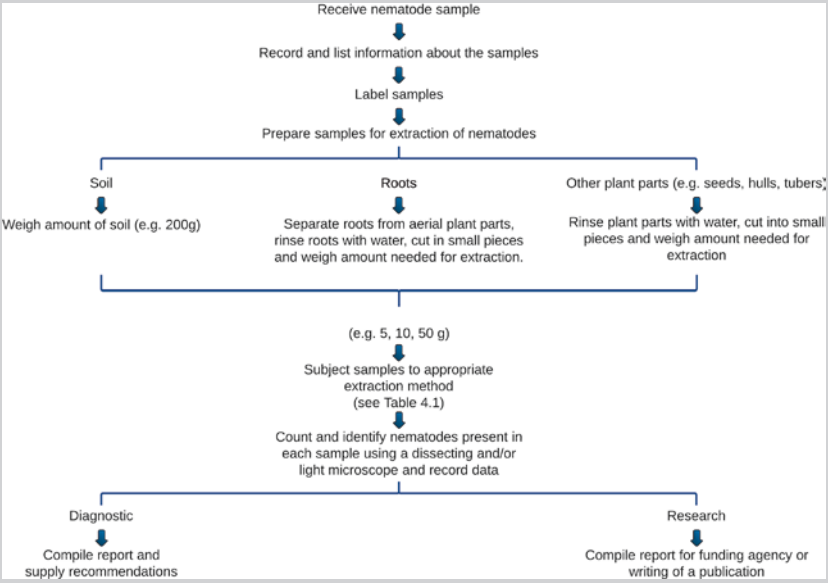
Staining of roots or other plant parts is a useful tool to study, e.g. the penetration and development of a target plant-parasitic nematode species over time. To do such research, root systems infected with plant-parasitic nematodes (e.g. root-knot nematodes) are obtained, washed free of soil and stained with an acid fuchsin-lactoglycerol solution (875 ml of lactic acid, 63 ml of glycerol, 62 ml of water and 0.1 g acid fuchsin) to facilitate nematode counting (Byrd et al. 1983; Hooper et al. 2005). The solution is heated on a warm plate that is placed inside a fume hood to avoid inhaling the lactic acid vapour. Root-knot nematode-infected root pieces are wrapped inside a muslin cloth and submerged into the solution as soon as it starts to boil. After 1–2 min, the muslin cloths with the stained roots are removed and rinsed with running tap water to get rid of the excess stain solution. The next step entails the transfer of the stained root systems to a solution that contains equal volumes of glycerol and distilled water to allow destaining of the roots, but not of the nematodes inside the roots. A few drops of lactic acid can be added to the glycerol:distilled water solution to enhance destaining of the roots. Nematodes are usually visible as red-coloured organisms within the roots and can be counted, using a dissection microscope, after the roots were left for 2–5 days in the destaining solution. Chemicals other than acid fuchsin, e.g. methyl blue, can also be used to stain plant tissue to enable identification and counting of nematodes within plant tissues (Bridge et al. 1982)

This chapter concludes with an inventory (Box 4.3) of the basic and important infrastructure and apparatus needed to set up and run a small nematology laboratory. Box 4.4 demonstrates the basic order and flow of activities from the moment a nematode sample is received by personnel of a nematology laboratory until the identification and nematode counts have been completed.

Box 4.3 An Inventory of the Basic Infrastructure and Equipment Needed to Set Up and Run a Small Nematology Laboratory for Diagnostic and Research Purposes

Glasshouse, field and microplot research	Preparation of nematode samples for extraction, extraction and counting of nematodes	Staining of plant tissues, killing, fixation and mounting of nematodes	Inoculation of plants for experiments
Infrastructure: Glasshouse or tunnel; microplots; room for the storage of chemicals including nematicides; vehicle to access trial sites; automated irrigation system for glasshouses and microplots *Tools/small apparatus*: Knapsack sprayer; plastic measuring cylinders; field-operatable balance to record crop yield and weight of aerial or below-ground plant parts; water cans; garden spades; hoes; oil augers; arden rakes; plastic pots (1, 5, 10 or 20 l); plot markers; pegs to mark field plots; insulated cool boxes, field book; protective clothing for when using nematicides *Consumables*: Plastic bags for soil and root samples; cable ties; measuring tape, string; permanent marker pen; fertiliser; disposable gloves	*Infrastructure*: Room to use as laboratory; working area; cool room or refrigerator to store samples at 5–10 °C; two to four basins with taps fitted with latex tubing (basins must be deep enough to place stacked sieves); mistifier; running water; sand-trap outside the laboratory *Equipment/big apparatus*: Dissecting- and light microscopes; inverted microscope; camera system that fits on microscopes; centrifuge; orbital shaker; kitchen blender; balance (4 decimals); two sets of sieves (1000-, 750-, 500-, 250-, 75-, 63-, 38-, 25- and 20-µm-apertures); elutriator; Baermann funnels or trays; water distiller *Small equipment/apparatus*: Stop watch; multiple or single tally counters (e.g. blood-cell counter); De Grisse counting dishes; Peter's counting slides; laboratory clock; measuring cylinders; flasks; pipettes; 5 l plastic buckets; plastic wash bottles; plastic sample bottles; plastic pot trays; plastic funnels; marking tape; scissors; Syracuse dishes; needles to fish nematodes; tissue paper *Chemicals*: Sugar; kaolin, NaOCl; other chemicals needed for specific extraction methods	*Infrastructure*: Room to use as laboratory *Equipment/big apparatus*: Fume cupboard; oven *Smaller equipment/ apparatus*: Hot plate; dissection set (needles, scalpel, etc.); Petri dishes; Syracuse dishes; glassware (e.g. beakers, measuring cylinders) *Consumables*: Microscope slides; cover slips; transparent nail polish; labels; holders for slides *Chemicals*: Acid fuchsin/cotton blue; glycerol, lactic acid; ethyl alcohol, formaldehyde; lactophenol; others	*Smaller equipment/ apparatus*: Micropipettes and tips; Socorex 411 Stepper used for accurate preparation of inoculum; plastic beakers; magnetic stirrer; magnetic stir bars (different sizes)

Box 4.4 The General Process Followed by Personnel of a Nematology Laboratory from the Receiving of Nematode Samples Until Recording of Data and Report Writing



References

Adams BJ, Dillman AR, Finlinson C (2009) Molecular taxonomy and phylogeny. In: Perry RN, Moens M, Starr JL (eds) Root-knot nematodes. CAB International, Wallingford, pp 119–138

Basson S, De Waele DGMA, Meyer AJ (1991) Population dynamics of *Ditylenchus destructor* on peanut. J Nematol 23:485–490

Bedding RA (1981) Low cost *in vitro* production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. Nematologica 27:109–114

Bedding RA, Akhurst RJ (1975) A simple technique for the detection of insect parasitic rhabditid nematodes in soil. Nematologica 21:109–110

Been TH, Schomaker CH (2013) Plant growth and population dynamics. In: Perry R, Moens M (eds) Plant nematology. CAB International, Wallingford, pp 301–330

Bekker S (2009) Assessment of the identity, distribution and control options for seed-and leaf-gall nematodes on grass seeds in South Africa. MSc Dissertation. North-West University, Potchefstroom

Bekker S, Fourie H., Rashidi M et al (2016) Discriminating between the eggs of two egg-mass-producing nematode genera using morphometric and molecular techniques. Nematol 18:1119–1123. doi:10.1163/15685411-00003022

Berry SD, Fargette M, Spaull VW et al (2008) Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. Mol Cell Probes 22:168–176

Blok VC, Powers TO (2009) Biochemical and molecular identification. In: Perry RN, Moens M, Starr JL (eds) Root-knot nematodes. CAB International, Wallingford, pp 98–118

Bolton C, De Waele D, Basson S (1990) Comparison of two methods for extracting *Ditylenchus destructor* from hulls and seeds of groundnut. Rev Nématol 13:33–235

Bridge J, Starr JL (2007) Plant nematodes of agricultural importance. A color handbook. Academic Press, Boston

Bridge J, Page S, Jordan S (1982) An improved method for staining nematodes in roots. Report of the Rothamsted Experimental Station for 1981. Part 1. Rothamsted, Harpenden

Byrd DW, Kirkpatrick T Jr, Barker KR (1983) An improved technique for clearing and staining plant tissues for detection of nematodes. J Nematol 15:142–143

Carneiro RMDG, Almeida MRA, Quénérhervé P (2000) Enzyme phenotypes of *Meloidogyne* spp. populations. Nematol 2:645–654

Carta LK (1991) Technical methods for collection and preparation of nematodes. Part 3. Preparation of nematodes for transmission electron microscopy. In: Nickle WR (ed) Manual of agricultural nematology. Marcel Dekker Inc, New York, pp 97–106

Çimen HC, Lee M-M, Hatting et al (2014a) *Steinernema innovationi* n. sp. (Panagrolaimomorpha: Steinernematidae), a new entomopathogenic nematode species from South Africa. J Helminthol 89:415–427

Çimen HC, Lee M-M, Hatting J et al (2014b). *Steinernema tophus* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from South Africa. Zootaxa 3821:337–353

Coolen WA, D'Herde CJ (1972) A method for the quantitive extraction of nematodes from plant tissue. State Agricultural Research Centre, Merelbeke

Coyne DL, Nicol JM, Claudius-Cole B (2007) Practical plant nematology: a field and laboratory guide. SP-IPM Secretariat, International Institute of Tropical Agriculture (IITA), Cotonou

De Grisse A (1963) A counting dish for nematodes excluding border effect. Nematologica 9:162

De Grisse AT (1965) Redescription ou modifications de quelques techniques ultilisees dans l'etude des nematodes phytoparasitaires. Medelingen Rijksfaculteit Landbouwetenschappen Gent 34:351–359

De Waele E, De Waele D, Wilken R (1987) Effect of root mass on the efficacy of methods for extracting root-lesion nematodes from maize roots. Phytophylactica 19:473–474

Ehlers R-U, Shapiro-Ilan DI (2005) Mass production. In: Grewal PS, Ehlers R-U, Shapiro-Ilan DI (eds) Nematodes as biocontrol agents. CAB International, Wallingford, pp 65–78

Eisenback JD (1985) Techniques for preparing nematodes for electronmicroscopy. In: Barker KR, Carter CC, Sasser JN (eds) An advance treatise on *Meloidogyne*, Vol III, Methodology. A cooperative publication of the Department of Plant Pathology and the United States Agency for International Development. North Carolina State University Graphics, Raleigh, pp 79–105

EPPO (2013) Diagnostics PM 7/119 (1) Nematode extraction. European and Mediterranean Plant Protection Organization, European and Mediterranean Plant Protection Organization. Bull OEPP/EPPO Bull 43:471–495. doi:10.1111/epp.12077

Esbenshade PR, Triantaphyllou AC (1985a) Use of enzyme phenotypes for identification of *Meloidogyne* species (Nematoda: Tylenchida). J Nematol 17:6–20

Esbenshade PR, Triantaphyllou AC (1985b) Identification of major *Meloidogyne* species employing enzyme phenotypes as differentiating characters. In: Sasser JN, Carter CC (eds) An advanced treatise on *Meloidogyne*. Volume I: Biology and control. A cooperative publication of the Department of Plant Pathology and the United States Agency for International Development. North Carolina State University Graphics, Raleigh, pp 135–140

Esbenshade PR, Triantaphyllou AC (1987) Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). J Nematol 19:8–18

Ferreira T, Malan AP (2014) *Xenorhabdus* and *Photorhabdus*, bacterial symbionts of the EPNs *Steinernema* and *Heterorhabditis* and their *in vitro* liquid mass culture: a review. Afr Entomol 22:1–14

Ferreira T, Addison MF, Malan AP (2014) *In vitro* liquid culture of a South African isolate of *Heterorhabditis zealandica* for the control of insect pests. Afr Entomol 22:80–92

Ferreira T, Addison MF, Malan AP (2016) Development and population dynamics of *Steinernema yirgalemense* and growth characteristics of its associated *Xenorhabdus* symbiont in liquid culture. J Helminthol 90:364–371

Flegg JJM (1967) Extraction of *Xiphinema* and *Longidorus* species from soil by a modification of Cobb's decanting and sieving technique. Ann Appl Biol 60:429–437

Fourie H (1998) The influence of plant-parasitic nematodes on soybean production in South Africa with special reference to root-knot nematodes (*Meloidogyne* species). MSc Dissertation, Potchefstroom University for Christian Higher Education, Potchefstroom

Fourie H, Zijlstra C, Mc Donald AH (2001) Identification of root-knot nematode species occurring in South Africa using the SCAR – PCR technique. Nematol 3:675–680

Fourie H, Venter GA, Mc Donald AH (2003) Optimum temperature and inoculation requirements for *in vitro* production of *Pratylenchus brachyurus* on carrot discs. S Afr J Plant Soil 20: 44–45

Fourie H, Mc Donald AH, Mothata TS et al (2012) Indications of variation in host suitability to root-knot nematode populations in commercial tomato varieties. Afr J Agric Res 7:2344–2352

Fourie H, Mc Donald AH, De Waele D et al (2013) Comparative cellular responses in resistant and susceptible soybean cultivars infected with *Meloidogyne incognita*. Nematol 15:695–708

Griffin GD, Barker KR, Pederson GA et al (1998) Plant and nematode interactions. Number 36 in the Agronomy series. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, USA, p 381–397

Han R, Ehlers R-U (1998) Cultivation of axenic *Heterorhabditis* spp. dauer juveniles and their response to non-specific *Photorhabdus luminescens* food signals. Nematologica 44:425–435

Hernandez A, Fargette M, Sarah J-L (2004) Characterization of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) from coffee plantations in Central America and Brazil. Nematol 6:193–204

Hodda M, Eyualem-Abebe (2006) Techniques for processing freshwater nematodes. In: Eyualem-Abebe, Traunspurger W, Andrássy I (eds) Freshwater nematodes: ecology and taxonomy. CAB International, Wallingford, pp 31–45

Hominick WM, Briscoe BR, del Pinto EG et al (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. J Helminthol 71:271–298

Hooper DJ (1998) Use of the scanning electronmicroscope to identify nematodes. In: Fortuner R (ed) Nematode Identification and Expert System Technology. Plenum Press, New York, pp 301–311

Hooper DJ, Evans K (1993) Extraction, identification and control of plant-parasitic nematodes. In: Evans K, Trudgill DL, Webster JM (eds) Plant parasitic nematodes in temperate agriculture. CAB International, Wallingford, pp 1–59

Hooper DJ, Hallman J, Subbotin S (2005) Methods for extraction, processing and detection of plant and soil nematodes. In: Luc M, Sikora RA, Bridge J (eds) Plant parasitic nematodes in subtropical and tropical agriculture. CAB International, Wallingford, pp 53–86

Hunt DJ, Handoo ZA (2009) Taxonomy, identification and principal species. In: Perry RN, Moens M, Starr JL (eds) Root-knot nematodes. CAB International, Wallingford, pp 55–97

Hussey RS, Boerma HR (1981) A greenhouse screening procedure for root-knot nematode resistance in soybeans. Crop Sci 21:794–796

Hussey RS, Jansen GJW (2002) Root-knot nematodes: *Meloidogyne* species. In: Starr JL, Cook R, Bridge J (eds) Plant resistance to parasitic nematodes. CAB International, Wallingford, pp 43–70

Jenkins WR (1964) A rapid centrifugal-flotation technique for separating nematodes from soil. Plant Dis Rep 48:692

Jones B, De Waele D (1990) Histopathology of *Ditylenchus destructor* on peanut. J Nematol 22:268–272

Karssen G, Van Hoenselaar T, Verkerk-Bakker B et al (1995) Species identification of cyst and root-knot nematodes potato by electrophoresis of individual females. Electrophoresis 16:105–109

Kaya HK, Stock SP (1997) Techniques in insect nematology. In: Lacey SA (ed) Manual of techniques in insect pathology. Academic, London, pp 281–324

Keetch DP (1982) Notes on the field evaluation of nematicidal materials. In: Keetch DP, Heyns J (eds) Nematology in Southern Africa, Science Bulletin No. 400. Department of Agriculture and Fisheries, Pretoria, pp 151–157

Khan MR (2008) Plant nematodes: methodology, morphology, systematics, biology and ecology. Science Publishers Inc, Enfield

Kleynhans KPN (1991) The root-knot nematodes of South Africa. Technical Communication 231. Department of Agricultural Development, Pretoria

Kleynhans KPN (1997) Collecting and preserving nematodes. Manual for a SAFRINET course in practical Nematology, 1st print. SAFRINET, the Southern African (SADC) LOOP of BioNet-International, Pretoria

Kleynhans KPN, Van den Berg E, Swart A et al (1996) Plant nematodes in South Africa. Plant Protection Research Institute Handbook No 8. ARC-Plant Protection Research Institute, Pretoria

Knoetze R, Burger JT, Meyer AJ (2000) Discrimination of some *Xiphinema* species from South Africa by rDNA-RFLP analysis. Afr Plant Prot 6:25–30

Knoetze R, Malan AP, Mouton C (2006) Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. Afr Plant Prot 12:103–110

Knoetze R, Swart A, Tiedt LR (2013) Description of *Globodera capensis* n. sp. (Nematoda: Heteroderidae) from South Africa. Nematol 15:233–250

Koen H (1969) Die voorkoms en beheer van plantparasitiese aalwurms in Suid-Afrika, Bladskrif No 35. Departement van Tegniese Dienste, Pretoria

Lax P, Doucet ME (2002) New process for preparing heteroderid cysts for scanning electron microscopy. Nematol 4:445–447

Le Roux S (2000) Life cycle, epidemiology and control of the golden cyst nematode *Globodera rostochiensis* (race: Ro1) under South African conditions at Ceres, Cape. ARC-Report 1999–2000. Project No. 103402. ARC-Vegetable and Ornamental Institute, Pretoria

Lesufi MM (2007) A study of the systematics and implications of the presence of the testa nematode, *Aphelenchoides arachidis* Bos, 1977 in South Africa. MSc Dissertation. North-West University, Potchefstroom

Lesufi M, Swart A, Mc Donald AH et al (2015) Morphological and molecular studies on *Aphelenchoides arachidis* Bos, 1977 (Tylenchina: Aphelenchoididae) from groundnuts in South Africa. Nematol 17:433–445

Lille RD, Fullmer HM (1976) Histopathologic techniques and practical histochemistry, 4th edn. McGraw-Hill Inc., New York

Malan AP, Nguyen KB, De Waal JY et al (2008) *Heterorhabditis safricana* n. sp. (Rhabditida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. Nematol 10:381–396

Malan AP, Knoetze R, Moore SD (2011) Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. J Invertebr Pathol 108:115–125

Malan AP, Knoetze R, Tiedt L (2014) *Heterorhabditis noenieputensis* n. sp. (Rhabditida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. J Helminthol 88:138–151

Malan AP, Knoetze R, Tiedt LR (2016) A new entomopathogenic nematode from South Africa, *Steinernema jeffreyense* n. sp. J Helminthol 90:262–278

Manzilla-López RH (2012) Methodology and symptomatology. In: Manzilla-López RH, Marbán-Mendoza N (eds) Practical plant nematology. BiBliotheca Básica de Agricultura, Monticillo, pp 89–129

Marais M, Shubane A (2012) 'n Tegniek om nematodes uit geelwortels te onttrek. In: Abstracts of the Annual Symposium of the Biological Sciences Division of the South African Academy of Arts and Science, North-West University, Potchefstroom, 25–26 October 2012

McClure MA, Kruk TH, Misaghi I (1973) A method for obtaining quantities of clean *Meloidogyne* eggs. J Nematol 5:230

Mc Donald AH (1998) Management of *Ditylenchus africanus* (Nematoda: Tylenchida) ongroundnut (*Arachis hypogaea*). PhD Thesis, Potchefstroom University for Christian Higher Education, Potchefstroom

Minagawa N (1986) Taxonomic studies of Criconematidae (Nematoda: Tylenchida) of Japan. Genera *Neolobocriconema*, *Paralabocriconema* n.gen. and *Macrocriconema* n.gen. Bulletin of the National Institute of Agro-Environmental Sciences, Japan No 1, pp 95–126

Murphy JA, Pappelis AJ, Thompson MR (1974) Morphological aspects of *Diplodia maydis* and its role in the stalk rot of corn. Scanning electron microscopy. Part II. In: Abstracts of of the workshop of scanning electron microscope and the plant sciences. IIT Research Institute, Chicago

Nguyen KB (2007) Methodology, morphology and identification. In: Nguyen KB, Hunt DJ (eds) Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. Nematology monographs and perspectives, vol 5. Brill, Leiden

Nguyen KB, Hunt DJ (2007) Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. Nematology monographs and perspectives, vol 5. Brill, Leiden

Nguyen KB, Malan AP, Gozel U (2006) *Steinernema khoisanae* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. Nematol 8:157–175

Nthenga I, Knoetze R, Berry S et al (2014) *Steinernema sacchari* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. Nematol 16:475–494

Ntidi N, Fourie H, Mc Donald AH et al (2012) Plant-parasitic nematodes associated with weeds in developing agriculture in South Africa. Nematol 14:875–887

Onkendi EM, Moleleki LN (2013a) Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* spp.) in potatoes from South Africa. Plant Pathol. 62:1184–1192

Onkendi EM, Moleleki LN (2013b) Detection of *Meloidogyne enterolobii* in potatoes in South Africa and phylogenetic analysis based on intergenic region and the mitochondrial DNA sequences. Eur J Plant Pathol 136:1–5

Oostenbrink M (1960) Estimating nematode populations by some selected methods. In: Sasser JN, Jenkins WR (eds) Nematology. University of North Carolina Press, Chapel Hill, pp 85–102

Powers TO, Ramírez-Suárez Á (2012) Molecular diagnostics of nematodes: some practical considerations. In: Manzilla-López RH, Marbán-Mendoza N (eds) Practical plant nematology. BiBliotheca Básica de Agricultura, Monticillo, pp 131–145

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electronmicroscopy. J Cell Biol 17:208–213

Riekert HF (1995) An adapted method for extraction of root-knot nematode eggs from maize root samples. Afr Plant Prot 1:41–43

Riekert HF (1996) Economic feasibility of nematode control in dryland maize in South Africa. Afr Crop Sci J 4:477–481

Seacor T, Striluk M, Havek E (2015) Classification of wild isolate nematode strains using classical systematics and molecular sequencing. J Student Res 4:45–52

Seinhorst JW (1950) De betekenis van de toestand van de grond voor het optreden van aantasting door het stengelaaltjie (*Ditylenchus dipsaci* (Kühn) Filipjev). Tijdschrift over Plantenziekten 56:289–348

Seinhorst JW (1959) A rapid method for the transfer of nematodes from fixative to anhydrous glycerine. Nematologica 15:67–69

Seinhorst JW (1964) Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. Nematologica 10:87–94

Shaham S (2006) Methods in cell biology. In: Shaham S (ed) Wormbook: the online review of *C. elegans* biology. http://wormbook.org.doi:101895/wormbook.1.49.1

Shapiro-Ilan DI, Han R, Qiu X (2014) Production of entomopathogenic nematodes. In: Morales-Ramos JA, Rojas MG, Shapiro-Ilan DI (eds) Mass production of beneficial organisms. Academic Press, Amsterdam, pp 321–356

Shepherd AM (1962) New blue R, a stain that differentiates between living and dead nematodes. Nematologica 8:201–208

Southey JF (1965) The incidence of stem eelworms on onion seed. Plant Pathol 14:55–59
Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43
Stoffelen R, Jimenez MI, Dierckxsens C et al (1999) Effect of time and inoculum density on the reproductive fitness of *Pratylenchus coffeae* and *Radopholus similis* populations on carrot disks. Nematol 1:243–250
Stokwe N, Malan AP, Nguyen KB et al (2011) *Steinernema citrae* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. Nematol 13:569–587
Subbotin SA, Inserra RN, Marais M et al (2011) Diversity and phylogenetic relationships within the spiral nematodes of *Helicotylenchus* Steiner, 1945 (Tylenchida: Hoplolaimidae) as inferred from analysis of the D2-D3 expansion segments of 28S rRNA gene sequences. Nematol 13:333–345
Subbotin SA, Wayenberge L, Moens M (2013) Molecular taxonomy and phylogeny. In: Perry R, Moens (eds) Plant nematology. CAB International, Wallingford, pp 40–72
Subbotin SA, Chitambar JJ, Chizkov VN et al (2014) Molecular phylogeny, diagnostics, and diversity of plant-parasitic nematodes of the genus *Hemicycliophora* (Nematoda: Hemicycliophoridae). Zool J Linn Soc 171:475–506
Swart A (1997) Description of *Laimaphelenchus patulus* sp. n. (Nematoda: Aphelenchoididae) from *Pinus pinaster* Ail in South Africa. Afr Plant Prot 3:23–28
Swart A, Subbotin SA, Tiedt L et al (2004) *Anguina woodi* sp. n. (Tylenchida: Anguinidae) from dune grass, *Ehrharta villosa*, in South Africa. Nematol 6:129–144
Swofford D (2002) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland
Tamura K, Stecher G, Peterson D et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729
Thompson JD, Gibson TJ, Plewniak F et al (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
Todd WJ (1986) Effects of specimen preparation on the apparent ultrastructure of microorganisms. In: Aldrich HC, Todd WJ (eds) Ultrastructure techniques for microorganisms. Plenum Press, New York, pp 87–100
Turner SJ (1998) Sample preparation, soil extraction and laboratory facilities for the detection of potato cyst nematodes. In: Marks RJ, Brodie BB (eds) Potato cyst nematodes: biology, distribution and control. CAB International, Wallingford, pp 75–90
Van den Berg E, Furstenberg JP (1982) Extraction and handling of plant and soil nematodes. In: Keetch DP, Heyns J (eds) Nematology in Southern Africa, Science Bulletin No. 400. Department of Agriculture and Fisheries, Pretoria, pp 158–161
Van den Berg E, Subbotin SA, Handoo ZA et al (2009) *Hirschmanniella kwazuna* sp. n. from South Africa with notes on a new record of *H. spinicaudata* (Schuurmans Stekhoven, 1944) Luc & Goodey, 1964 (Nematoda: Pratylenchidae) and on the molecular phylogeny of the genus *Hirschmanniella* Luc & Goodey, 1964. Nematol 11:523–540
Van den Berg E, Subbotin SA, Tiedt LR (2010) Morphological and molecular characterisation of *Hemicycliophora lutosa* Loof & Heyns, 1969 and *H. typica* de Man, 1921 from South Africa (Nematoda: Hemicycliophoridae). Nematol 12:303–308
Van den Berg E, Tiedt LR, Subbotin SA (2012) Morphological and molecular characterisation of *Criconemoides brevistylus* Singh & Khera, 1976 and *C. obtusicaudatus* Heyns, 1962 from South Africa (Nematoda: Criconematidae) with first description of a male *C. obtusicaudatus* and proposal of new synonyms. Nematol 14:961–976
Van den Berg E, Tiedt LR, Coyne DL et al (2013) Morphological and molecular characterisation and diagnostics of some species of *Scutellonema* Andrássy, 1958 (Tylenchida: Hoplolaimidae) with a molecular phylogeny of the genus. Nematol 15:1–27
Van den Berg E, Tiedt LR, Subbotin SA (2014) Morphological and molecular characterisation of several *Paratylenchus* Micoletzky, 1922 (Tylenchida: Paratylenchidae) species from South Africa and USA, together with some taxonomic notes. Nematol 16:323–358

Van den Berg E, Palomares-Rius JE, Vovlas N et al (2016) Morphological and molecular characterisation of one new and several known species of the reniform nematode, *Rotylenchulus* Linford & Oliveira, 1940 (Hoplolaimidae: Rotylenchulinae), and a phylogeny of the genus. Nematol 18:67–107

Van der Walt P, De Waele D (1989) Mass culture of the potato rot nematode *Ditylenchus destructor* on groundnut callus tissue. Phytophylactica 21:79–80

Van Zyl C, Malan AP (2014a) Optimisation of inoculation techniques for *in vivo* mass culture of entomopathogenic nematodes through nematode and insect host manipulation. Afr Entomol 24:405–416

Van Zyl C, Malan AP (2014b) The role of nematodes as biological control agents, the history of nematode mass culture and *in vivo* nematode production. Afr Entomol 22:236–249

Vrain TC, Wakarchuck DA, Lèvesque AC et al (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundam Appl Nematol 15:563–573

Wendt KR, Swart A, Vrain TC et al (1995) *Ditylenchus africanus* sp. n. from South Africa; a morphological and molecular characterization. Fundamen Appl Nematol 18:241–250

White GF (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302–303

Windham GL, Williams WP (1987) Host suitability of commercial corn hybrids to *Meloidogyne arenaria* and *Meloidogyne incognita*. J Nematol 19(S):13–16

Zijlstra C (2000) Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. Eur J Plant Pathol 106:283–290

Zijlstra C, Donkers-Venne DTHM, Fargette M (2000) Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region(SCAR) based PCR. Nematol 2:847–853