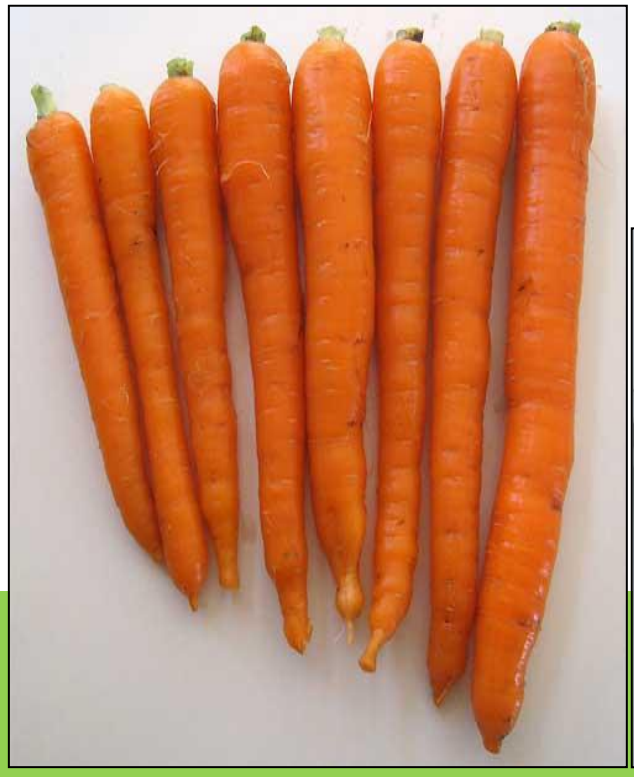

PROTOCOL

for

in vitro culturing of lesion nematodes: *Radopholus similis* and *Pratylenchus* spp. on carrot discs

D.L. Coyne, O. Adewuyi and E. Mbiru

About IITA

The International Institute of Tropical Agriculture (IITA, www.iita.org) is an Africa-based international not-for-profit research-for-development organization, established in 1967, and governed by a board of trustees. IITA is Africa's lead research partner facilitating agricultural solutions for hunger and poverty in the tropics. It is a member of the CGIAR Consortium, a global research partnership for a food secure future.

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A PDF version of this document is also available on the IITA website.

FORWARD

This document has been produced by the International Institute of Tropical Agriculture (IITA) in order to provide a clear and simple, repeatable mechanism to establish and maintain cultures of lesion nematodes for use as inoculum in experiments.

The manual presents a traditional protocol to culture lesion nematodes for use in screening and efficacy exercises. The protocol is focused on lesion nematodes, using carrot discs as a food source/culturing medium. Carrot discs allow the *in vitro* rearing of high numbers of *Pratylenchus* spp. and *Radopholus similis,* in particular, under sterile conditions to provide a clean, uniform and pure source of inoculum. The protocol builds upon previous technical guidelines, such as by Speijer and De Waele (1997), aligning descriptive instructions with visual figures as much as possible, to create an easy-to-follow guide.

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The authors are grateful to colleagues Inge Van den Bergh and Jenna Ross for assistance, advice and help in producing this document. The document was produced to support activities supported by the Federal Ministry for Economic Cooperation and Development (BMZ) Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) GmbH, on behalf of the Government of the Federal Republic of Germany, within the framework the project: '*Local Focus: safe and effective pest and crop management strategies to strengthen the vegetable value chain in the humid tropics*'.

CONTENTS

INTRODUCTION

Nematodes can feed as migratory ectoparasites and endoparasites, thus their feeding habits determine the type of plant tissue required for their culture. Nematodes that feed on vascular tissue, inducing a specific host response, require differentiate tissue for reproduction in dual callus. This is the case of the sedentary endoparasites *Meloidogyne, Heterodera* and *Globodera*. In contrast, migratory nematodes do not require vascular elements, and reproduce readily on undifferentiated callus tissue or material, such as carrot discs. Carrot has been shown to be a suitable medium for nematode multiplication of a number (though not all) of migratory species.

Economically important nematode species that multiply well and can be cultured *in vitro* on carrot discs include *Radopholus similis* and *Pratylenchus* spp.: *P. brachyurus*, *P. coffeae, P. scribneri, P. sudanensis, P. vulnus*, *P. zeae*. Carrot discs enable the rearing of high numbers of these nematodes for timely use in experiments and for screening purposes (e.g. O'Bannon and Taylor, 1968; Mudiope *et al*., 204; Kagoda *et al*., 2010).

The procedure outlined here provides a descriptive method for multiplying pure cultures of lesion forming endoparasitic nematodes. The procedure is based on previous descriptions, but here we avoid using strong sterilants and further, aim to provide a clearly outlined and visually informative guide, to enable an easy-to-follow procedure. We describe the recovery of the nematodes from the tissue of plants that the nematodes naturally infect, the preparation of the nematode extract for inoculation onto carrot discs, that themselves have been prepared to receive the nematodes. It is necessary to use sterile techniques and to sterilise the nematodes to prevent contamination during incubation (3-4 weeks+), which would otherwise destroy the nematode cultures. Some descriptions use a strong, toxic sterilant, such as mercuric chloride ($HgCl_2$) or similar, in addition, or as an alternative to the streptomycin sulphate described here.

We have avoided this however, as these tend to be highly toxic. We find that by carefully following the sterilisation process and sterile techniques outlined here, the use of

streptomycin as a sterilant is sufficient and contamination can be limited. Different nematode populations, however, can vary in their sensitivity to streptomycin sulphate and so while we present a useful and practical protocol, adaptations may be necessary depending on the target nematode population and indeed the local conditions.

The initiation and maintenance of *in vitro* cultures require five steps:

1. Isolating target nematode species. This involves extracting the target species from the infected source material of roots or plant tissue.

2. Preparation of *in vitro* plant tissue (carrot discs). Selection, cleaning, peeling and sterilisation of the carrot to reduce contamination and cutting into discs.

3. Sterilisation of target nematodes. Preparing nematodes to enable the generation of pure, uncontaminated cultures.

4. Transfer of nematodes to *in vitro* plant tissue (carrot discs). Inoculating prepared, sterile nematodes onto carrot discs for incubation in Petri dishes.

5. Culture maintenance through sub-culturing. Regular renewal of cultures.

EQUIPMENT AND MATERIALS

- Nematode inoculum of target species (e.g. *Radopholus similis*, *Pratylenchus* spp. etc.)
- Small glass /disposable Petri dishes 3-5 cm diam.
- Microscope (e.g. compound or dissection microscope)
- Paper towel/kitchen paper/milk filter
- Measuring cylinder and beaker
- Incubator
- Laminar flow cabinet
- Autoclave
- Blender
- Analytical balance
- Sink with running water
- Sterilized distilled water
- Glass block
- Streptomycin sulphate
- 70%, 96% Ethanol
- Aluminum foil
- Pasteur pipette
- Parafilm
- Surgical gloves
- Canister can
- Sterile forceps
- Sterile vegetable peeler
- Sterile knife
- Spirit lamp or Gas lamp
- 0.2 µm microfilter
- Wash bottle
- Raw carrots

PROCEDURE

1.0 Extraction of nematodes from infected roots

1. Choose infected tuber/roots for extraction of the target nematode species to be cultured, such as the necrotic banana root below left, pre-identified as being caused by *Radopholus similis* (burrowing nematode), or maize roots infected with *Pratylenchus zeae*.


2. Chop up the roots/tuber cortex/plant tissue with a knife on a chopping board or with scissors, and weigh out a sub-sample (e.g. 5 g).


3. Prepare a modified extraction plate using a plastic sieve lined with milk filter/tissue paper for nematode extraction and place the chopped root material into the centre of the tissue paper inside the sieve.


4. Carefully pour water under the sieve onto the collection plate where it will soak up into the paper and root material. Ensure there is sufficient water for the root sample to remain fully submerged for 12-48 hours.


5. Carefully remove the sieve from the collection plate after the extraction period and pour the nematode extraction from the collection tray into a beaker/cup.

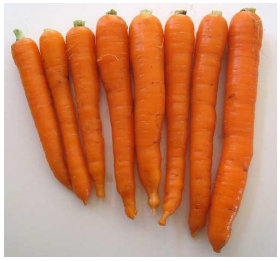

6. Label beakers clearly with details, such as date, sample name, location. Leave samples to settle until ready to use, then reduce the excess volume by carefully decanting the excess, or pouring through a 28 µm (or similarly small) aperture sieve. Rinse with distilled water and collect into a beaker.

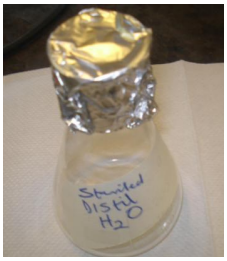
7. Keep nematodes in a beaker or tube in distilled water until required, but without leaving for too long. Preferably use within one day. Store overnight on the bench or in the fridge.

2.0 Preparation of in vitro plant tissue (carrot disc)

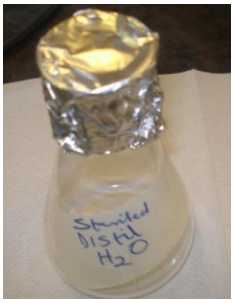
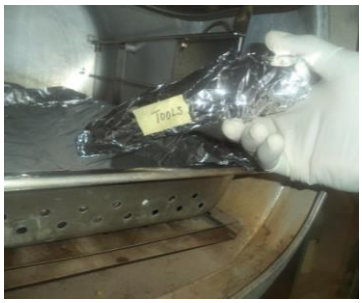
1. Select clean, unblemished carrots with a cylindrical rather than tapering shape. Carrots should be without cracks and not overly thick. Choose a cultivar that is less succulent (e.g. cv. Nantes), which tend to be less susceptible to rot during incubation compared with less dense, more succulent types.

2. Wash selected carrots under running water and clean off any soil or debris. Then clean with sterilised distilled water before use.





3. Sterilise plenty distilled water, tools, Petri dishes, glass blocks, glass plate, kitchen paper and Pasteur pipettes by autoclaving at 121˚C for 15 minutes. Cover distilled water and wrap tools and materials with aluminum foil.

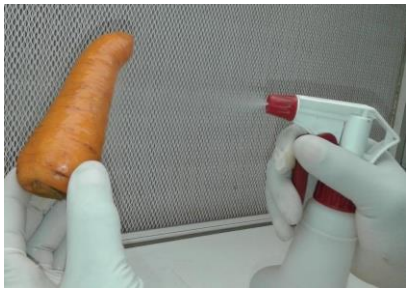




4. Open the laminar flow cabinet and swab down the surface with 70% ethanol to sterilise the working surface. Then sterilise the tools and tongs by dipping in or spraying with ethanol and flaming over the spirit lamp.

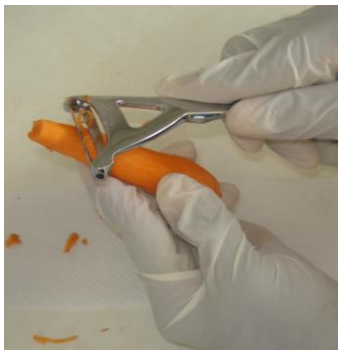
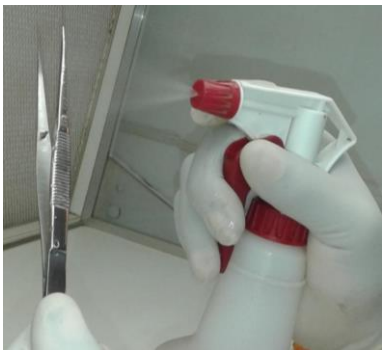
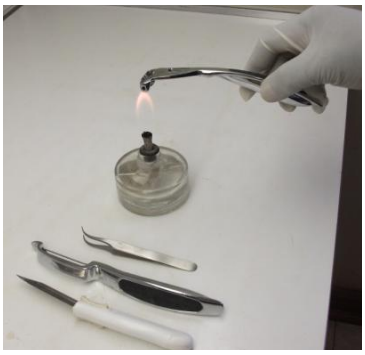
NB: use 96% ethanol for the spirit lamp, which has a longer flaming span than 70%.



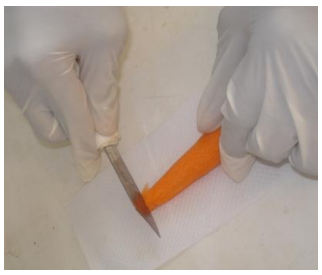
5. Inside the laminar flow sterilise the carrot by flaming. Hold the carrot with the sterilised tongs /forceps, spray it with 70% ethanol and flame over spirit lamp. Repeat this two more times. Sterilise equipment (forceps, peeler and knife) with ethanol and flaming each and every time the equipment is used.



6. Working on the autoclaved paper towel and glass plate, remove the crown end of the carrot with a sterile knife and dispose of it; do not use the crown end for inoculation. Peel the carrot with the sterilised vegetable peeler. Sterilise knife and peeler after every use.

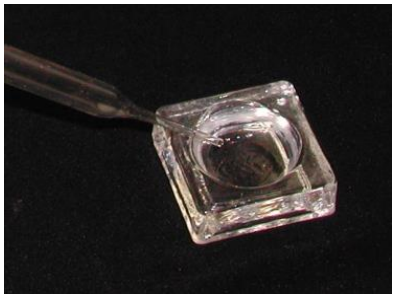
7. Cut the peeled carrots into 0.5 cm thick sections of 3-4 cm diam., using the sterile knife. Discs must not be less than 2 cm diam. Sterilise the knife after cutting each disc. Using the sterilised forceps transfer the cut carrot discs into sterilised glass or sterile disposable Petri dishes (3-5 cm diam.) and cover. Sterilise the forceps after each transfer.



*****NOTE: use of glass Petri dishes tend to result in lower rates of contamination*****

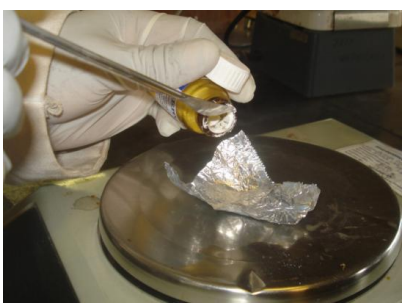
3.0 Selection and sterilisation of nematodes

1. Place a concentrated volume of the nematode extract into a small (3-5 cm diam.) Petri dish under a dissecting microscope. Use a fine pick or bamboo splinter to 'fish' (pick) 60-100 nematodes – but this depends on how many carrots are to be established/ required. Select **only females** of the target species of nematode and place them into a glass block containing sterile distilled water. If only a few nematodes are available, juveniles may also be selected. Place a cover slide over the glass block and store carefully on the bench until ready for use.

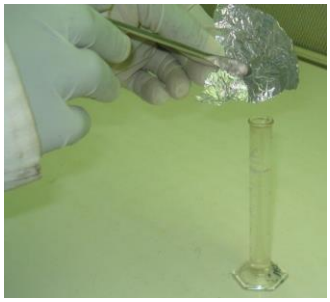
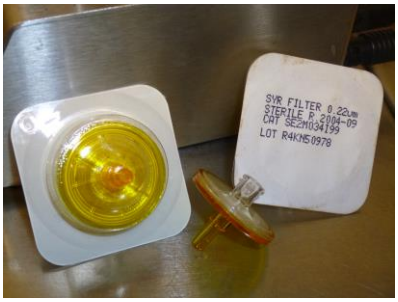



2. Swab down the laminar flow with 70% ethanol then transfer the picked nematodes from the glass block using a sterile pipette into a sterilised glass measuring cylinder containing 10 ml of sterile distilled water.


3. Weigh out 0.06 g (6 mg) of streptomycin sulphate onto sterile aluminum foil.



4. In the laminar flow transfer the 6 mg of streptomycin to the glass measuring cylinder containing 10 ml of water, providing 6000 ppm of streptomycin. Gently mix the solution to dissolve the streptomycin. *If available* filter the 10 ml streptomycin solution through a 0.2 µm microfilter with a sterile 10 ml syringe, which will further reduce/prevent contamination, and then transfer to a fresh sterile measuring cylinder.

5. Allow the nematodes to settle to the bottom in the measuring cylinder for about 1 hour. With a micro-Pasteur pipette reduce the volume in the measuring cylinder from the surface, to about 5 ml, taking care not to disturb or remove the nematodes at the bottom. From the prepared 10 ml streptomycin solution, pipette 5 ml into the nematode suspension to surface sterilise the nematodes and reduce contamination. Leave for another 1 hour to settle, then reduce the volume again, replenish with sterilised distilled water to 10 ml and leave for 1 hour. Repeat the process for a third time, leave for 30 min and finally reduce the volume to 2-3 ml.

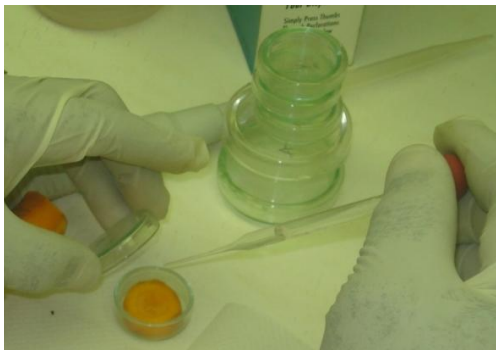
****NB**: streptomycin concentration can be increased if contamination is experienced, or reduced if nematodes are weak and dying; adapt according to local conditions.

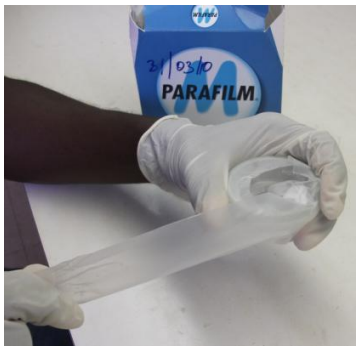
*****NOTE: Reduction of surface water should be carefully conducted so as not to disturb and remove nematodes*****

4.0 Inoculation of the carrot discs with nematodes

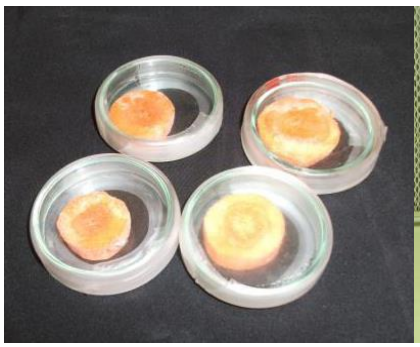
1. Retrieve the Petri dishes with the prepared carrot discs and place in the laminar flow cabinet. Place the surface-sterilised nematodes from the measuring cylinder close at hand and using a sterile Pasteur micropipette transfer them onto the surface of the carrot.

2. Gently place small drops of nematode suspension onto the margin/edge of the carrot discs. The aim is to transfer the nematodes in as little water as possible; the smaller the drops the better. Aim to deliver 15-50 nematodes per disc in a maximum of three drops of nematode suspension per carrot disc. The number of nematodes in the suspension will determine the number to transfer. This is a good number for culture maintenance. For mass multiplication though, use about 100-150 nematodes. Replace the Petri dish lid and continue with the next carrot disc.





3. Seal the Petri dishes containing inoculated carrots with Parafilm and label accurately. Labels should include the date of inoculation, nematode species, origin, crop of origin.



4. In the laminar flow cabinet place the Petri dishes with the inoculated carrot discs into a canister or plastic box. This helps to prevent mite infection. Place the canister in an incubator for 3-4 weeks at 25-28°C – although the temperature depends on the species to culture. Incubation in the dark is also necessary to replicate underground conditions. Check the discs periodically and dispose of any discs that are contaminated. Callus (whitish matter) occurring on the surface of the carrot discs is a good indicator of healthy cultures during incubation.

5. After 3-4 weeks, check for nematodes emerging onto the surface of the carrot or collecting on the Petri dish glass surface around the edges of the carrot. Do this by placing the dish under a dissection microscope **without removing the Parafilm** or the lid. Nematodes will begin to exit the carrot when nutrients start to become depleted and at this point the nematodes are ready for harvesting. This can continue over a number of weeks until the carrot is fully depleted and then needs sub-culturing.

5.0 Sub-culturing

The initial inoculum and the nematode species or strain, will affect how soon the nutrients become depleted and initiate nematode migration from the carrot. Incubation time will vary with the aggressiveness of the nematode species or strain being cultured – e.g. some strains of *R. similis* can take just 3-4 weeks but other, less aggressive, populations can take up to 1-2 months before they emerge. For *P. zeae* and *P. sudanensis* incubation time tends to last 3 months before nematodes emerge (Mudiope *et al*., 204; Kagoda *et al*., 2010). Once the nematodes emerge, they can be harvested for experimental purposes or sub-cultured for maintenance if there is no immediate use.

When required for an experiment, emerging nematodes can be rinsed from the Petri dish and from off the carrot surface into a collection beaker, using a water bottle.

The nematodes can be harvested and stored in the fridge at 4^{o}C until ready for use, preferably within 1 week, so that nematodes remain fresh. The nematodes can, however, remain viable for up to 2-3 weeks. To rinse nematodes from the carrot cultures it is essential to work inside the laminar flow cabinet, and use the sterile techniques described above in Sections *3.0* and *4.0*. Remove carrot discs with sterilised tongs and rinse the carrot with sterile distilled water over a beaker. Place the carrot disc back into the Petri dish, replace the lid and re-apply the Parafilm around the Petri dish. Repeat the process with the remaining carrot discs, bulking the nematodes in the beaker. Store the harvested nematodes in the fridge at 4^{o}C.

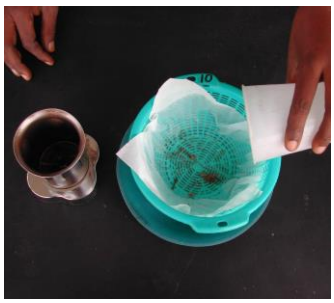
Nematodes will continue to emerge from the carrot disc provided that a food source (nutrients) remains available. This can be observed from the color of the carrot which gradually changes from the original orange, to a brownish color, at which point it is necessary to sub-culture.

Remove the carrots from the incubator to sub-culture the nematodes onto fresh carrot discs. For sub-culturing, it is best to use those discs from which many nematodes are

emerging. Prepare the carrot discs according to the procedure outlined in Section *2.0* above.

1. To extract the nematodes from the carrot discs cut the discs into small pieces or chop roughly and place into a blender and extract nematodes using the method set out above in Section *1.0*.



2. After extraction, collect the nematodes into a beaker and sterilise them according to Section *3.0* above, followed by the procedure for inoculation onto carrot discs in Section *4.0*.

3. Picking the nematodes individually is not necessary when sub-culturing as the culture is already a pure population. Therefore, collect the nematodes in a beaker, agitate to equilibrate nematode distribution in the beaker and remove a known aliquot volume (e.g. 1 ml) using a pipette. Place the aliquot into a counting dish and, under the microscope, count the number of nematodes in order to determine nematode density and the volume required to transfer to the sterilisation process (Section *3.0*). If there are too many nematodes to count using e.g. 1 ml, then reduce the amount (e.g. 0.5 ml) and add distilled water to the counting dish to dilute and enable ease of counting. Assess nematode density using 2-3 separate aliquots and calculate the mean.

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