



***PROTOCOL***  
*for*  
**Nematode Resistance Screening**  
**Root-Knot Nematodes**  
***Meloidogyne spp.***

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## About IITA

The International Institute of Tropical Agriculture (IITA, [www.iita.org](http://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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## FOREWORD

This document has been produced by the International Institute of Tropical Agriculture (IITA) in order to provide a clear and simple mechanism to identify and establish resistance of suitable cultivars of vegetables against root-knot nematodes.

The document was produced to support activities supported by the Federal Ministry for Economic Cooperation and Development (BMZ) and Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) GmbH, on behalf of the Government of the Federal Republic of Germany within the framework of two complementary projects: *'Beating Begomoviruses: Integrated crop management and disease-resistant hot pepper, tomato and mungbean to improve livelihoods in tropical Asia'* led by AVRDC-the World Vegetable Center, and the *'Local Focus: safe and effective pest and crop management strategies to strengthen the vegetable value chain in the humid tropics'*, and also by the Africa-Brazil Marketplace for the project: *'Species identification of root-knot nematodes (RKN) through improved diagnostic techniques aimed at durable resistance in vegetables grown in peri-urban systems in Africa'* both led by IITA.

The protocol presents a procedure for screening for resistance against root-knot nematodes using plants grown in pots, and inoculated with root-knot nematodes. The protocol is focused on root-knot nematodes, using tomato as a target host to screen for resistance but can be modified for use with other nematodes and hosts. The protocol provides an outline for a method that can be consistently implemented across different situations, while appreciating that materials and conditions may differ depending on circumstances and conditions at various sites.

A PDF version of this document is also available on the IITA and The World Vegetable Center websites.

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# 1. INTRODUCTION

Plant parasitic nematodes can feed as 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 differentiated tissue for reproduction in dual callus. This is the case of sedentary endoparasites such as species of *Meloidogyne*. These nematodes enter the roots at juvenile infective stages, usually behind the root tip, where they find a feeding site and then remain in position, immobile, until their lifecycle is completed. They feed from the specialised (modified) cells situated around the female head.

Root-knot nematodes (genus *Meloidogyne*) are obligate plant parasites. They are highly polyphagous and among the most important biotic constraints affecting crop production. They are likely the most economically important group of plant parasitic nematodes. The infective second-stage juveniles hatch from eggs that are contained within an egg mass of between 500-1000 eggs. The juveniles are microscopic and motile, moving within a film of water to locate root tips, penetrate and migrate towards the vascular tissue. Here they establish themselves for the duration of their lifecycle, feeding from several enlarged cells that they manipulate. The host reaction to this infection, depending on crop, cultivar and *Meloidogyne* species, is the characteristic symptom of swollen roots, or galling, where cells neighbouring the giant cells divide and enlarge. Some host roots however, upon infection, do not react by galling, but instead are affected in other ways, such as becoming stunted, stubby or alter root shape. However, infection with root-knot nematodes invariably leads to suppressed root development and consequently reduced crop productivity – unless resistant. The root-knot nematode life cycle is completed in 3-6 weeks, depending on *Meloidogyne* spp., host, conditions and temperature.

Root-knot nematodes can be cultured *in vitro* on transformed tomato roots or alternatively on host plant roots in pots. Tomatoes are regarded as the universal host for *Meloidogyne* spp. Although not all species will infect and multiply on tomato, many will, especially the more economically damaging and common species, such as *M. arenaria*, *M. enterolobii*, *M. hapla*, *M. incognita*, *M. javanica* etc. Tomato plants are suitable hosts for multiplication of root-knot nematodes because they are highly susceptible and are easy to cultivate in pots in the screenhouse. Other plants similarly perform this role, but tomatoes tend

to be more commonly used to rear and multiply nematode inoculum and also to maintain species populations for experimental use.

The protocol presented here is focused on root-knot nematodes, using tomato as a target host to screen for resistance, it can be modified for use with other nematodes and hosts. The method focuses on plants with small seeds. Plants with large seeds, such as pumpkin or cowpea, support extended seedling growth with minimal nutrient supplement, which can be assayed in paper pouches, held in racks and in the absence of soil (see Atamian *et al.*, 2012).

#### **PRE-EXPERIMENT CONSIDERATIONS**

- The availability of sterilised soil or sterile potting media.
- The availability of nematode inoculum for inoculation at the suitable time.
- Sufficient time to conclude the experiment.
- Sufficient space in screenhouse/ shadehouse/ glasshouse.
- Timing of harvest – sufficient hands to conduct assessments at harvest.

## **2. OBJECTIVES**

The main consideration in this protocol is the identification of root-knot nematode resistance in cultivars, or breeding lines, of target crops. The protocol outlines the basic requirements and procedures to screen plants for resistance, with tomato as the target crop. A secondary consideration however, concerns the assessment of resistance against a range of *Meloidogyne* spp. or different populations to emphasise the genetic variability between species and within species even. It is important to note that results of screening exercises as outlined here, are reflective of resistance against the species used and in some cases against the specific populations of the species.

### 3. THE SCREENING PROCESS

1. Define a timeline
2. Prepare materials and equipment
3. Soil Sterilization
4. Define data collection, basis of resistance
5. Establish seedlings
6. Prepare nematode inoculum
7. Maintain infected plants/experiment
8. Harvest plants for assessment

#### STEPS TO FOLLOW

- 1. Soil sterilisation** - The soil/sand is sterilized to ensure all nematodes are removed.
- 2. Seedbed preparation** - Seed trays or pots with sterile soil are set up for planting clean and viable seeds of required tomato cultivars.
- 3. Transplantation and experimental setup** - Transplant two seedlings (two-three weeks old) to small one litre pots filled with sterilised soil/sand. A suitable experimental design should also be determined (e.g. Complete Randomised Design (CRD) or Completely Randomised Block Design (CRBD)).
- 4. Preparation of nematode inocula** - Nematode eggs from infected roots, are collected and allowed to hatch into juveniles (J2 stage).
- 5. Nematode inoculation** - The nematode inoculum is introduced to the tomato root zone.
- 6. Experimental maintenance** - Provide suitable conditions to ensure good plant growth, such as irrigation and nutrient application, and to protect against pests and diseases.
- 7. Data collection and analysis** - Collect target experimental data and analyse using appropriate statistical methods.

#### *3.1 Define a timeline*

Provided that the inoculum is available for the experiment, this procedure can be completed within about 10 weeks, from sowing to harvest. Depending on conditions, the level of infection suitable for differentiating resistance between treatments may vary. Use of excess inoculated

plants of the 'check' susceptible cultivar will enable galling development to be monitored and therefore help determine when plants are suitable for harvest.

### **3.2 Equipment and materials**

#### **Equipment**

- ❖ Steam steriliser or autoclave
- ❖ Microscope (e.g., compound or dissection microscope)
- ❖ Blender
- ❖ Balance
- ❖ Working area with sink, running water and bench
- ❖ Screenhouse

#### **Materials**

- ❖ Nematodes of target species (e.g., *Meloidogyne* spp.)/ infected plants
- ❖ Certified tomato seeds (check cultivar(s) and experimental lines)
- ❖ Sterilised soil and sand (potting media)
- ❖ Plastic sieve and collection tray/plate
- ❖ Milk filter/tissue paper
- ❖ 250 µm, 25 µm sieves
- ❖ Beakers
- ❖ Distilled water
- ❖ NaOCl (sodium hypochlorate/bleach)
- ❖ Phloxine B, or erioglauanine (stains)
- ❖ Measuring cylinder
- ❖ 10/20 litre buckets
- ❖ Seedling trays
- ❖ 1 litre pots
- ❖ Pipette
- ❖ Nematode counting dish
- ❖ Fungicide/insecticide
- ❖ Labels
- ❖ Data collection forms

### **3.3 Soil sterilisation**

Ensure that the soil used as potting media is sterilised and free of nematodes and pathogens. Sterilise soil and sand at least one week prior to use using an autoclave, or steam steriliser. When using an autoclave place soil and sand in autoclave bags and seal ends with autoclave tape. Items should then be autoclaved for 20 minutes at 121°C.



*Sterilisation of soil and sand using autoclave or larger steam steriliser.*

Following sterilisation leave soil for a minimum of one week to settle and allow gases to dissipate and equilibrate.

Periodically, the sterilisation process should be assessed. For this follow the soil extraction procedure in Appendix 1, or see Practical Nematology Manual (Coyne *et al.*, 2007) for a detailed description.

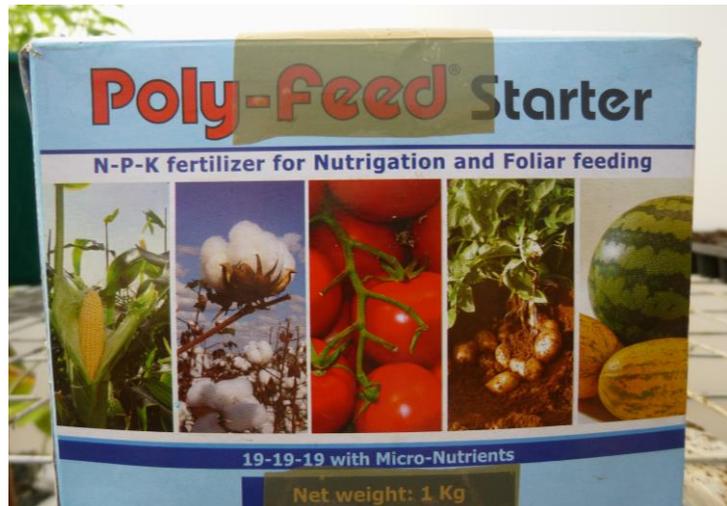
### **3.4 Define data collection, basis of resistance**

Before sowing seeds, it is essential to prepare a plan of the experimental layout of the varieties/lines under assessment (Appendix 2), following a randomized arrangement. When screening in trays, one tray should ideally represent a single replication. Use a pre-printed plan of each replication for guidance when sowing seeds and follow it accurately, labeling correctly. When using pots, varieties can be grouped together until transplanting or inoculation, after which the experimental layout should be followed. Arrange cultivars for assessment according to the layout using suitable design (e.g., Complete Randomised Design (CRD) or Completely Randomised Block Design (CRBD)). Label pots by sequential number, not treatment, in order that assessments are unbiased.

Use 5-10 replications per treatment (cultivar), depending on space and resources, but more is better to reduce variability in analysis.

### ***3.5 Establish seedlings***

Screening assays can be undertaken in pots or in seedling trays. For pots, the seeds can be planted into trays / seed beds for transplanting later into pots or can be sown directly into pots. For higher throughput screening assays using trays, sow seeds into trays of sufficient capacity to support tomatoes for the period of the exercise. Upon germination, apply locally available fertilizer once a week at appropriate rates.



#### **Pots**

For pot assays, plant the seeds into single pots in suitable soil-sand mix or a commercially available organic rich potting mixture. Sow two seeds per pot if sufficient seed is available but reduce to a single plant one week after emergence. Maintain in a greenhouse at 22-28°C.



*Healthy and viable seedlings of tomato planted in large bucket/basin and seedling trays for transplanting.*

If transplanting to pots, do this at 3-4 weeks after sowing, at the two true-leaf stage, in sandy soil (two parts soil to one part sand) and maintain in a greenhouse at 22-28°C for 10-14 days. Apply fertilizer to plants according to recommendations but generally once a week. Pots must have drainage holes at the bottom to drain off excess water.

### **Trays**

For high-throughput screens, plant seeds directly in trays in sandy soil according to experimental layout and maintain as above; ensure individual wells will support tomato development for the duration of the experiment.

**\*\*\*NOTE: label treatments/cultivars CLEARLY\*\*\***

**\*\*\*NOTE: always include additional pots in the experiment of the check/ regular cultivar, inoculated with nematodes in order to assess for nematode development by destructive sampling – without disrupting plants in the screen test\*\*\***

### **3.6 Preparation of nematode inoculum**

There are two principal procedures for collecting inoculum: using sodium hypochlorate (NaOCl) or picking egg masses off. Collecting egg masses takes more time, and involves manually removing the egg masses from the roots and hatching the juveniles for live infection in water. The procedure with NaOCl is more immediate and time efficient. It is based on semi-dissolving the gelatinous egg mass thus releasing the eggs into suspension, which are then hatched out (or can be used directly as inoculum). The NaOCl can affect egg viability though, so it is necessary to limit exposure to the chemical and rinse the eggs well. If using the eggs directly after collection, then a hatch assay should be performed to provide an accurate indication of viable inoculum.

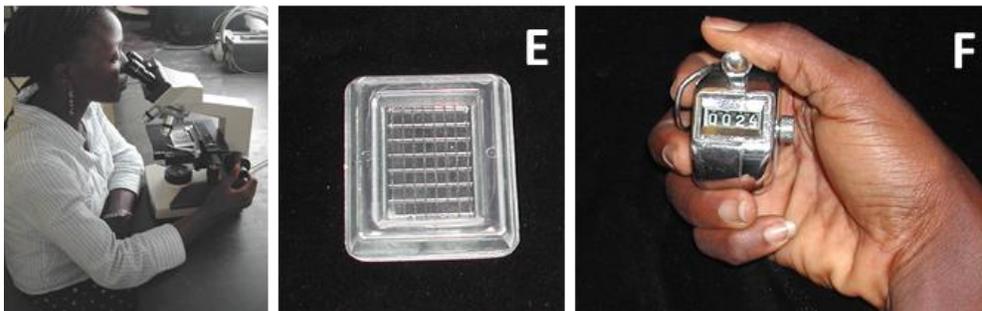
#### **Egg mass collection**

- Collect tomato plants infected with root knot nematodes of the target species (A).
- Remove from pots and gently rinse roots in a bucket filled with water or under a running tap.
- Gently, take galled root segments, place on Petri dish with some water and under the dissection microscope remove egg masses using scalpel and fine tweezers (B).

- Carefully place egg masses into distilled water in ink blocks (C) or other small vessels and incubate in the dark until the juveniles emerge - approx. 48 h but good to check after 24 h however. Hatch will depend on a range of factors, such as temperature.



- Egg masses can be stained with 0.05% Phloxine B or 1 mg/L erioglaucine to increase their visibility.
- After 48 h, or once majority of the eggs have hatched, collect juveniles into a measuring cylinder. Determine the nematode number (E) by taking a measured aliquot (e.g., 1 ml) after shaking and place in a counting dish (F) and accurately determine the number under a microscope using a counter (G). If nematode density is too large to count easily, dilute the volume in the measuring cylinder, or remove a smaller aliquot (e.g., 0.1 ml) and add distilled water in the counting dish.



#### *Sodium hypochlorate method*

- Remove the tomato plants infected with root-knot nematodes of the target species (A) from the pot and gently rinse roots in a bucket filled with water or under a running tap.
- Cut roots into 1-2 cm sections (B).

- Place roots in a large beaker or conical flask with 0.5% NaOCL and shake for 2-3 min. (C) or place in blender and blend for 1-2 min (D).



- Pour the suspension through a 250  $\mu$ m sieve nested over a 25  $\mu$ m sieve to collect the eggs (E) or pour through the 250  $\mu$ m sieve into a bucket and then pour this through the 25  $\mu$ m sieve, if easier. Retain the root material from the 250  $\mu$ m sieve to repeat.
- Quickly rinse the eggs and juveniles, captured on the 25  $\mu$ m sieve, under running water (F) then wash them off the sieve into a beaker using a water bottle and distilled water (G).



- Place the roots from the 250  $\mu$ m sieve into a large beaker or conical flask with distilled water and repeat the above process to release any remaining eggs/juveniles.
- Collect eggs and juveniles from the 25  $\mu$ m sieve as above and combine (G).
- Determine egg number as above.
- Store in a beaker on the bench or place into a tube ready for use as inoculum.



### *Hatching nematode eggs from NaOCl method*

Line a clean small sieve with a few layers of Kimwipe/milk filter/tissue paper and place on a glass Petri dish. Allow a small distance between the bottom of the sieve and the dish. Similarly, a plastic sieve and plate will suffice for this procedure.



Pour the extracted nematode eggs onto the paper in the sieve. Add enough distilled water so that the bottom of the sieve touches the water surface but is not immersed. Cover the top with a plastic lid.

Check the water level regularly for evaporation and add whenever necessary to ensure the bottom of the sieve remains in contact with the water to enable nematodes to pass through and prevent eggs from drying out.

Check daily, to assess nematode densities. Collect the water from the Petri dishes on daily basis or every two days depending on nematode hatch. Juveniles may be collected over a period of 6-8 days. If not used immediately, aerate the collected inoculum at room temperature using an aquarium pump or similar, or alternatively add 1-2 drops of hydrogen peroxide ( $H_2O_2$ ), which helps oxygenate the water. Consolidate juveniles and use not more than 2 days after collection if possible.

Once the nematode density and overall number has been determined, standardise the number of juveniles per unit volume and inoculate onto the plants at an appropriate rate (see below).

**\*\*\*Note: use of chlorinated tap water will reduce nematode hatch and infectivity\*\*\***

## Nematode Inoculation

A typical rate of inoculation is 500 juveniles per plant in seedling trays and 1000 per plant in pots. However, for higher pest pressure and plant stress, higher inoculum rates can be used. The rate may depend on the crop being evaluated, conditions or the aggressiveness of the *Meloidogyne* species or population being used.



*Healthy plants in pots and trays ready for inoculation.*

- Ensure the soil is moist, but not saturated or too wet, before inoculation.
- Make 2-3 holes around the healthy plant using a pencil or fine rod.
- Agitate the inoculum or stir using a magnetic stirring plate at low speed to ensure even distribution of inoculum.



- Apply nematode inoculum evenly to the plant through the 2-3 holes using a pipette.



- Cover the holes with soil.
- Leave pots to settle for at least 24 h before watering.
- Maintain plants for 6-8 weeks to allow nematode stress reaction. Nematode lifecycle is approximately 3-4 weeks depending on conditions.
- After 6 weeks assess nematode galling damage on the extra control plants by removing plants and examining roots.

### ***3.7 Maintain infected plants/experiment***

Apply an equal amount of water to each plant without overwatering and keep them suitably irrigated according to the soil volume and conditions. This will vary depending on the geographical location and the season. Document the volume and time of water application. Apply locally available, suitable fertiliser at recommended rates and frequency, preferably as a liquid nutrient solution when watering. A slow or controlled release fertiliser product, otherwise, would be convenient. It is important to follow recommended rates of application to avoid adversely affecting the plants.

Monitor pests and diseases regularly and if necessary apply appropriate insecticide or fungicide using non-systemic, as opposed to systemic, products if possible. Document the application rate and time of pesticide and fungicide application.

### ***3.8 Harvest plants for assessment***

Collect experimental data. This will vary depending on the target objectives and crop. For evaluation of nematode infection, a range of parameters can be assessed, depending on the level of information required. Beginning with the simplest and least time demanding:

- a) **Galling damage** assessment provides a rapid visual indication of the root reaction to nematode infection, and a relative indication of resistance. This is useful but should be supported by additional evaluation. Galling damage is best assessed according to a defined and established gall index (Appendix 2). Scale can be defined but most common are scales of 1-5 or 1-10, where 1 is no damage and 5 (or 10) is most severe.

- b) **Egg mass number** per root system (or unit weight) is also quick and efficient, but provides a higher level of resistance indication through evaluation of the number of nematodes able to reach maturity on a given plant/cultivar.
- c) **Egg number** per egg mass takes more time, but is useful to establish the ability of developed females to reproduce: providing fecundity data of mature females.

Observations of plant development will provide additional information on the effect of nematodes on growth of roots and aerial parts. Growth measurements, such as plant height, can be useful to help determine crop/cultivar response.

For evaluation at harvest, gently remove the plants from the pots and rinse the roots as above.

- a) Score galling damage according to a standard scoring sheet as in Appendix 2.
- b) Count egg mass number per root system. Staining the egg masses first will help to speed up the process as it helps to identify the egg masses. Use either 1 mg/L erioglaucine to stain them blue or Phloxine B 0.1% to stain them pink, by submerging the roots for 15 min.

Rinse the roots in water and against a white background evaluate roots by counting the stained egg masses per root system such as in a white tray or on a white plate. Use of an illuminated desk magnifier can help to visualize the egg masses for counting.

- c) Egg number per egg mass determines fecundity of the mature females. Collect 10 egg masses per root system and using the NaOCl system described above, release eggs into suspension and count egg number from an aliquot to estimate total egg number and thus egg production per egg mass.

Examples of experimental data, which will vary by crop and objectives.

<b>EXPERIMENTAL DATA</b>	
Height (recorded every two weeks)	
Date of flowering	
Girth	
Number of standing leaves	
Number of fruit	
Fruit weight	
Shoot weight	
Fresh root weights	
Dry shoot weights	
Dry root weights	
Number of juveniles per kg soil	
Number of egg masses	
Number of eggs	
Galling index	

After recording data, enter into spread sheets (Appendix 3) without delay, and then process, analyse and report as soon as possible upon conclusion of the experiment.

## 4. DEVELOPMENT AND MAINTENANCE OF PURE SPECIES CULTURES

In order to screen plants for resistance against a pest or disease a source of inoculum is necessary. In the case of nematodes, 'field' populations, which have been retrieved from infected plants from the field can be collected and used directly. This will enable an assessment against populations that occur naturally in the field, but will usually involve multiple species. For accuracy therefore, it is important to establish pure populations of single species and use these in screening, in order to determine single species reactions.

Root-knot nematodes may occur in mixed species populations in the field. It is necessary therefore to establish pure populations from one single, individual female to ensure purity of species. If plant roots are in a good condition when removed from the field, egg masses can be removed directly from the root tissue for use in developing single species cultures.

To establish pure species populations:

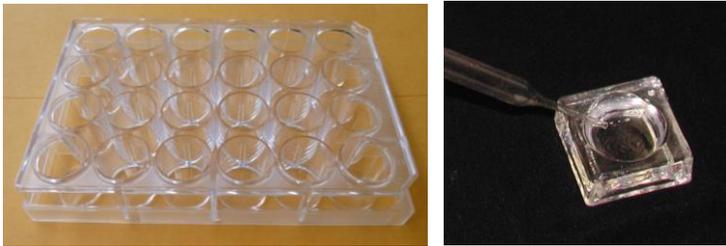
- Collect plants infected with root-knot nematodes from the field. Root galling present symptoms in a variety of forms, which can depend on the crop, cultivar, root-knot nematode species etc. and the various host-pest interactions (see below).



- Remove plants and gently rinse roots in a bucket filled with water or under a running tap.
- Gently, take galled root segments, and place on a Petri dish with some water to observe egg masses.
- Carefully, remove egg masses using scalpel and fine (watch-makers) tweezers. Egg masses can be seen protruding from the root surface (centre), which may sometimes darken with age (below right).



Carefully place single egg masses, individually, into distilled water in ink blocks or in single wells in a multiple well tray. It is important to place only one egg mass in each separate well or block. Egg masses can also be stained with 0.05% Phloxine B or 1 mg/L erioglaucine for increased visibility.



- After removing each egg mass, rinse tweezers in distilled water to remove any attached eggs and prevent contamination between wells.
- Incubate egg masses in the dark to allow juveniles to emerge (approx. 48 h). Check after 24 h by placing blocks under the dissection microscope.
- When the majority of juveniles have hatched they are ready for inoculating onto tomato plants using a pipette – juveniles from one egg mass for one tomato plant.
- Rinse the pipette after each inoculation to prevent contamination between plants/populations.
- Maintain host plants in the greenhouse ensuring a reasonable space between plants to prevent contamination between pots.
- Upon population development, identify species within each pot.
- In addition to morphological identification it is necessary to extract DNA from the nematodes to provide molecular identification of the species and ideally additionally use serological techniques to ensure accurate species identification.

Sometimes it is difficult to find egg masses or egg masses that are intact or suitable to use from root material brought from the field. This can be due to deteriorated root material, but is especially the case for woody roots and tuber material, with egg masses often enclosed within the plant tissue.



*Galled tubers with egg masses not easily discernible or accessible on beetroot (left) and yam, and buried within the tissue on sweet potato (right).*



*Galled woody root tissue of guava (left), from which egg masses are difficult to find, as they also are on the aged and deteriorated capsicum pepper roots (right).*

In the case where egg masses cannot be sourced from the root material it is necessary to culture the field populations on a suitable plant host in the screenhouse, from which egg masses can be easily removed. Tomatoes are mostly used, as many species will infect and develop on tomato. However, not all species will and it may be necessary to grow plant species of the original host sample crop to culture the nematode, for example coffee. Roots or tuber material should be chopped and introduced to ~3 week-old tomato plants grown in sterilised soil in the screenhouse. Maintain the plants for 6-8 weeks under suitable conditions after application of the infected material, until egg masses have developed and can be easily identified for use.

The host tomato plant pots in the screenhouse need to be maintained with a reasonable space between them to avoid splash and contamination between the pots, in order to maintain species purity.

Once single species cultures have been established and their identity confirmed they can be used for screening purposes. To identify species, samples effectively need to be sent to a suitable laboratory where the expertise exists for identifying root-knot nematodes. The species populations need to be maintained and when required for an experiment can be multiplied by infecting numerous sterile tomato plants grown in pots in sterilised soil with the target root-knot nematode species. Multiply and prepare inoculum for experiments by preparing well ahead of time and using a sufficient number of pots to ensure enough inoculum will be available and at the appropriate time. When preparing inoculum for experiments, nematodes can be extracted from root material (Appendix 1) to inoculate plants to multiply the inoculum, or can be infected using chopped root material placed within pots, or alternatively a portion of soil from the infected pots can be added. It is also best not to use too much inoculum, which can over-stress the plant, resulting in poor multiplication, but use a relatively low infection rate. A population of about 1000 juveniles would be suitable.

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## APPENDICES

### ***APPENDIX 1: Extracting nematodes from soil to assess sterilization***

To test the effect of the sterilisation of soil and sand prepare a plastic sieve and plate for nematode extraction using a modified Baermann technique.

1. Place tissue/filter paper in sieve then place sieve onto the collection plate.



2. Place soil or sand sample onto the tissue/filter paper inside the sieve.



3. Carefully pour water into the collection tray under the sieve. Ensure there is enough water for the sample to be fully submerged. Leave sample for 48 h.



4. Carefully remove the sieve from the collection tray, discard the soil and pour the extraction from the collection tray into a beaker and rinse the plate. Examine under a microscope.



5. Repeat sterilisation process if nematodes are present.

*APPENDIX 2: Root-knot galling score on tomato*

**Root-knot galling score on tomato**



1. No galling damage



2. Slight galling damage



3. Mild galling damage



4. Heavy galling



5. Severe galling damage

**APPENDIX 3: Example of experimental layout with randomized distribution of 60 cultivars in a randomized block design**

30	21	54	35	24	11	49	40	58	10
42	57	14	2	44	9	36	20	25	56
51	13	28	18	23	31	46	59	50	39
29	7	3	22	38	1	53	41	26	19
60	34	17	45	8	37	12	55	32	4
6	43	16	52	27	15	47	5	48	33

**Numbering of pots for record taking**

1	2	3	4	5	6	7	8	9	10
20	19	18	17	16	15	14	13	12	11
21	22	23	24	25	26	27	28	29	30
40	39	38	37	36	35	34	33	32	31
41	42	43	44	45	46	47	48	49	50
60	59	58	57	56	55	54	53	52	51

**Appendix 4: Example of spreadsheet recording sheet for de-randomizing layout of Rep 1**

Pot no.	Treatment	Replication		Data 1	Data 2	Data 3	etc.
1	30	1					
2	21	1					
3	54	1					
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