THE INTERNATIONAL PLANT DIAGNOSTIC NETWORK

# Standard Operating Procedure for Plant Diagnostic Laboratories

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**Root-knot nematodes (*Meloidogyne* spp.)**

# Acknowledgements

The authors would like to acknowledge the contributions made by various institutions towards the completion of this Standard Operating Procedure (SOP) for root-knot nematodes (RKN) (*Meloidogyne* spp.). Thanks are extended to: International Plant Diagnostic Network (IPDN); Kenya Agricultural Research Institute (KARI), Kenya and the Integrated Pest Management – Collaborative Research Support Programme (IPM CRSP).

The authors appreciate the contributions made by all the participants during the IPDN workshops held at Kenya Agricultural Research Institute, KARI-Kabete in January 2012.

The development of the SOP for RKN was financially supported by the United States Agency for International Development (USAID) through the Integrated Pest Management Collaborative Research Support Program (IPM CRSP).

The majority of protocols have been adopted from the manual 'Practical Plant Nematology: A field and laboratory guide' by Coyne *et al*., (2007) and where appropriate we have acknowledged accordingly in the text. This SOP is tailored towards understanding protocols involved in RKN sampling, extraction and identification with special emphasis on tomato. We therefore strongly recommend that readers consult the field guide manual for further details on the same regarding other plant parasitic nematodes.

# List of Acronyms

| | |
|---|---|
| DGO | Dorsal esophageal gland orifice |
| IPM CRSP | Integrated Pest Management Collaborative Research Support Program |
| ID | Identification |
| IITA | International Institute of Tropical Agriculture |
| J2 | Second-stage juvenile |
| KARI | Kenya Agricultural Research Institute |
| KEPHIS | Kenya Plant Health Inspectorate Service |
| KU | Kenyatta University |
| NARL | National Agricultural Research Laboratories |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| RKN | Root-knot nematodes |
| Rpm | Revolution per minute |
| SCAR | Sequence characterized amplified region |
| SOP | Standard operating procedure |
| UoN | University of Nairobi |
| UoP | University of Pretoria |
| USAID | United States Agency for International Development |
| WLB | Worm Lysis Buffer |

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# 1.0 ROOT-KNOT NEMATODES

## 1.1 Introduction

Root-knot nematodes (RKN) are an especially important group of polyphagous, highly adapted obligate plant pathogens within the genus *Meloidogyne* Göldi, 1892 (*Meloidogyne* = apple-shaped female). Considered as the most important plant parasitic nematodes, they pose a significant threat to crop production globally (Jones *et al*., 2013).

## 1.2 Distribution

Root-knot nematodes have a wide distribution globally and parasitize virtually every species of higher plants. Some species are quite host specific, many are able to parasitize a range of host plants, while some have extensive host ranges. Although over 90 species of *Meloidogyne* have been described to date (Perry *et al*., 2009), four species, which are particularly polyphagous, are of particular economic importance: *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Eisenback *et al*., 1981). Of 1000 RKN populations collected in 75 countries, 53% were identified as *M. incognita*, 30% as *M. javanica*, 8% as *M. arenaria*, 8% as *M. hapla* and 2% as *M. exigua* or other species (Taylor & Sasser, 1978). However, discovery of *Meloidogyne* species previously unknown will continue to appear as farming practices evolve and our knowledge and expertise extends, especially into resource-poor areas, where information on species identity and distribution is less than optimal. *Meloidogyne* spp. is also a difficult group of nematodes to identify, but as diagnostic techniques improve, our ability to accurately identify species and their distribution improves.

## 1.3 Lifecycle

The infective stage is the vermiform second-stage juvenile (J2), which, after molting once in the egg, emerges, penetrates the plant root behind the growing tip, and migrates towards the vascular cylinder. Here, they establish a feeding site where they remain for the duration of their lifecycle, feeding from several enlarged multinuclear cells called 'giant cells' which are formed from cells that underwent karyokinesis (repeated mitosis) without cytokinesis. Neighboring pericycle cells divide and enlarge in size resulting in the typical root deformations or galls that give rise to the term of root knot, reflecting the characteristic root damage symptoms of *Meloidogyne* spp. infection. Upon feeding, J2's undergo three additional molts before maturing into the swollen adult females. The adult female lays 500-1000 eggs into a protective gelatinous matrix or egg mass, which may protrude and be exposed from the surface of the root, or may form and remain below the surface of the root. New infective J2's hatch from the eggs repeating the cycle. Depending on *Meloidogyne* species, host and environmental conditions, the duration of the life cycle will vary, but is usually completed in 4-6 weeks at 26-28$^{o}$C. Males, when present are vermiform, and there is no evidence that they feed. Males can be found in parthenogenetic species

when conditions are unfavorable, such as when food resources are limited and when population densities are high.

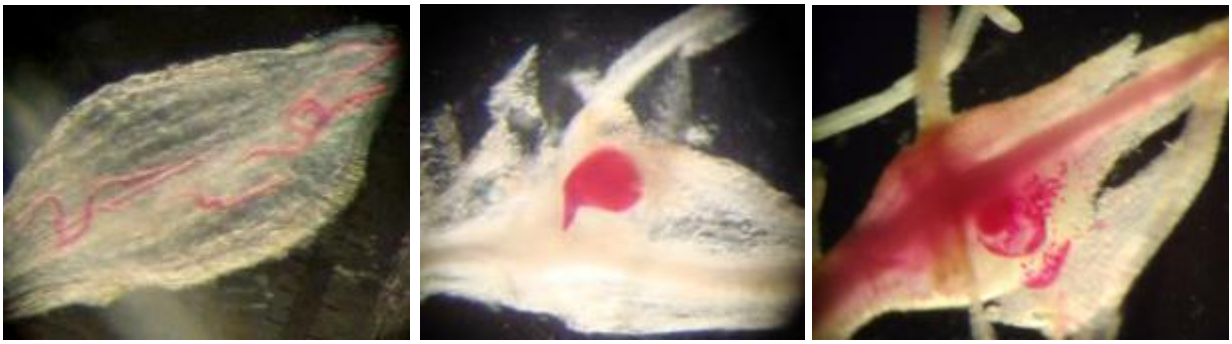


Figure 1: J2 enter roots behind the root tip and migrate to the central cortex creating gall formation (left), where they remain feeding from specialized cells and develop into swollen adult females (center) to produce eggs in a gelatinous egg mass contained within (right) or extruding from the root surface (Photo: E. Galon)

### 1.4 Damage

Due to their endoparasitic mode of feeding, RKN disrupt the host crop physiology , reducing root efficiency and affecting their ability to uptake nutrients and water. Typically, RKN reproduce and feed within plant roots, deforming root growth and inducing small to large galls or root-knots (Coyne *et al*., 2007). Plant hosts however, vary in their reaction to RKN infection, with some hosts exhibiting massive galling / knotting damage and others with no or only limited galling. Their disruption to roots result in reduced performance, reducing crop development, yield and product quality (as seen in Figure 2) and, therefore, are of immense economic importance (see Perry *et al*., 2009). RKN will, over time, result in the deterioration of the root system and under severe infections will ultimately lead to the total breakdown of the root system, with secondary necrosis contributing to this. Infected roots gradually become infected with additional soil-borne pathogens, contributing to extensive root necrosis and breakdown of roots. Infection with RKN also predisposes hosts to a greater likelihood of infection from other pests and diseases, and in some cases rendering otherwise resistant hosts susceptible to pests and diseases, through a breakdown of resistance following RKN infection.

# 2.0 DIAGNOSIS OF ROOT-KNOT NEMATODE DAMAGE IN THE FIELD

## 2.1 Above-ground symptoms

The damage caused by RKN to roots of host plants can be very characteristic. The above-ground symptoms observed on infected plants though, are quite generic and similar to those produced on plants having a damaged and malfunctioning root system. However, if one is to carry out an effective diagnosis especially for advisory purposes then field diagnosis is equally important.

Generally these symptoms include:

i) Suppressed shoot growth and accompanying decreased shoot-root ratio;

ii) Symptoms of nutrient deficiency, such as chlorosis;

iii) Temporary wilting during periods of mild water stress or during the midday heat, even when adequate soil moisture is available;

iv) Early senescence or die-back.

Figure 2: A tomato (left) and carrot crop showing die-back due to root-knot nematode infection

The common explanation for these above-ground symptoms is that RKN infection affects water and nutrient uptake and upward translocation by the root system. Infected plants are also more susceptible to additional pests and disease.

## 2.2 Below-ground symptoms

The presence of galls on the root system is the primary below-ground symptom associated with RKN infection. The size, shape, frequency and form of galls may vary with the RKN species involved, the number of nematodes in the tissue, plant host species and/or variety and plant age (Figure 3). Contained within the galls are swollen white females accompanied by egg masses.

Figure 3: Varied root galls and deformations on (from left) tomato seedlings, sweet potato, banana and rice following infection with root-knot nematodes.

For root and tuber crops that are susceptible to RKN, their infection not only reduces yield and productivity, but can disfigure and distort crop products reducing their quality and market value (Figure 4). Tuber crops that are additionally used for vegetative propagation and which are infected with RKN pose an additional problem, because if used as seed material will transfer infection to the new crop and to new fields (e.g. yam and potato).

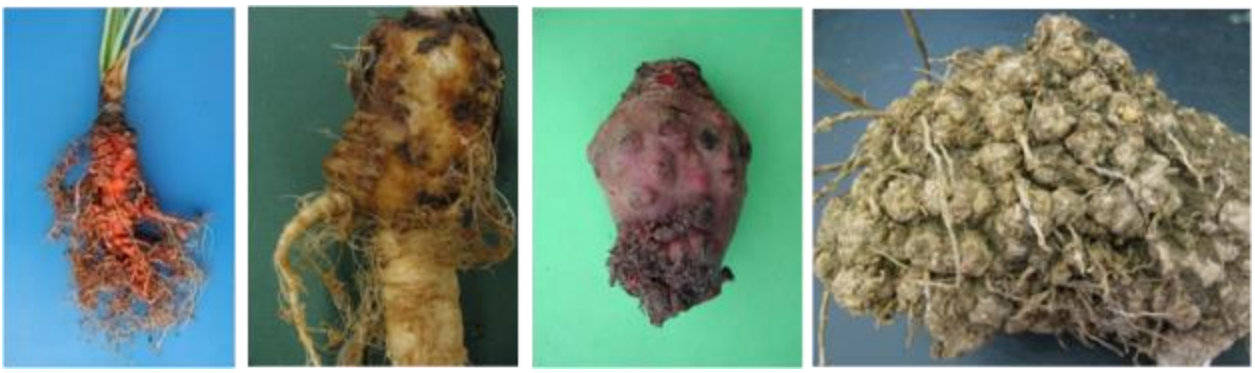


Figure 4: Quality of tuber crops affected by root knot nematodes from left: carrot, parsnip, beetroot, and yam

# 3.0 SAMPLING, HANDLING AND PROCESSING OF SAMPLES

## 3.1 Field sampling

After observing symptoms of RKN in the field, samples should then be collected from the affected plants and from the soil around the roots (rhizosphere). The amount of root damage based on the galling can be estimated visually at the same time as field sampling using a standard scoring procedure. A typical galling scale is provided in Coyne *et al*. (2007) ranging from 1-5 and 1-10, where 1= no galls; and 5 (or 10) = severe galling damage.

When sampling, it is important to consider that nematodes are more often distributed unevenly within a field, and samples should therefore be collected from several areas of the field to ensure a good coverage of the area. Sampling pattern can be random or systematic, but usually the systematic pattern (such as a zigzag pattern) is preferred as it takes into consideration the

irregular distribution of nematodes (Figure 5). Collecting separate samples from both a poor growth area and an area of relatively good growth will enable a comparison.

The soil and root samples should be stored carefully to prevent death of the nematodes and deterioration of the roots. Store the collected samples in a shaded area, out of direct sunlight, and preferably in an insulated cool box. **Do not** leave samples in a closed vehicle in the heat. Samples should be taken to the laboratory for analysis as soon as possible, to determine if nematodes are present and their infestation level. Store samples in the fridge (~10°C) if unable to process immediately.

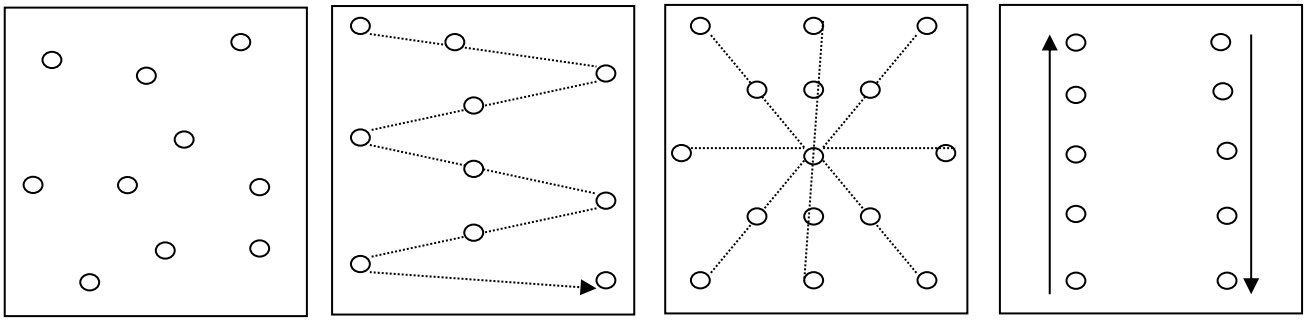


Figure 5: Different sampling patterns

### 3.2 Sampling tools

This can include a range of tools, but is not limited to a spade, a hand trowel, a screwdriver, a soil auger (corer), knives (for cutting roots), scissors, polythene sample bags, tags. Marker pens for labeling the sample bag and a pencil and notebook are also necessary for recording information.

Figure 6: Range of tools useful in sampling for root-knot nematodes

### 3.3 Number of samples

One should take sufficient samples to ensure they are representative of the situation in the field. The greater the number of sub-samples/cores combined for each field the more accurate the assessment is likely to be. A balance between available time and resources is however necessary.

### 3.4 Time of sampling

- The ideal sampling time depends on the type of host crop, its growth stage and the reason for sampling i.e. for predictive or diagnostic purposes;
- For tomato farming, predictive sampling is often conducted early in the season, such as shortly before scheduled planting, or at the end of the previous cropping season to determine the nematode density and damage potential and thus enable a decision to be made on whether to plant or take suitable management action;
- For diagnostic needs, samples should ideally be collected at mid-season or at final harvest. Under heavy infestations, roots may be too deteriorated and rotted at harvest to provide suitable material.

### 3.5 Taking soil samples

- Whether for predictive or diagnostic purposes one should avoid sampling when conditions are very wet or very dry;
- Divide fields larger than 1 hectare into 1 hectare (10,000 $m^2$) plots and sample these plots separately;
- Take 10 to 50 sub-samples (cores) and combine them to make a composite sample that weighs up to 1.0 kg;
- Remove the soil sub-sample from the root zone using a trowel, auger, corer, spade or similar implement that is suitable for the job.

### 3.6 Taking root samples

Roots should be collected at the same time and from the same locations as for soil, and in general should be combined in the same sample bag, so that the soil helps to preserve the roots.

- Lift the plants and their roots from the soil using a spade or trowel, so that a sizeable proportion of the root system is unearthed intact, taking care not to break off the roots; **do not** just pull the plant out of the ground;
- Generally, take 25-100 g of roots per total sample;
- After tapping soil free, randomly remove roots from large root systems with a knife or scissors.

### 3.7 Care of samples

- Samples should be collected in strong plastic bags, and **labeled** clearly and systematically/sequentially to prevent confusion;
- Plastic labels marked with a water-resistant permanent marker or pencil can be placed in the sample bag or alternatively write directly on the plastic bag with a permanent marker pen the sample number or reference;
- Paper labels can be attached to the outside of the bag with wire or twine. Do not place inside the bag as the paper will be destroyed. Do not place paper labels inside the bag as they will deteriorate and likely become unreadable;
- If using paper labels, use a pencil, not a pen but remember that paper labels will deteriorate quickly during wet conditions;
- Record, where possible, the crop and cultivar, the sampling date, the farmer, the location (and GPS coordinates), a reference number (or plot) if within an experimental trial, the previous crop(s), soil type;
- After collection, samples should be placed in a cool box, or packed in strong cardboard boxes and placed in a shaded area where conditions are cool;
- If unable to process immediately, samples can be stored in a refrigerator (approx. $10^{o}$C) for up to 2 weeks. Nematode survival decreases with time however and they should not be kept for a long period of time.

# 4.0 NEMATODE EXTRACTION

To assess the level of RKN infestation, it may be necessary to extract the nematodes and determine the root and/or soil density. In order to do this, samples need to be collected and processed to extract the nematodes for counting and observation under the microscope. The choice of which method to use for nematode extractions will vary from one laboratory to the other and largely depends on the conditions and materials available, the sample type, and the urgency of the extraction results.

## 4.1 Preparation of samples

- Soil samples should be properly mixed before sub-sampling for nematode extraction;
- For dry soil, break up clumps and remove stones, roots and debris;
- Pass (dry) soil through a coarse sieve with holes of approx. 1-2 mm into a suitable container and then mix thoroughly;
- Remove a sub-sample using a beaker or container of known volume e.g. 100 ml;
- From each bulked field sample, two × 100 ml soil samples should ideally be processed and the mean taken;
- Roots should be separated from soil and any soil adhering removed by gently tapping off, or rinsing gently under a tap or in a container of water. The roots should be patted dry with paper towel, and then chopped finely for extraction according to the chosen extraction method.

## 4.2 Extraction of second-stage root-knot juveniles

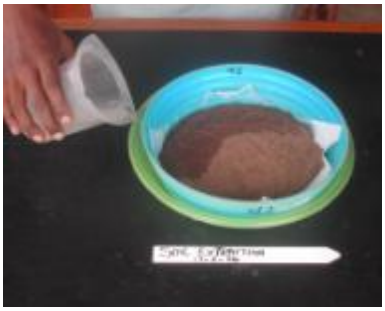
### 4.2.1 *The extraction tray method (Baermann technique*)

- This method largely targets the extraction of RKN from infected roots and soil. This method (or variations of it) is sometimes also referred to as the modified Baermann technique, the pie-pan method, or the Whitehead tray method;
- The method is easy to adapt to basic circumstances using locally available materials and is a simple technique;
- The extractions can sometimes be quite dirty and therefore difficult to count while the proportion of nematodes extracted can vary with temperature, causing potential variation in results between samples extracted at different times;
- The maximum recovery period can take 3–4 days, but 1-2 days should be enough;
- It is very important to ensure good, **consistent labeling** of all trays used for each sample, as it is very easy to make mistakes. Root and soil extractions should be labeled separately.

***For soil samples* (after Coyne *et al*., 2007)**

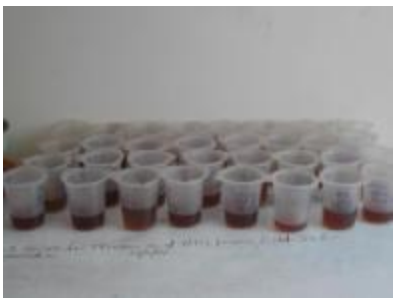
- Remove roots from sample and place in a separate dish. Follow the below procedure to extract from roots, after chopping and weighing a specific amount of roots (e.g. 10 g). Using a coarse sieve, remove stones and debris from soil and break up soil lumps;
- In an open plastic container (basin, bucket) thoroughly mix the soil sample by hand. Remove a known measure of soil (e.g. 100 ml using a 100 ml beaker);
- Place tissue paper (milk filter, paper napkin, etc.) in the plastic sieve/basket (placed on a plastic plate) ensuring that the base of the sieve is fully covered by the tissue;
- Place the soil sample (measured) on the tissue in the sieve. It is important that the soil remains on the tissue paper – spillover results in dirty extractions;





- Add water to the extraction plates. Take care to gently pour water into the plate (dish) between the edge of the mesh and the side of the tray and not directly onto the tissue paper or soil. Add a set volume of water to each dish to wet but not cover the soil, ensuring there is sufficient water and that it will not dry out. **More water is needed for soil samples than root material**. Add more water later if necessary;
- Leave (preferably in the dark) undisturbed for a set period (48 hours if possible) adding more water after 24 hours if it is likely to dry out. By gravitation, nematodes will move from the soil in the sieve through the tissue paper into the water below, resting on the tray/plate;





- After the extraction period, gently drain excess water from the sieve and the soil into the extraction tray;
- Remove the sieve and dispose of soil;
- Pour the water from the plate into a labelled beaker (or cup), using a water bottle to rinse the plate;
- Leave samples to settle.




- To concentrate the nematode extraction for counting purposes, gently pour off or siphon the excess water (taking care not to lose nematodes and sediment) or better still, pass the extract through a small aperture sieve (e.g. 20–30 µm) sieve and collect in a beaker ready for assessing and counting nematodes;
- Rinse the nematodes off the small aperture sieve into a beaker (or tube) for counting, or for preserving, if sending away or counting later;
- If sending away for assessment (identification), the nematodes can be removed from the bottom of the large tube with a pipette, after leaving them to settle, for storage/dispatch in small container such as an Eppendorf tube.

### 4.2.2 *The density centrifugation technique*

**Extraction from soil**

- Put a known amount of soil (after sieving through a large mesh sieve) water in a 500 ml or 1000 ml beaker;
- Add water to approximately half full and stir thoroughly;
- Pour the suspension through a 2 mm mesh sieve collecting the suspension in a large beaker and discard the large debris from the 2 mm sieve;
- Re-agitate the suspension and pour the suspension through nested sieves of 250 µm, 90 µm and 38 µm mesh sieves;
- Rinse the nematodes and residues from the 90 µm and 38 µm mesh sieves into a beaker;
- Place beaker contents into centrifuge tubes and spin at 3000 rpm for 4 min.;
- Discard supernatant and retain nematodes and debris;
- Fill the tube with sucrose solution 454 g (1 lb)/I L water. Stopper the tube and shake until the pellet is suspended;
- Fill the tubes with sugar solution and centrifuge at 3000 rpm for 30 sec. to 2 min. Nematodes remain in suspension;
- Pour the supernatant through a 38 µm sieve, ensuring all sucrose is rinsed off using tap water ;
- Gently wash nematodes off the sieve into a beaker.
- Reduce (or top up) volume of the nematode suspension to a set volume (e.g. 50 ml) in a sample bottle for examination and counting.

### 4.3 Extraction of root-knot nematode females

- Employ the use of a dissecting microscope;
- Carefully tease away the galled root tissue with forceps and a fine needle to release the head and neck of the swollen female;
- Avoid puncturing the body;
- Dissect and store in 1.0% NaCl which prevents females absorbing water and bursting through the osmotic effect of water.

### 4.4 Extraction of root-knot nematode eggs from roots

#### 4.4.1 *Incubation method*

- Select roots of tomato heavily infected with RKN and rinse free of debris;
- Cut/chop roots finely and remove a sub-sample of a set weight;
- Place sub-sample of roots in a jar, or conical flask with water and leave for 2-7 days. Take care not to fully seal the container, but loosely cover to allow for aeration;
- Each day, agitate/swirl the container and gently pour the suspension into a beaker, retaining the plant material in the container;
- Replenish fresh water daily into the container with the root material;
- Collect the suspension after each successive day into the same beaker until final collection is obtained. Concentrate accordingly, and reduce the volume after final collection to a set volume for further assessment/counting/experimental work.

#### 4.4.2 *Use of bleaching agent (sodium hypochlorite; NaOCl)* **(after Hussey and Barker, 1973)**

- Rinse roots free of soil debris;
- Cut/chop infested roots finely and remove a sub-sample of a set weight;
- Place sub-sample of roots in a jar, or conical flask with 1% NaOCl and shake vigorously for 3-4 minutes;
- Pass the solution through nested 150, 90 and 25 µm sieves and rinse through with tap water;
- Dispose of root debris on 150 µm sieve; collect eggs from the 90 and 25 µm sieves into a beaker;
- Collect the suspension and concentrate to a set volume for further assessment/counting/experimental work.

# 5.0 HANDLING NEMATODES

Upon the extraction of nematodes, they can be counted under a dissecting microscope, or further processed for further identification and diagnosis. Individual nematodes need to be selected for preparing identification slides and therefore need to be 'picked'. Individual live female egg sacs also need to be selected and isolated when establishing pure cultures of individual populations or species for experimental or identification purposes.

To be able to morphologically identify RKN, it is necessary to observe specimens under the microscope and measure certain features. RKN are among the more difficult species of nematode to identify accurately, and so it is often necessary to combine observations from J2, adult vermiform males as well as measuring perineal patterns cut from the base of females. Sometimes not all forms are available. The vermiform nematodes need to be individually 'picked' from the extraction suspension and placed on a glass slide.

## 5.1 Picking or 'fishing' nematodes

Various instruments can be used for picking vermiform nematodes, for example a fine insect pin, a bamboo splinter, an eyelash or bristle glued to the end of a mounted needle, a sharpened toothpick, or feather spine.

To view nematode(s) on a slide with a compound microscope, it must first be briefly heated (about 55-60$^{o}$C) on a hotplate to 'heat relax' them. In their relaxed position they take on a species characteristic position.

## 5.2 Fixing nematodes

- It is important to kill nematodes quickly, as each species assume a characteristic 'death shape' when killed quickly, which aids identification;
- Nematodes are best killed with gentle heat (55-65$^{o}$C), which retains the nematode body content; if the temperature is too high or they are heated for too long protein will be denatured and cause difficulty for identification;
- Nematodes can either be killed first and then fixed (preserved) with a suitable fixative (such as formalin or TAF, see Appendix II), or killed and fixed in the same process;
- A simple and efficient method for killing nematodes is to add an equal volume of boiling water to the nematode suspension;
- It may be easier to kill and fix the nematodes in larger tubes then place a reduced volume into smaller tubes, or remove nematodes from the bottom of the tube with a pipette and place in the tubes for transportation.

### 5.3 Other methods

- Nematodes can also be killed by holding the tube containing a small volume of nematode suspension in near-boiling water for 1-2 minutes;
- The simplest method for fixing or preserving samples is to pipette a few drops of formaldehyde (formalin) into recently heat-killed samples;
- Two or three drops into a 7 ml sample bottle are sufficient; larger sample bottles will require more;
- This is a quick and easy method, which will prevent samples from deteriorating during transit and storage before identification, however it does not provide good quality specimens for long-term preservation and can also cause difficulty for identification, especially if not examined immediately. Take great care with formaldehyde as it is dangerous to health;

### 5.4 Preserving root-knot nematodes in root or tuber tissue

- Place a small sub-sample of infected plant tissue into a sample bottle containing lactophenol or lactoglycerol;
- Staining before preserving can help identification;
- Lactophenol can be purchased ready-made, or made by mixing equal volumes of glycerol, lactic acid and distilled water (lactoglycerol) and dissolving a small amount (1%) of phenol into it (lactophenol);
- **Phenol is very toxic** however, so it is best to avoid it and to just use lactoglycerol, although this will help preserve samples for only short periods.

# 6.0 DETECTION OF ROOT-KNOT NEMATODES THROUGH STAINING

Sometimes it is important to observe nematodes while they remain in the root tissues. Alternatively, it may be useful to assess nematode infection levels by counting females and egg masses on root tissue. Observation of nematodes embedded in plant tissue can be made easier by using appropriate stains. The nematode 'acquires' the stain while the plant tissue remains relatively clear. Thick or bulky roots should be sliced thinly before staining.

Stain in lactoglycerol + 0.1% cotton blue or 0.1% acid Fuchsin, then de-stain plant tissue in a beaker containing a solution of equal volumes of glycerol and distilled water + a few drops of lactic acid. Nematodes will remain stained. De-staining is most effective if left overnight or for 1-2 days for larger root material.

### 6.1 Staining nematodes inside the root tissues

- Gently rinse plant material free of soil and other debris and gently dab dry using paper towels;
- Cut or slice thick or wide roots or tubers into small lengths;
- Place in muslin cloth, tie up the corners with a piece of cotton string, and **label** clearly with labels attached to each separate muslin 'bag';
- Bring stain solution, using a glass beaker on a hot plate, to near boiling;
- Place muslin bags into boiling stain solution and leave for approximately 1-3 minutes, depending on root thickness. Use a deep beaker, approx. half full of stain solution, as it will froth up when plant tissue is added;
- Remove the muslin bags and rinse in running water;
- Place the muslin bags in the clearing solution and leave overnight or longer;
- Examine under the microscope. Placing roots side-by-side on a microscope slide and gently squashing them using another slide placed on top enables the stained nematodes to be seen more clearly. Nematodes will be stained red with acid Fuchsin or blue with cotton blue.

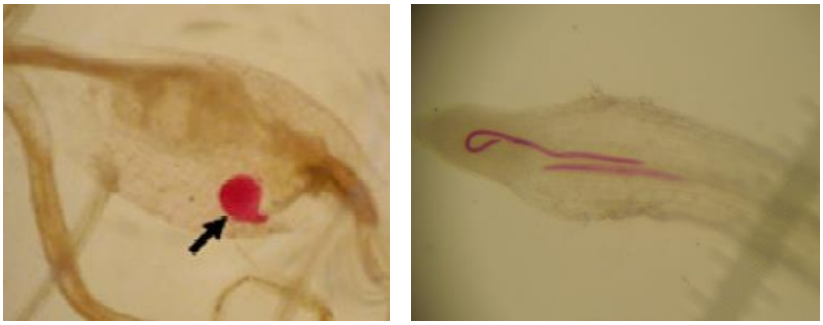


Figure 5: A female and J2 root-knot nematode inside tomato root tissue stained with acid Fuchsin. (Photo: G. Ngundo; E. Galon)

### 6.2 Staining root-knot nematode egg masses

- Gently rinse the plant roots free from soil or debris.
- Place the clean roots in a tray or dish (preferably white) containing Phloxine B (0.15 g/l water) solution and leave for 15-20 min. Count stained (pink-red) egg masses.

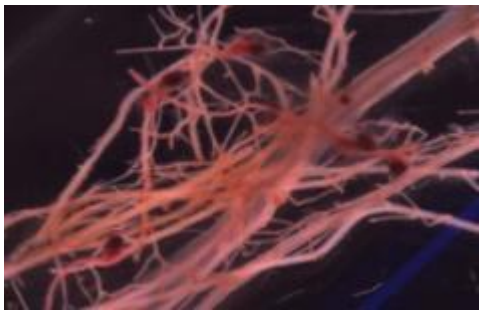



Figure 6: Staining roots in Phloxine B (left) and stained egg masses. (Photo: G. Ngundo)

# 7.0 ESTIMATION OF NEMATODE DENSITY/ ENUMERATION

Extracted nematodes can be viewed and counted using a dissecting or a compound microscope.

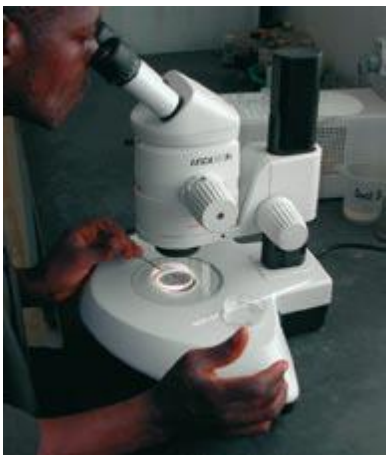



Figure 7: Viewing nematodes under dissecting stereomicroscope (left) and compound microscope (right)

Extract nematodes from a known weight of plant tissue or volume of soil using one of the previously described methods, then:

- Concentrate the extracted nematode suspension to a precise known volume in a measuring cylinder or graduated tube (e.g. 10 ml);
- Shake or stir the suspension to achieve homogeneity immediately before removing aliquots;
- Use a wide mouth pipette (to prevent blockage by debris) to remove aliquots. Pipette tips can be cut if they are too narrow;
- Carefully pipette aliquots into the counting dish, avoiding splashing;
- If only a few nematodes are present, count them in the total suspension volume. If nematode density is high, count the nematodes from an aliquot (e.g. 1 or 2 ml). Dilution of the suspension may be necessary to aid counting, for example doubling the volume with tap water or distilled water;
- Count all the nematodes in the counting dish in a systematic way following the gridlines on the dish. Sometimes nematodes may float on the surface, but adding a tiny spot of detergent/liquid soap helps overcomes this;

Figure 8: Graduated nematode counting dishes and counter

- Use a tally counter to count the number of J2 present, or score using the Roman tally system if no tally counter is available;
- Return the counted aliquot to the suspension after counting;
- Repeat using 2-3 aliquots per sample and then calculate the mean for the combined aliquot score before calculating the total nematode number per sample.

The mean number of nematodes (per ml) calculated from the aliquots should be multiplied by the total volume of the suspension to calculate the total number in the plant tissue or soil that they were extracted from (e.g. 100 ml soil or 5 g root).

# 8.0 ROOT-KNOT NEMATODE IDENTIFICATION

To identify RKN, the best approach is to combine a number of methods which provides for greater accuracy of diagnostics. Information on reproduction on different hosts can be useful. Traditional morphometric methods remain important, but need also to be supported with molecular and/or isozyme techniques, which tend to be more exact. However, this is not possible for all species. As RKN are a difficult group to identify, it is usually best to send samples away for identification to a specialized laboratory. Placing a few galled roots in lactoglycerol is a suitable method, while extracted nematodes can be killed and fixed and additionally sent to trained taxonomists. Samples for molecular analysis are mostly preserved in absolute ethanol but for enzymatic analyses nematodes need to be live. For these analyses contact should first be made with the receiving lab, to establish their preferred mode of preservation and /or transport, and to ensure correct import/export regulations are adhered to.

## 8.1 Use of morphological and morphometric characters

### 8.1.1 Morphometric characters of second-stage juveniles, males and females

Morphometric characters are especially useful to identify the different RKN present in a mixed population. Measurements in µm (Linear) with corresponding standard deviations indicated, is commonly used.

For morphometric characters, at least 25 J2, males, and females should be examined.

- Live specimens should be narcotized with low heat and mounted in water agar (Esser, 1986);
- For females - measure the body shape, stylet length, knob shape and perineal patterns;
- For J2's - measure the body length, tail and hyaline tail length, Dorsal Esophageal Gland Orifice size (DGO), stylet knob length, hemizonid position and tail shape;
- For males - measure the head shape, stylet length, knob shape, and DGO size.


Figure 9: Female head region, whole root-knot nematode juvenile (centre) and characteristic tail region. (Photo: L. Al Banna, J. Mwangi)

**8.2 Preparation of perineal patterns from female root-knot nematodes** (for full details refer to: http://plpnemweb.ucdavis.edu/NEMAPLEX/Methods/Perineal%20Patterns.htm)

- Using needles and a scalpel, pick out RKN females under a dissecting microscope from sections of RKN-infected roots placed in 1.0% NaCl;
- Place the females in a small drop of 45% lactic acid in a Petri dish, or on a Perspex plate;
- Separate a single female away from the main drop of lactic acid, into a small isthmus of its own, held in place by surface tension;
- Using the end of an eye scalpel or razor blade fragment or surgical needle tip cut off the posterior of the nematode;
- Gently clear away the body tissue from the sectioned posterior end and trim the cuticle into a square shape for slide mounting; try to ensure the perineal pattern is in the center;
- Mount the perineal pattern onto a microscope slide in a small drop of glycerin. Place about 5-10 patterns from the same root system (or RKN population) on the slide and seal with a cover slip;
- View the patterns made under a compound microscope and take a photo or make a drawing.

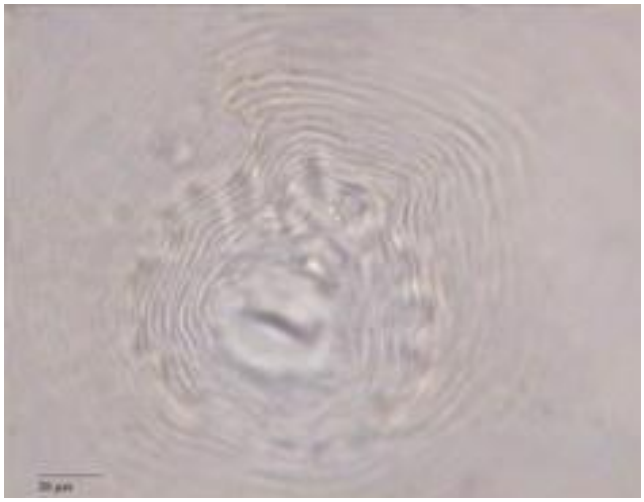


Figure 10: A typical perineal pattern of *Meloidogyne incognita* at X 40 magnification (Photo: L. Al Banna)

### 8.3 Use of enzyme phenotypes

Enzyme phenotypes (isozymes) are used to resolve the identity of various species of *Meloidogyne* (Carneiro *et al.,* 2000). Usually, the adult female stage is the preferred choice since it is associated with the expression of a given target gene product.

1. Malate dehydrogenase (mdh) is *M. hapla* specific.

2. Glutamate dehydrogenase is *M. incognita* specific ( Esbenshade & Triantaphyllou, 1990; Muturi *et al*., 2003).

In surveys targeting *Meloidogyne* species, isozymes can be used as a convenient preliminary stage in species identification.

**Protocol**

- Isolate females from root tissue under a stereomicroscope and rinse them in reagent grade water;
- Transfer them to an ice bath containing 60 µl extraction buffer (20% sucrose, 2% Triton X-100 and 0.01% Bromophenol blue);
- Macerate the nematodes to release body contents;
- Perform polyacrylamide gel electrophoresis (PAGE) using the Phast system (Amershan Pharmacia) on 12% straight gels and native buffer strips;
- Cool the system to a standby temperature of $50^{o}$C;
- Draw the resulting solution from the macerated individual female nematodes into a well sample applicator;
- Using the samples, load them on two Phast system devices and use *M. javanica* females as a standard in each gel for reference;
- After electrophoresis, stain the gel for enzymatic activity in a Petri dish at $37^{o}$C with different staining solutions. Malate dehydrogenase staining solution should contain 0.05 g β-NAD, 0.03 g Nitro Blue Tetrazolium, 0.02 g PhenazineMethosulfate, 50.0 ml 0.5 M Tris pH 7.1 and 7.5 ml stock (10.6 g $Na_2CO_3$ + 1.34 g L-malic acid in 100 ml water) dissolved in 70 ml of reagent-grade water;
- For Esterase enzymes (EST) activity, use staining solution containing 100 ml 0.1 M Phosphate buffer pH 7.3, 0.06 g Fast Blue RR salt, 0.03 g EDTA and 0.04 g α-Naphthyl acetate dissolved in 2 ml acetone and ensure incubation for MDH lasts for 5 min;
- Wash the gels twice with distilled water and stain for EST activity for 30 min. When isozyme phenotypes patterns are clearly visible the enzymatic reaction should be stopped by rinsing gels with distilled water and fixed for 5 min. in a solution of 10% acetic acid, 10% glycerol and 80% distilled water;
- Photograph the gels with a digital camera for interpretation.

### 8.4 Molecular Identification

***DNA Extraction and PCR***

Individual J2 or female stages from each sample are commonly used to provide DNA using the method and procedure adopted by Adam *et al*. (2007). See protocols in Appendix I.

Sequence Characterized Amplified Region (SCAR) markers have been adopted in a number of studies (see Table 1 below) to identify various *Meloidogyne* species including those in mixtures (Zijlstra *et al*., 2000; Randig *et al*., 2002; Meng *et al*., 2004; Adam *et al*., 2007; Tigano *et al*., 2010).

**Table 1: List of SCAR Markers**

| Target spp. | Primer set (5'-3') | Amplicon Size | Code | Reference |
|---|---|---|---|---|
| *M. incognita* | GTGAGGATTCAGCTCCCCAG<br>ACGAGGAACATACTTCTCCGTCC | 955bp | Fi/Ri | Meng *et al.*,2004 |
| *M. javanica* | GGTGCGCGATTGAACTGAGC<br>CAGGCCCTTCAGTGGAACTATAC | 670bp | Fj/Rj | Zijlstra *et al.*, 2000 |
| *M. arenaria* | TCGGCGATAGAGGTAAATGAC<br>TCGGCGATAGACACTACAACT | 420bp | Fa/Ra | Zijlstra *et al.*, 2000 |
| *M .hapla* | TGACGGCGGTGAGTGCGA<br>TGACGGCGGTACCTCATAG | 610bp | Fh/Rh | Zijlstra *et al.*, 2000 |
| *M. chitwoodi* | TGGAGAGCAGCAGGAGAAAGA<br>GGTCTGAGTGAGGACAAGAGTA | 800bp | Fc/Rc | Zijlstra *et al.*, 2000 |
| *M. fallax* | CCAAACTATCGTAATGCATTATT<br>GGACACAGTAATTCATGAGCTAG | 515bp | Ff/Rf | Zijlstra *et al.*, 2000 |
| *M. enterolobii* | GAAATTGCTTTATTGTTACTAAG<br>TAGCCACAGCAAAATAGTTTTC | 322bp | Ff/Rf | Blok *et al*., 2002 |
| *M. exigua* | CATCCGTGCTGTAGCTGCGAG<br>CTCCGTGGGAAGAAAGACTG | 562bp | Ff/Rf | Randig *et al*., 2002 |

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# 10. APPENDICES

## Appendix I: DNA Extraction Protocol (Adam *et al*., 2007)

**Requirements**

1. Worm Lysis Buffer (WLB): 50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 60 µg/ml proteinase K (Roche), 0.45% $NP_4O$ (Fisher Scientific), 0.45% Tween 20 (Sigma) and 0.01% gelatin.

**Procedure for PCR**

1. Pick individual J2's or males using a small needle and place it in 15 µl of WLB on a glass microscope slide and cut into 2 pieces under a stereomicroscope;
2. Transfer the cut nematode in 10 µl of WLB by a pipette into a 0.5 ml centrifuge tube containing another 10 µl of WLB;
3. Apply the same procedure to females but first squash them with forceps;
4. Centrifuge the tubes at 13500 rpm for 2 min;
5. Place at -80$^o$C for 15 min and add mineral oil (7 µl) to each tube and incubate at 60$^o$C for 1 hr followed by 90$^o$C for 10 min;
6. Remove the mineral oil using a pipette after the aqueous sample has been frozen at -20$^o$C
7. Carry out PCR amplifications using rDNA or SCAR primers in 25 µl reactions with 0.5 µl of DNA extract, 0.5 µl of each 10 µm primer, 2.5 µl of X10 buffer, 1.5 µl of 50 mM $MgCl_2$ and 2.5 µl of 200 mM of each dNTPs and 2 units of enzyme (Promega Taq polymerase). All amplification tests include a no-template control;
8. Amplification for the Ff/Rf SCAR primers, can be carried out using the following thermocycler regime: 94$^o$C for 2 min, 45 cycles of 94$^o$C for 30 sec, 50$^o$C for 30 sec, 72$^o$C for 90 sec and final extension at 72$^o$C for 7 min. For Fh/Rh/Fc/Rc SCAR primers, use the same parameters but with 60$^o$C as the annealing temperature. For Fj/Rj SCAR primers, amplifications can be done using cycling conditions of 94$^o$C for 2 min, 45 cycles of 94$^o$C for 30 sec, 64$^o$C for 30 sec, 72$^o$C for 1 min and one final cycle of 72$^o$C for 7 min. For Fi/Ri SCAR primers, use the same parameters but with 62$^o$C as the annealing temperature;
9. Size-separate the PCR products using 1% agarose (v/v) in Tris Borate EDTA (TBE) buffer at 50 V followed by UV illumination after staining with ethidium bromide.

## Appendix II: Fixatives

The most suitable fixatives to use are:

**TAF**
**T**riethanol**a**mine 2 ml
**F**ormalin (40% formaldehyde) 7 ml
Distilled water 91 ml
The fixative remains stable for a long time and nematode appearance remains lifelike because the specimens do not dry out.

**FA 4:1**
**F**ormalin (40% formaldehyde) 10 ml
Glacial **a**cetic acid (propionic acid) 1 ml
Distilled water 89 ml
In FA 4:1, nematodes maintain their structure though they may become discolored after some time.

**Formalin glycerol**
**F**ormalin (40% formaldehyde) 10 ml
**G**lycerol 1 ml
Distilled water 89 ml