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Towards management of *Musa* nematodes in Asia and the Pacific

Technical manual of the training workshop on enhancing capacity for nematode management in small-scale banana cropping systems held at the Institute of Plant Breeding, University of the Philippines Los Baños, Laguna, Philippines, 1-5 December 2003

F.S. dela Cruz, Jr., I. Van den Bergh, D. De Waele, D.M. Hautea and A.B. Molina, editors

VVOB

The mission of the **International Network for the Improvement of Banana and Plantain** (INIBAP) is to sustainably increase the productivity of banana and plantain grown on smallholdings for domestic consumption and for local and export markets.

The programme has four specific objectives:

To organize and coordinate a global research effort on banana and plantain, aimed at the development, evaluation and dissemination of improved banana cultivars and at the conservation and use of *Musa* diversity.

To promote and strengthen collaboration and partnerships in banana-related activities at the national, regional and global levels.

To strengthen the ability of NARS to conduct research and development activities on bananas and plantains.

To coordinate, facilitate and support the production, collection and exchange of information and documentation related to banana and plantain.

INIBAP is a network of the International Plant Genetic Resources Institute (IPGRI), a Future Harvest center.

The **International Plant Genetic Resources Institute** (IPGRI) is an independent international scientific organization that seeks to advance the conservation and use of plant genetic diversity for the well-being of present and future generations. It is one of the 16 Future Harvest Centres supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and proverty, improve human nutrition and health, and protect the environment. IPGRI has its headquarters in Maccarese, near Rome, Italy, with offices in more than 20 other countries worldwide. The Institute operates through three programmes: (1) the Plant Genetic Resources Programme, (2) the CGIAR Genetic Resources Support Programme and (3) the International Network for the Improvement of Banana and Plantain (INIBAP).

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Cover Photos: (counterclockwise starting from the top) a. participants of the training workshop; b. fruit-bearing banana plant; c. banana field in the Philippines,planted with different accessions; d. root and soil sample collection for nematode evaluation; e. (center photo) banana root showing necrosis. Outer photos provided by UPLB-IPB while center photo by Dirk De Waele.

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Editorial note
Some references have been submitted without complete publishing data. They may thus lack the full names of journals and/or the place of publication and the publisher. Should readers have difficulty in identifying particular references, staff at INIBAP-Asia Pacific regional office will be glad to assist.

Contents

Foreword

Plant-pathogenic nematodes are considered one of the major constraints in banana production. While nematodes can be controlled by the use of chemicals, these are expensive and thus beyond the reach of small-holder farmers. They also accumulate in the environment and are highly toxic.

Alternative strategies to combat these pests include the use of banana varieties that are resistant or less susceptible to nematodes, the use of clean planting materials and the use of soil amendments and biocontrol agents. These strategies, including the need for more information on the status of nematode problems in the region, were last stressed at the conference-workshop on nematodes and weevil borers affecting bananas in Asia and the Pacific in Malaysia in 1994.

In addition, several levels of training requirement were identified: (1) specialized nematology training, to address the shortage of skilled nematologists; (2) extension training, to raise the awareness of farmers of nematodes; and (3) training of trainers, to ensure that the two first training requirements can be met.

The University of the Philippines Los Baños, Philippines (UPLB) and the Katholieke Universiteit Leuven, Belgium (K.U.Leuven) are currently undertaking a project entitled 'Enhancing Capacity for Nematode Management in Small-Scale Banana Cropping Systems' financed by the *Vlaamse Interuniversitaire Raad* (VLIR), Belgium. The project aims to: (1) improve banana production by identifying varieties which are either resistant or less susceptible to nematodes and evaluate their usefulness in small-scale, low-input banana cropping systems; (2) strengthen the nematological training and research capacity at the College of Agriculture of UPLB (CA-UPLB); and (3) train Southeast Asian nematologists in banana nematology.

Training in banana nematology is given special emphasis because there is currently a lack of trained scientists and technicians to address problems of nematology. In the Philippines, for example, several senior nematologists of CA-UPLB have already retired without being replaced by young nematologists.

The training held at UPLB aimed to: (1) enhance capacity for nematode research in the region, specifically on nematode management in *Musa*; (2) compile up-to-date information on the status of nematodes in *Musa* and ongoing nematological activities in the region; and (3) bring together and work out future collaborations among partners in the region.

The training workshop offered an opportunity to summarize the status of *Musa* nematode research in the region through country reports. Technical presentations and hands-on exercises on nematode survey, collection and culture techniques and field trips to the laboratories were also undertaken. The activity concluded with a workshop to discuss and come up with a set of protocols for survey, collection and culture of *Musa* nematodes. The country status reports are published in the accompanying proceedings of this technical manual.

As the project will end in 5 years time, we also plan to organize a follow-up workshop within the next years to evaluate the progress made by each country in the region.

The editors

Technical Presentations

Nematode survey and collection of samples

Romulo G. Davide*

Introduction

According to Thorne (1961), nematodes are so universally distributed that if plant life of any form grows in the soil, it is almost certain to contain several species.

Cultivated fields, with their high fertility and heavy plant covers, are especially favourable habitats for a wide range of species, and populations of several billions per acre are not unusual. A pound (0.45 kg) of soil from one of these fields generally contains 10 to 30 species belonging to many and diverse genera, including endoparasites, ectoparasites, predators, saprophages and other free-living forms. Forest soils contain a great variety of ectoparasitic tylenchs and dorylaims, as well as predaceous mononchs, saprophagous cephalobs and rhabditids.

Naturally, we are generally most interested in those species of economic importance. These we shall find in and about the roots of their host plants or in the stems, leaves and seeds of the plants themselves.

Nematodes are difficult to study and it is essential that all materials collected for identification should be given the best care.

Nematode distribution in the soil

Population densities for plant-parasitic nematodes in the soil vary greatly in time and space. Patchy or aggregated spatial distribution of nematodes and changes of the typical polyspecific communities over time pose major sampling problems. The inherent tendency of nematode populations to oscillate, which is often enhanced by climate and other factors, is partly responsible for their patchy distribution (Jones and Kempton 1978).

Some of these ecological factors that affect nematode population density and distribution in the soil are soil texture, soil moisture, soil pH, plant age and host-plant resistance.

Soil texture

Most plant-parasitic nematodes show higher population densities in sandy-loam soils than in clay soils. Davide (1980) has shown that the banana nematodes *Meloidogyne incognita* and *Radopholus similis* have the highest population density in sandy-loam soil and the lowest in clay soil.

Soil moisture

This is a very important factor for nematode survival in the soil. A study by Davide (1980) indicated that the population densities of both *M. incognita* and *R. similis* on banana were much higher during the rainy season (June, July and August) than during the dry season (February, March and April).

Soil pH

Davide (1980) showed that *M. incognita* and *R. similis* on banana had the highest population densities in soils with pH 5.0 - 5.6 and the lowest at pH 7.1 - 7.7.

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Plant age

Usually, the highest population density of plant-parasitic nematodes is detected when the plants are in the flowering stage. A low population is usually found in newly planted crops and in the harvesting stage when root growth has already stopped.

As indicated in a study by Davide (1980), *R. similis* population density was highest in banana plantations of 4-5 years old and lowest in plantations of less than 1 year old. On the other hand, *M. incognita* was already high in banana plantations of 1-2 years old and later declined when *R. similis* increased.

Host-plant resistance

In crop cultivars with some degree of resistance, the nematode population density is generally low compared within those which are susceptible. Davide and Marasigan (1992) showed that *R. similis* and *M. incognita* population densities were much higher in the susceptible cultivar Giant Cavendish than in resistant cultivars like Amas, Tanggung, Katsila and others.

Survey and collection of soil and root samples

Since plant-parasitic nematodes are considered a limiting factor in crop production because they cause serious root diseases resulting in decline of plant growth and yield, it is of paramount importance to gather information through survey and collection of soil and root samples. The data obtained can be used in predicting nematode crop damage and yield losses. If one knows the threshold or damaging level of the nematode population in a given crop, a timely application of control measures can be made before the damage can occur. For instance, the banana companies in Davao base their *R. similis* control application on population counts: the threshold level is reached when counts are 25 000 nematodes per root sample of 25 g.

The survey data are also very useful for the identification of nematode species associated with different crops and their distribution in a given locality. In quarantine work, collection of samples for nematode detection is necessary to prevent entry of infected plants into the country and other localities.

Given the survey data, studies on the biology, ecology and control of plant-parasitic nematodes can be initiated, especially their role in the development of disease complexes in plants caused by other pathogens like fungi, bacteria and viruses. For instance, survey data showed that citrus plants with virus infection had a high population of the citrus nematode, *Tylenchulus semipenetrans* (Trinidad and Davide 1969). Likewise, in the presence of root-knot nematodes, *M. incognita*, the bacterial wilt of tomato becomes more severe.

Survey data also indicated that a cropping system like crop rotation or planting of resistant varieties can be designed to prevent crop damage caused by nematodes, especially when the population densities have already reached the damaging level.

Collection of samples

Before the conduct of the survey and collection of samples, a detailed sampling plan including the collection pattern, as well as the number and size of samples, must be developed based on available funds and resources.

Primary considerations for sampling must focus on the life cycle of nematodes, their horizontal and vertical distribution and their interactions with the host plants and environment (Cochran 1977).

Equipment for collecting soil and root samples for nematodes includes trowel, spade, shovel, soil auger, tube or you can make your own. Timing of sampling depends on the objectives. For advisory purposes, collection of samples right after land preparation should be done to determine whether the initial population densities of the plant-parasitic nematodes are already at the damaging level. If so, advise the farmers to apply control measures like the use of crop rotation, planting of resistant varieties or treatment of the soil with nematicides before planting. If the objective is to identify plant-parasitic nematode genera and species associated with different economic crops and their distribution in different parts of the country, sampling can be done on areas where crops are actively growing, especially at flowering stage.

In established banana plantations, periodic sampling is usually done every 3-6 months for monitoring purposes to determine the threshold level of the nematode population to serve as basis for application of control measures.

Sample size, depth and distance of sampling from the plants

Soil and root samples are usually collected not far from the base of actively growing plants, where roots are still growing and functional and where the nematodes are feeding. The depth of sampling depends on how deep the root system of the crop is. In case of banana plants, the samples are collected 30-40 cm from the base of the plant. The samples are usually collected from three sampling sites at 15-20 cm depth. About 200 g of soil and 50-100 g roots may be collected per site. According to Barker and Campbell (1981), the sampling area and number of sampling sites vary widely for different purposes. For advisory use, a sampling area of about 2 hectares or less is recommended. For very large farms, randomly selected 2ha units with uniform soil and cropping histories should provide a useful basis for sampling. Ten sampling units may provide an adequate estimate of a large nematode population, while 100 units may be necessary for a low population density.

Bulking or mixing together of soil and root samples from different sites may mask the degree of variability between sampling sites. Therefore, it is better to examine the nematodes from each site.

Sampling patterns

Possible sampling procedures for nematodes include simple random, stratified random, systematic, haphazard, cluster and two-stage sampling (Goodell 1979). The factors influencing nematode distribution in the soil should be considered. Generally, systematic sampling gives more reliable results than random sampling (Goodell 1979).

However, a major source of variation in sampling is the mechanics or the way the samples are collected by different individuals. To reduce such variations, there should be a standardization of the collection sites: distances from plants, depth, angles, inclusion of roots, moisture conditions and other considerations are most important (Moriarty 1960).

Handling of samples during collection and transit

Soil samples must be handled carefully to maintain the nematodes in the state in which they were collected (Barker and Nusbaum 1971). Nematodes should be in good condition for easy identification. Soil samples are usually placed in plastic bags or in containers where moisture can be maintained. Each sampling bag should have labels with important information. The samples should be carefully transported to the laboratory for nematode extraction. Improper transport and storage often results in death and deterioration of nematodes, and the data obtained are no longer reliable and useful.

Temperature and soil moisture are the two most critical factors that affect the survival of nematodes in storage. Excessive moisture can also result in rapid decline of nematode population in storage. A storage temperature of 10-15 °C usually provides maximum recovery rates of nematodes from the soil samples (Barker and Nusbaum 1971).

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Nematode extraction from roots and soil

Rustico A. Zorilla*

Introduction

Plant-parasitic nematodes can be extracted from soil and plant parts in many different ways. Processing of samples consists of separating the nematodes from the soil or plant materials in order to identify and count them. Some methods are more effective than others for a particular type of nematodes or for a particular kind of sample. Also, some techniques of processing samples are better adapted than others to specific purposes, such as nursery-stock inspection or quarantine enforcement. Some techniques require expensive equipment or are too labourious to be practical, except in exacting research work. For instance, a combination of gravity screening and mist extraction has proven to be more effective in processing soil than the Baermann- funnel technique. However, since a number of laboratories are not equipped for mist extraction, gravity screening and Baermann-funnel methods may be used alternatively until mist equipment is available.

The following guidelines may be helpful in your choice of an extraction technique for a particular situation.

Extracting nematodes from soil samples

There are numerous methods of processing soil samples, all of which have advantages and disadvantages. To extract nematodes from soil, one of the funnel-extraction methods is usually used. If the sample is small (one cup or less), the soil may be directly placed in a funnel setup; larger soil samples should first be processed by the combined screening-funnel technique or by gravity-screening technique followed by mist extraction of the nematodes.

The following methods will give a satisfactory yield of the common plant-parasitic nematodes.

Cobb's sifting-and-gravity method (Cobb 1918)

The Cobb's sifting-and-gravity method consists of a series of operations by which heavy particles are settled out and discarded. Lighter fractions containing the nematodes are passed through a series of screens or sieves, which separate the nematodes and small particles of light organic materials from the muddy solution.

- Stir 300 g soil in a bucket with water. Nematodes become suspended in the water.
- After about 20-30 seconds (depending on the soil texture), pour the water through a coarse sieve (100 μm) into a second bucket. Nematodes will pass through the sieve, while heavy particles remain in the first bucket. Large particles of debris are caught by the sieve.
- Stir the muddy solution in the second bucket and allow to stand for 20-30 seconds, then pour the water with nematodes through a fine sieve (usually 40 μm mesh sieve). Nematodes remain on the sieve with small particles of debris.
- Wash the fine sieve with a gentle stream of water to remove finer particles.
- Wash the nematodes from the inverted fine sieve into a cup or beaker.
- Pour the nematodes into a shallow dish for examination under a dissecting microscope. Nematodes may be difficult to see because they are mixed with many fine particles.

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Advantages

- Since the method is not dependent on nematode movement, sluggish nematodes are recovered as effectively as active ones.
- The method allows recovery of most nematodes from large soil samples.
- Nematodes are available for direct examination in less than half an hour.

Disadvantages

- The method requires expensive sieves and an experienced worker.
- Nematodes are difficult to see because of fine particles.

Baermann-funnel method (Baermann 1917)

The Baermann-funnel method is an excellent system for separating specimens from soil and condensing them for examination. The apparatus consists of a regular funnel with a short piece of rubber tubing attached to the stem and closed by a clamp or pinchcock. The funnel is supported in an upright position and filled with water. A wire screen is fitted across the funnel.

Procedure

- Place about a handful (50 g) of soil on top of a two-layered tissue paper (arranged in crisscross manner) on top of the wire screen. Fold the sides of the tissue paper towards the center to cover the soil.
- Place the 'enveloped' soil sample on top of a Baermann funnel in the rack.
- Fill the funnel up to the rim with water. The nematodes move through the tissue and the screen into the water in the funnel. They settle at the bottom of the funnel by gravity, where they are collected (5-10 ml suspension) after 1-2 days. The nematodes are now ready for counting and identification under a dissecting microscope.

Advantages

-The technique is simple and the equipment is inexpensive.
-Recovery of active nematodes from small samples is fairly good.

Disadvantages

-Lack of aeration in the water reduces nematode movement, hindering recovery. Recovery may be reduced when the tissue and other materials obstruct nematode movement.
-Recovery of active nematodes from large samples is poor.
-Because the funnel capacity is small, the samples in it may be too small to be representative.

Combination of Cobb's sifting-and-gravity method and the Baermann- funnel method (Cobb 1918)

After the sieving process described in the first method, nematodes may be separated from the remaining soil particles by the use of the Baermann funnel as described in the second method.

Advantages

- The combination method allows recovery of most nematodes from large soil samples.
- The resultant sample contains less silt and debris than it does with the gravity-screening technique alone and is therefore easier to examine under the dissecting microscope.

Disadvantages

- Although recovery of mobile nematodes is good, sluggish nematodes recovered during the sieving part of the technique may fail to pass through the tissue in the funnel.
- Processing takes longer and requires considerable and expensive equipment.

Centrifugal flotation (Jenkins 1964)

Another method of extracting nematodes from soil samples is by centrifugal flotation, which technique requires centrifugation of the nematodes and addition of sucrose and $MgSO_4$ or $ZnSO_4$. The nematodes are floated out into solution of specific gravity greater than their own.

Extracting nematodes from plant tissues

Isolation of nematodes from plant tissues, such as roots, bulbs, leaves or stems can be done in a number of ways, the choice depending upon the kind of nematodes involved, the type and size of the samples and the quantity of the nematodes required. The following are some of the commonly used methods.

Teasing out (Ayoub 1977)

The diseased plant parts or roots with lesions and galls are placed in a Syracuse watch glass or Petri dish containing water. With the use of a dissecting needle, tease the tissue apart. Examine material under a dissecting microscope for the presence or absence of nematodes. This method can determine the presence of both migratory and sedentary nematodes.

Staining of tissues (Daykin and Hussey 1985)

Research on nematode species often involves study of the penetration and development of specimen within intact roots. Many procedures for staining and clearing nematode-infected plant tissues have been developed and are satisfactory for various kinds of tissues. The acid-fuchsin–lactophenol method, developed in 1941, has been the most widely used procedure. However, the two other procedures which are noted as being reliable, relatively simple and superior to the former are the sodium-hypochlorite-acid fuchsin method and the McBryde method.

Baermann-funnel method (Baermann 1917)

This technique is applicable to plant tissue in the same manner as with the soil samples.

Procedure

- Cut the roots in short sections, about 1 cm in length. If the roots are of large diameter (e.g. banana roots), you can cut them lengthwise also. Place the roots in a muslin or tissue paper and fold it around the roots to make an 'envelope'.
- Gently submerge the envelope in water on the Baermann funnel.
- Leave the apparatus for 1-2 days and recover the nematodes in vials or test tubes by opening the pinchcock to allow 5-10 ml water to escape.
- Examine in a counting dish and/or preserve for future use.
- This method can extract only the migratory nematodes.

Incubation method (Young 1954)

This was found to be more efficient than the Baermann-funnel technique for recovering burrowing, lesion and other migratory nematodes from avocado roots (Young 1954).

Procedure

- Wash the roots gently to remove soil particles.
- Place the roots while still wet in a glass jar and add a small amount ofwater. Close the jar lid loosely.
- Leave the sample for 1-2 days at room temperature.
- Add 50 to 100 ml water, replace the screw cap and shake gently, inverting the jar several times to wash off nematodes clinging on the surface of the root tissues.
- Pour the water through a coarse sieve mesh (100 μm) on top of a very fine sieve mesh (40 μm opening). Wash gently with water.
- Collect the residue from the very fine sieve in a beaker by washing with a small amount of water.
- Place the suspension in a Syracuse watch glass or counting dish and count the nematodes with the use of a dissecting microscope.
- This method can be used to extract migratory endoparasites only.

Blender or maceration and sieving method (Southey 1970)

This method was initially used for extracting both migratory and sedentary endoparasites of abaca roots. The method is rapid because it does not depend upon the nematodes moving out of the tissue by their own activity. The method should be used with caution. If infections

are light, some of the nematodes may be destroyed by the action of the blade or may not be freed from the plant tissue. This is a quick and useful method of examining roots for the presence of *Pratylenchus*, *Hirschmanniella* and *Radopholus*. However, *Meloidogyne* females are mostly broken. The suspension also contains debris particles which will interfere in the counting.

Procedure

- Wash the roots gently to remove soil particles.
- Cut 10 g of roots into short pieces (1-2 cm) and place them with 50 ml water in a blending jar. Run the motor for 10 to 30 seconds intermittently for 3 times. The time and frequency will vary according to the amount and kind of materials being processed.
- Pour the mixture through a coarse sieve (100 μm) into a plastic pail.
- Wash the macerated tissue with water. Discard the material on the sieve.
- Stir the mixture in the pail until the residue is all in suspension.
- Pour gently through a 40 μm sieve into a pail. Wash gently with water. Discard the residue on the sieve.
- Stir the mixture in the pail and pour it gently through a 40 μm sieve.
- Wash gently with a stream of water and collect the residue on the sieve into a 100-ml beaker. You can still set up the residue in a Baermann funnel for 48 hours prior to observation and counting under the dissecting microscope.

NaOCl technique (Hussey and Barker 1973)

Extraction of *Meloidogyne* eggs in roots to be used for inoculation.

Procedure

- Place galled roots in a beaker.
- Shake roots vigorously in 200 ml of a 0.5-1.0% (a.i.) NaOCl solution for 2 to 4 minutes.
- Quickly pass the NaOCl solution through a 200 mesh (75 μm) sieve, nested over a 500 mesh (26 μm) sieve to collect freed eggs.
- Quickly place the 500 mesh sieve with the eggs under a stream of cold water to remove the residual NaOCl (rinse for several minutes).
- Rinse the remaining roots with water to remove additional eggs; then collect them by sieving.

Standardize the number of eggs per unit volume and inoculate plants directly with a known number of eggs.

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Processing and mounting of nematodes

Joey I. Orajay*

Introduction

Nematodes obtained from a survey need to be characterized and properly identified to give valuable information about what pest to deal with. While they can be examined alive, it is usually necessary to inactivate them to facilitate detailed studies and to avoid distorting them. Storing specimen will allow future examination and verification. For all these processes, due to the very delicate and complex structures of nematodes, proper means of killing, fixing and mounting of nematodes on slides must be observed to ensure their usefulness for identification. Improper handling may result in distortion and total loss of the quality of specimen.

Killing and fixation

Processing of newly extracted nematodes involves two phases, which can be done at the same time or successively. Killing is the process resulting to sudden and permanent termination of the life processes in all cells of specimen. Fixation, on the other hand, is the preservation of all cellular and structural elements in a condition as near to the living condition as possible. This process may cause tissue hardening which is necessary for good fixation and tissue preservation. Once fixed, nematodes may remain in the solution for a long time.

Live nematodes in a given suspension are usually killed rapidly by heat. Rapid killing results in a characteristic relaxed shape of the nematode body; relaxed in the sense that they assume a natural straight, curved or spiral form.

What to do

1. Heat killing before addition of fixative – nematodes are best killed by gentle heat (55-60°C) directly over a flame, hot plate, in an oven or by partially immersing containers in a large volume of water at nearly boiling point (80-90°C) for 2-3 minutes. At these conditions, body contents are not disrupted. Then, an equal volume of double-strength (2x) cold fixative is added to the suspension.
2. Addition of hot fixative – this is a one-step procedure for killing and fixing nematodes. Double-strength fixative is heated to 80-90°C and then poured into a vessel containing an equal volume of nematode suspension. However, caution must be observed in using this method. All fixatives have formalin as a component and when heated, fumes are emitted. This is HAZARDOUS, so one should work in a fume hood. If not available, the first method is more recommended.

What not to do

1. Boiling/overheating of nematodes – the objective of immersing the nematodes in a hot water bath is to kill them and not to 'cook' them. Cooked nematodes are soft and will be easily distorted when picked prior to mounting.
2. Adding cold fixative to a suspension containing live nematodes – exposure to fixative solution will not immediately kill the nematodes. They will resist and move until they die. Resistance will cause them to die in an unnatural position, instead of relaxed.

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Commonly used fixatives and their formulation

Formalin

2-5% final concentration, buffered to pH > 7
Advantage: simplest fixative yet giving very good result
Disadvantage: carcinogenic and highly denaturing, so handle with extra care and always work in a fume hood

Formalin-glycerol (FG)

Reagent	Normal strength (ml)	Double strength (ml)
formalin (40% formaldehyde)	10	10
glycerol	1	1
dH_2O	89	45

Advantage: nematodes won't dry out due to the glycerol, even if the vials are not properly sealed

Formalin - Acetic Acid (FAA)

Reagent	Normal strength (ml)	Double strength (ml)
formalin (40% formaldehyde)	6	6
ethanol (96%)	20	20
glacial acetic acid	1	1
dH_2O	40	20

Advantage: due to the ethanol, a certain degree of shrinkage may occur on the specimen, but this is sometimes useful in highlighting annulations and incisures

TriethanolAmine-Formalin (TAF)

Reagent	Normal strength (ml)	Double strength (ml)
triethanolamine	2	2
formalin	7	7
dH_2O	91	45

Advantages: the nematodes appear remarkably life-like
the solution remains stable for a long time
triethanolamine neutralizes free formic acid
being hygroscopic, it prevents the specimen from drying out, even if the fixative evaporates
Disadvantage: causes the cuticle to degenerate with time

Picking out nematodes

Nematodes are picked out from the suspension under the low magnification of a dissecting microscope. The picking implements that can be used are:
- bamboo splinters or coconut midrib sharpened to have a fine point;
- eyelashes or cactus spines mounted on needles with water-resistant glue;
- fine insect needles (# 00) with a hooked tip.

The fine point of the needle is positioned under the nematode, which is then gradually worked upwards to the surface of the water before being removed by draping over the end of the point. The level of suspension in the dish should be shallow and constant refocusing of the microscope is necessary as the nematode is worked upwards. Picking requires some practice before nematodes can be removed quickly.

Processing for mounting

Temporary mounts

Nematodes are mounted alive. Many valuable observations can only be made on this type of specimen. Also, refractive structures such as the spear, head skeleton, lumen of the esophagus, excretory pore, spicules, etc. appear much more distinct than in processed specimen. This type of mount also allows a quick and preliminary identification of the nematodes, at least to the genus level, which is important when one wants to determine the composition of a nematode community in a given sample.

Selected nematodes are carefully picked and transferred to a drop of water on a glass slide. Normally, three short pieces of glass fiber with thickness similar to that of the specimen are arranged radially and equidistantly from each other. This will provide support for the cover slip and will prevent the nematodes from being smashed. Once the specimen is checked, the cover slip is placed over the slide.

Semi-permanent mounts

Lactophenol

Fixed nematodes are transferred to a drop of stained or unstained lactophenol on a glass slide, which has been heated to 60 °C. This process is not recommended due to the hazardous fumes from the lactophenol.

Formulation:	phenol crystals	1 part (CAUTION! Hazardous chemical)
	lactic acid	1 part
	glycerine	2 parts
	distilled water	1 part

Lacto-glycerol

This method is basically the same as above except for the exclusion of the hazardous chemical, phenol. This is therefore preferred over the first one under normal circumstances.

Formulation: equal parts by volume of lactic acid, glycerol and distilled water

Permanent mounts

The primary difference between the semi-permanent and the permanent mount is the dehydration step. Water is completely removed in the latter and replaced with glycerine. Absence of water in the specimen allows the long-term storage without deterioration. Specimen deposited in collection centers must be prepared using this method.

Glycerol-ethanol (Seinhorst 1959)

This is a rapid method of processing nematodes through 96% ethanol to glycerine. The whole process takes only 1-2 days.

Solution I:	ethanol (96%)	20 parts
	glycerine	1 part
	distilled water	79 parts
Solution II:	ethanol (96%)	95 parts
	glycerine	5 parts

Nematodes are directly transferred from the fixative to a watch glass (e.g. cavity glass block) containing 0.5 ml of Solution I. The watch glass is then placed in a closed dessicator containing about 1/10 of its volume 96% ethanol. It is left there for 12 hours in an incubator oven set at 35-40 °C. This removes nearly all water through vapor exchange and leaves the nematodes in a larger volume of a mixture of glycerol and ethanol. This should not be left inside for too long or it will be flooded with ethanol. Volume should be decreased if necessary. The watch glass with nematodes is then topped up with Solution II and placed in a partly covered Petri dish to allow slow evaporation of the ethanol. It is kept in the oven for at least 3 hours at 40 °C or until all the alcohol has evaporated. The nematodes are then in pure glycerol and are transferred to a drop of glycerol on a slide ready for mounting.

Glycerol-ethanol (De Grisse 1969)

This method is a modification of the above method, which lessens the labour in transferring the nematodes. Killing and fixing is done at the same time using 10 ml of hot (70-80°C) fixative (FG) replacing Solution I of Seinhorst. A third solution is added to prevent desiccation of the nematodes.

Solution I:	formalin (4%)	99 parts
	glycerine	1 part
Solution II:	ethanol (96%)	95 parts
	glycerine	5 parts
Solution III:	ethanol (96%)	50 parts
	glycerine	50 parts

Mounting

Materials needed

- Glass slides (25 mm x 76 mm x 1 mm) and cover slips (22 x 22 mm)
- Picking implements
- Copper cylinder (1.5 cm diameter) and paraffin wax (MP > 60°C) or nail polish and ringer (Fig. 1)
- Alcohol lamp
- Ethanol for soaking glass slides and cover slips
- Lacto-glycerol (mounting medium)

Creating cover slip support

Without support, the cover slip will exert pressure on the specimen. The specimen will either be flattened at a certain degree which will make it appear a bit more stout than its normal size or it will be completely smashed. There are many ways to support the cover slip. Previously described is the mounting of glass fibers with the same diameter as the specimen. This method is a bit labourious, however, as one needs to pick up three fragments and arrange them on the slide. The two methods described below introduce the use of a ring on the glass slide, which will not only act as support but will also confine the mounting medium at the centre of the mount.

Using paraffin wax

A wax ring can be made by heating the copper cylinder, dipping it in paraffin wax and introducing the melted wax onto the centre of the glass slide. The glass slide (and cover slip as well) must be previously soaked in ethanol to get rid of any grease and then wiped with clean tissue paper. Fixed nematodes are arranged in a small drop of mounting medium placed at the centre of the ring. They should not be floating. A cover slip is placed on top, and together, they are put over a hot plate (65°C) for a few seconds or until the paraffin wax melts. Melting of the wax causes the cover slip to settle down, and confines the mounting medium at the centre of the mount. Aside from cover slip support, the paraffin wax, once solidified, serves also as the sealant.

Using nail polish

A device called 'ringer' (Fig. 1) is needed in this method. A ringer consists of a rotating metal disc where the glass slide can be fixed by two clips. While holding a brush dipped in nail polish, the hand can be rested on the other disc (hand rest) so that it will not move once the ring is being made. By touching the surface of the glass slide with the brush and rotating the main disc, the nail polish is spread over, creating a ring on the glass slide. The mounting medium and the specimen are introduced in the same manner as above. The cover slip is placed and all sides and corners should be thoroughly sealed with nail polish.

Fig 1. The ringer device.

Labeling

Labels allow organized keeping of slides and quick retrieval once needed. A glass slide sticker is usually used to provide a contrasting surface for writing information. You may indicate as much information on the label as you want. The following are the basic ones and should never be overlooked:

- place and date of collection
- host name and cultivar (if applicable)
- identity of the specimen (once identified)
- number and life stage of the specimen mounted (in case there is more than one).

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Identification of plant-parasitic nematodes associated with *Musa*

Joey I. Orajay*

Introduction

Wherever bananas and plantains are grown, nematodes are a potential major problem. Very fundamental for their management is the correct identification. Before any particular management strategy can be formulated, one has to determine which are the nematode pests present in the farm. Knowledge on their identity also facilitates retrieval of a huge amount of specific valuable information available in literature, thus aiding the nematologist in precisely addressing the problem.

For nematologists working in diagnostic laboratories, it is a very essential skill to identify nematodes while counting them under a low magnification (not more than 100x). It is definitely impractical to mount all the nematodes present in a sample and examine them under the usual high magnification (400x or 1000x). A beginner would definitely find this a bit hard, because of the fact that most of the time, a nematode community consists of polyspecific populations and there are only few distinguishing characters clearly visible at low magnification. Identification then would rely on more obvious morphological characters.

The skills involved in distinguishing genera by using only a few characters are not easily acquired let alone by reading taxonomic books and references. They develop through constant practice. For beginners, it is advisable to first examine a suspension, try to have an overview of the composition and range of the nematodes present (based on body size and shape) and then mount representative individuals on a glass slide. Viewing under high-power objectives will facilitate a more detailed examination of the different morphological characters leading to a more precise identification. Once an overview is made, one will be able to associate the identity with the specific set of characters observable in the nematodes under low magnification.

Familiarity with the morphology of nematodes is very essential to identify them, whether at low or high magnifications. It helps a lot in identification if one knows to pinpoint which structures provide distinguishing characters. Because of the great diversity and uniqueness found in nematodes, the specific terms used to discuss them are defined and/or illustrated here. A simplified description of plant-parasitic nematodes, most commonly found in banana rhizospheres are presented as well.

Key morphological characters

Body shape

All soil nematodes are vermiform or wormlike, with the exception of the females of some genera that upon maturity become swollen (either kidney-shaped or globular). Nevertheless, they at least spend a part of their life cycle as vermiforms when they emerge from eggs.

The posterior region (from anus to terminus) is called the tail, while the anterior end is called the lip or cephalic/head region. The cephalic region can be low or raised, flattened or rounded, and continuous with the body contour or offset.

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When properly relaxed, the nematodes assume any of the following postures or habitus: straight, C-shaped (with varying degrees of curvature) or spiral (Figure 1). For C-shaped and spiral nematodes, the tail region can easily be distinguished from the anterior region because it is always curved while the latter is more or less straight. Variations in tail shape (Figure 2) are important distinguishing characteristics between nematode genera.

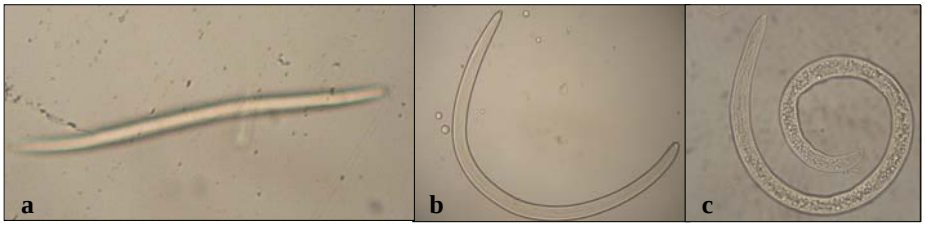

Figure 1. Body shapes of nematodes when relaxed. (a) straight, (b) C-shaped or curved, and (c) spiral.

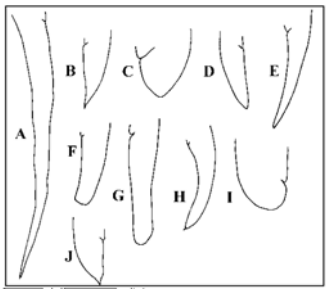

Figure 2. Variations in tail shape: (A) filiform; (B) conoid, pointed terminus; (C) conoid, short; (D) conoid; (E) moderately long, evenly tapered; (F) truncate; (G) cylindrical, terminus slightly bulbous; (H) conoid with terminal mucro; (I) hemispherical; (J) conoid with digitate terminus. (After Stirling *et al.* 2002).

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The cuticle is the nematode's outermost layer consisting its external body wall. This covers the entire body and intrudes in the openings of digestive, reproductive and excretory-secretory systems as well as in those of various sensory and/or secretory structures. This structure is elastic, relatively tough and more or less impermeable, and is shed off during molting.

The cuticle can be smooth (as in the Adenophoreans) or annulated (as in the Secernenteans). Annulation refers to the radial incisures or striations of varying depth and spacing, which are found starting beneath the lip region down to the tail region. A smooth cuticle does not really mean absence of radial striations, but they are very fine and shallow, appearing invisible under the light microscope.

Laterally, the body is generally marked by longitudinal striations or lateral field. These are longitudinal invaginations of the external layer of the cuticle, which may or may not be raised into ridges. Generally, these striations interrupt the radial striae. If the latter extend into the lateral field, it is said to be aerolated. The lateral field starts anteriorly as a narrow area/ridge and gradually broadens to become of normal width just behind the esophagus and finally ends near the tail tip. The number of lateral lines is determined at mid-body and is regarded useful in identification.

Stylet

Plant-parasitic nematodes are characterized by the possession of a protrusible feeding structure, called stylet (although not all stylet-bearing nematodes are necessarily plant parasites). They use their stylet to puncture their hosts and to withdraw cellular contents from them. Three distinct types of stylet are found within the plant-parasitic species: onchiostylet, odontostylet and stomatostylet.

The onchiostylet is a curved, solid and needle-like tooth possessed by the members of the Order Triplonchida (e.g. *Trichodorus* and *Paratrichodorus*).

The odontostylet on the other hand is hollow. It has two parts: the visible part or odontostylet and the posterior half or odontophore, which may have flanges and is much less distinct. Since the flanges of the odontostylet are often not visible at low magnifications, the stylet appears as a thin line without any obvious structure at the base. Anteriorly, the odontostylet is surrounded by a guiding ring. All dorylaimids have an odontostylet but the plant-parasitic species are confined to the genera *Longidorus, Paralongidorus* and *Xiphinema*.

The stomatostylet, like the odontostylet, is also hollow. Nematodes belonging to the Order Tylenchida have this type of stylet. It has three parts: a cone, a shaft and three basal knobs. Variations occur in the stylet length and in the size and shape of the knobs, which are helpful in recognizing a particular taxon within this order.

Esophagus (pharynx)

The morphology of the esophagus is very diverse, so its usefulness in identification cannot be discounted. For plant-parasitic nematodes, esophagus morphology falls under two types: dorylaimoid and tylenchoid.

The dorylaimoid esophagus, resembling a wine bottle, is characterized by two parts, a narrow anterior portion and a swollen posterior glandular portion.

The tylenchoid esophagus on the other hand has three parts, namely an anterior corpus, which is subdivided into a pro- and metacorpus, a narrow isthmus and a basal or glandular bulb. The pump apparatus or valve is situated in the metacorpus and appears very distinct under the microscope. Within the tylenchoid type of esophagus, three variations exist in the junction of the basal bulb and the intestine. It can be non-overlapping, overlapping dorsally or overlapping ventrally.

Vulva position

At low magnification, the vulva usually appears as a thin line perpendicular to the ventral side of the nematode. Female nematodes have one or two ovaries and therefore the vulva can be located in the middle of the body or towards the posterior end. Vulval position (V) is expressed V. It is the distance between the vulva and the anterior end as a percentage of the total length of the nematode. In nematodes with a vulva in the middle of the body, V = 50%, whereas a vulval position three quarters of the way towards the posterior end of the body would be designated as V = 75%. Vulval position can be a useful taxonomic character at both generic and species levels. For pragmatic purposes, a vulva located at mid-body implies didelphy (both the anterior and the posterior branches of the ovary are developed). On the other hand, a vulva position near the tail region generally means monodelphy (only the anterior branch is developed).

Other structures

Secretory-excretory pore

Located at the ventral side, the secretory-excretory pore (S-E pore) is the opening of the S-E system. Interesting disparity can be observed in the organization of the S-E system among different nematode groups, but these are considered rather unimportant, as they are not readily observed. In practice, only the longitudinal position of the S-E pore is used for identification. Generally, the S-E pore is located a little behind the nerve-ring region, but it may be situated behind or before the nerve ring.

Phasmid

Phasmids are chemoreceptors on the tail of the Suborder Hoplolaimina, but may also be located in the pre-anal region or erratically placed on the body. They are not always easy to see which lessens their taxonomic importance. Nevertheless, they are sometimes useful to distinguish genera within the Family Hoplolaimidae having differences in the size and location of the phasmids.

Bursa

The bursa or caudal alae is a male accessory genital structure. It may be adanal (just around anal region), subterminal (leptoderan) or terminal, enveloping the entire tail and not just confined to the terminal portion of the tail (peloderan). The bursa flaps may be simple, convex or modified to become lobed, pointed or rectangular. The bursa margins can also be smooth or crenate. The usefulness of this structure for identification is limited to male specimen. For many plant-parasitic nematodes, male individuals in the population are rare.

Characteristics of major nematode groups

The Rhizosphere and roots harbour a number of different nematodes, both free-living (predators and those that feed on organic matter and/or microorganisms) and plant-parasitic. While the interest of laboratories involved in nematode diagnosis are the nematodes of the latter group, it is very likely that the former are also present in the resulting suspension. Familiarity with the morphology of nematodes in both groups will guide you as to which ones should be and should not be included in the count.

Five major groups (Orders) of nematodes are found in the soil:

Tylenchida

Most of the known species of nematodes parasitic to plants belong to this group. They possess a stomatostylet with very distinct knobs, and have a three-part esophagus (with median bulb or metacorpus). Exceptions are specific stages of a few species where the stomatostylet is barely visible, indicating the non-functionality of the structure.

Not all tylenchids however are plant parasites. There are a number of nematodes in this group that are actually free living. These can be normally distinguished from plant-parasitic species through their weak (short and slender) stomatostylet and filiform tail.

Aphelenchida

The Aphelenchida are very similar to the Tylenchida, having a stomatostylet and a three-part esophagus. However, the basic differences are in their stylet, being much weaker (with small or no knobs) in the former and in the metacarpus. Aphelenchids have a very large metacorpus, which nearly occupies the body width. Soil-inhabiting aphelenchids are usually free living. Some species however are known to be plant parasitic but are infecting above-ground parts.

Dorylaimida

Most genera of the Dorylaimida are actually free living. Only three genera (*Longidorus*, *Paralongidorus* and *Xiphinema*) are plant parasitic and are particularly important for being vectors of plant viruses. These nematodes possess an odontostylet and have a two-part esophagus. They are also generally bigger and longer compared with the tylenchids.

Rhabditida

Rhabditida are very different from the first three groups for not possessing a stylet. Their cylindrical or cup-shaped mouth allows ingestion of bacterial cells. Their esophagus is characterized by a round basal bulb adjoining the intestine and has a distinct valvular apparatus. They may also have a median bulb like the tylenchids.

Mononchida

Of all the soil nematodes, these nematodes are probably the easiest to distinguish. They do not have a stylet but rather possess a wide buccal cavity lined with sclerotized walls which appears very distinct even at low magnification. Often, there is a large tooth or small teeth (denticles) in the stoma wall. The esophagus is characterized by being cylindrical and muscular all throughout. Mononchids are predators, feeding on small soil animals, including nematodes.

Nematodes associated with *Musa* spp.

Familiarity on the range of nematode pests attacking a particular crop will also help to further narrow down possible identities of populations present in the sample. Nematode genera/species not reported to infect a crop, say banana, can easily be dropped from the list of possibilities, thereby avoiding confusion. Identification will then be more straightforward.

The following are the major nematode pests of bananas (Gowen and Quénéhervé 1990):

- *Helicotylenchus multicinctus*
- *Meloidogyne incognita*
- *Meloidogyne javanica*
- *Radopholus similis*
- *Pratylenchus coffeae*
- *Pratylenchus goodeyi*
- *Helicotylenchus dihysteria*
- *Helicotylenchus nannus*
- *Scutellonema brachyurum*
- *Rotylenchulus reniformis*
- *Rotylenchus* sp.
- *Hoplolaimus* sp.

Among the nematodes listed above, only the endoparasites (those in the first column) are considered important. Their feeding habit causes necrosis or galls, which severely affects normal root function.

Generic identification

Since the important nematode pests on *Musa* are only limited to four genera, the description below will only focus on them. Furthermore, emphasis shall be given to stages that are likely to be encountered in suspensions obtained by the usual extraction methods (Baermann-funnel or maceration-sieving). That means, description of swollen females of a few genera are not included here for the simple reason that they will not be present in the suspension.

***Helicotylenchus multicinctus* (Tylenchida: Hoplolaimidae)**

Body C-shape (exception from other species of *Helicotylenchus* which are spiral); stylet well developed; esophagus overlaps intestine ventrally; both branches of the ovary developed though the posterior one sometimes appears reduced; vulva located on the posterior half; tail slightly tapering, with anus marked by a slight depression; terminus annulated and hemispherical in shape (Fig. 3 a-c).

***Radopholus similis* (Tylenchida: Pratylenchidae)**

All stages of this nematode are vermiform. There is a secondary sexual dimorphism on the anterior region. The juveniles and females are parasitic.

Female: Body straight to slightly curved; lip region low and not offset; stylet with well-developed round knobs; esophagus overlaps intestine dorsally; both genital branches developed; vulva slightly post-equatorial; tail elongate-conoid with a narrow rounded or indented terminus (Figures 4 a, b).

Male: Lip region raised and offset from the body contour by constriction; stylet weak with indistinct knobs; esophagus degenerate; bursa coarsely crenate, enveloping about two thirds of tail (subterminate) (Figigures 4 c, d).

***Pratylenchus* spp. (Tylenchida: Pratylenchidae)**

All stages vermiform; no secondary sexual dimorphism between males and females; body straight and short; lip region low and flattened anteriorly and continuous with body contour; stylet prominent with round to oblong basal knobs; esophagus overlaps intestine ventrally; vulva posterior; only the anterior branch of the ovary developed, the posterior branch being reduced to post-vulval sac; female tail subcylindrical to conoid; male tail completely enclosed by bursa.

There are two species of *Pratylenchus* that are important pests of bananas- *P. coffeae* (Figures 5 a-c) and *P. goodeyi* (Figures 6 a, b). The differences are summarized in the table 1.

Table 1. Characteristics of *Pratylenchus coffeae* and *Pratylenchus goodeyi* (Hunt 2000).

* position of the vulva from the anterior and expressed as percentage of body length. Higher value means more posterior.

Meloidogyne spp. (Tylenchida: Meloidogynidae)

Species of *Meloidogyne* are commonly known as root-knot nematodes. The second-stage juveniles, after starting feeding, become sedentary and swell. Females are globose or pear-shaped. Only the newly hatched second-stage juveniles and males are the vermiform and migratory stages.

Second-stage juveniles: body straight to arcuate upon death; cephalic region generally rounded with one to four course annules, a distinct labial disc, framework slightly sclerotized; stylet slender; tail with conspicuous hyaline region, tip narrow and irregular in outline (notched appearance) (Fig. 7 a, b)

Males: vermiform up to 2 mm long; lateral field with four incisures; cephalic region rounded, not sharply offset, with distinct labial disc and few (one to three) annules; stylet robust with large knobs; gland bulb ventrally overlapping intestine; tail with rounded and twisted end, spicules located near the tail end, bursa absent (Fig. 7 c, d)

There are at least two species regarded important to bananas- *M. incognita* and *M. javanica* (De Waele and Davide 1998). Species identification however is complicated by extensive inter- and intraspecific morphological variations. A combination of different approaches like biochemical, cytological, differential host test and electron microscopy may be needed for accurate identification. A quick and preliminary identification however can be provided by examining the perineal pattern morphology of the females (Eisenback 1985).

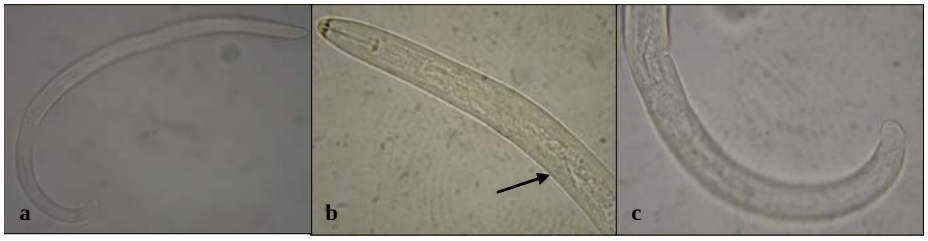

Figure 3. *Helicotylenchus multicinctus*: (a) whole body, (b) anterior region (arrow pointing at the ventral overlap of the esophagus to the intestine), and (c) vulva and tail region.

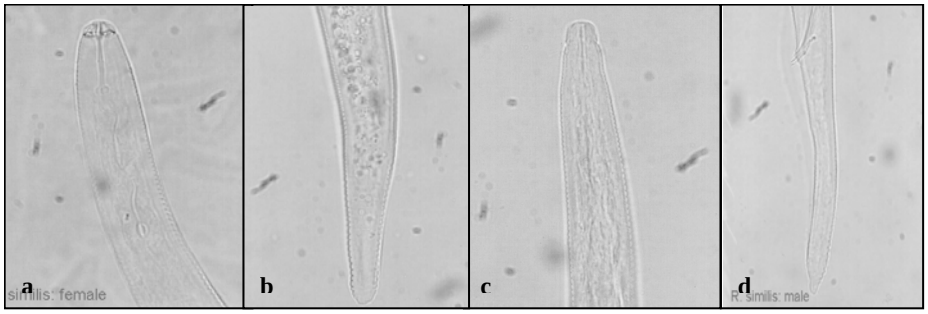

Figure 4. Head and tail region of *Radopholus similis* (a and b) female and (c and d) male (photograph courtesy of D. De Waele).

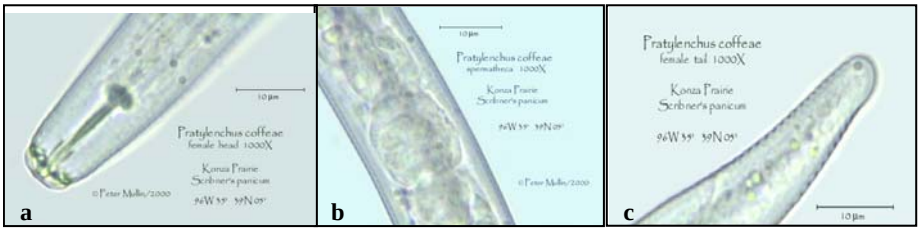

Figure 5. *Praylenchus coffeae* : (a) lip and stylet, (b) spermatheca and (c) tail regions (photograph downloaded from http://nematode.unl.edu).

Figure 6. *Pratylenchus goodeyi* female (a) head and (b) tail regions (photograph courtesy of D. De Waele).

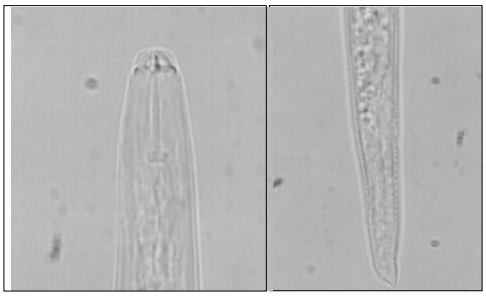

Figure 7. Head and tail region of *Meloidogyne incognita* (a and b) second stage juvenile and (c and d) male (photograph courtesy of D. De Waele).

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Culturing and mass propagation of *Musa* nematodes

Inge Van den Bergh*

Introduction

The most important banana nematodes, with the exception of *Helicotylenchus multicinctus*, can be cultured *in vitro* either on carrot discs (*Radopholus similis*, *Pratylenchus* spp.; Pinochet *et al*. 1995) or on transformed tomato roots (*Meloidogyne* spp.; Verdejo *et al*. 1988). Carrot discs allow the rearing of high numbers of *R. similis* and *Pratylenchus* spp. Transformed tomato roots are suitable for the maintenance of stock cultures of root-knot nematodes, but for the rearing of high numbers of root-knot nematodes, tomato plants in soil should be used. *In-vitro* culturing of *Helicotylenchus multicinctus* has so far been unsuccessful.

The initiation of *in-vitro* cultures of nematodes requires four steps:
1) preparation of the *in-vitro* plant tissues (carrot discs or transformed tomato roots)
2) extraction of the nematodes from infected roots
3) sterilization of the nematodes
4) transfer of the nematodes to the *in-vitro* plant tissues.

For the mass propagation of *Meloidogyne* spp. on tomato plants in the soil, step 3 (sterilization) is not necessary.

Radopholus similis and *Pratylenchus* spp. on carrot discs under *in-vitro* conditions

Preparation of the carrot discs

It will never be possible to completely sterilize the carrots, but they should be cleaned, surface-sterilized and peeled to reduce the risk that bacteria or fungi will develop in the carrot tissue. Then the carrots are cut into discs and the discs are placed into sterile Petri dishes.

Extraction of nematodes from infected roots

In order to establish a culture with as least as possible contamination, it is important to start with a clean nematode solution. Therefore, the Baermann-funnel method is the most appropriate technique to extract the nematodes from the infected roots.
The infected roots are washed thoroughly with tap water and cut into small pieces. They are macerated in a kitchen blender and passed through a 40 µm sieve. The mixture of blended roots and nematodes from the sieve is placed overnight on a Baermann funnel. The next morning, the nematodes are collected from the base of the funnel and poured through a 25 µm sieve. The residue in the sieve is rinsed with tap water to eliminate bacteria, etc. The nematodes are collected from the sieve with distilled water.

Selection and sterilization of nematodes

Actively moving, gravid female nematodes are the preferred nematodes to initiate a culture. After selection, the nematodes are transferred to sterile water. From now on, it is important to work under a laminar-flow cabinet. The nematodes should be sterilized twice: first in 0.01% $HgCl_2$ for 2 minutes and afterwards in 2000 ppm streptomycin sulphate overnight. The next morning, the nematodes are rinsed several times with sterile water.

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Inoculation of the carrot discs with nematodes

The carrot discs should be used not too long after preparation, before bacteria and fungi can develop. It is also recommended to use discs of different carrots for inoculation with a given population to spread the risk of contamination due to bacteria or fungi in the carrots.

The nematodes are transferred to the carrot discs with a sterile micropipet. Small drops of nematode solution are placed on the margin of the carrot discs. For culture maintenance, low inoculum levels (25 nematodes or less) are used, while for mass multiplication (e.g. for use in experiments), high inoculum levels (more than 25 nematodes) are used.

The Petri dishes are sealed well with parafilm, labeled (date, nematode species, origin of population, number of females) and placed in a plastic box in an incubator at 28°C.

Subculture

Depending on the initial inoculum and on the nematode species, the nutrients will be depleted after some weeks and the nematodes will leave the carrot discs. It becomes then necessary to subculture the nematodes onto fresh carrot discs. It is best to use these discs on which one can see many nematodes on the Petri dish around the carrot disc.
The carrot discs are prepared in the same way as described before. The nematodes are extracted from the old carrot discs using the Baermann-funnel method. For the sterilization of the nematodes, the sterilization in $HgCl_2$ is skipped to avoid a too high pressure on the nematodes. The sterilization in streptomycin sulphate is however done as described before. The inoculation of the carrot discs with the nematodes is also done in a similar way.

Meloidogyne spp. on transformed tomato roots under *in-vitro* conditions

Preparation of transformed tomato roots

Growing root tips from stock cultures are transferred to new Petri dishes with autoclaved Gamborg-B5 medium: Gamborg-B5 powder including vitamins 3.292 g/L, 20 g/L sucrose, 15 g/L agar, pH = 6.2.

The root tips are allowed to grow for 2 weeks before the nematodes are inoculated on the roots. Any contaminated dishes should be removed immediately.

Extraction of nematodes from infected roots

The infected roots are washed thoroughly with tap water and cut into small pieces. They are macerated in a kitchen blender in 0.12% NaOCl and passed through a 40 µm sieve. The mixture of blended roots and nematodes from the sieve is placed on a Baermann funnel. The mixture is left on the sieves for a few days, until the juveniles have hatched from the eggs. After some days, the nematodes are collected from the base of the funnel and poured through a 25 µm sieve. The residue in the sieve is rinsed with tap water to eliminate bacteria, etc. The nematodes are collected from the sieve with distilled water.

Selection and sterilization of juveniles

The procedure is the same as for the culture of *R. similis* and *Pratylenchus* spp. on carrot discs, but actively moving juveniles instead of females are used to initiate a culture. After selection, the nematodes are transferred to sterile water. From now on, it is important to work under a laminar-flow cabinet. The nematodes should be sterilized twice: first in 0.01% $HgCl_2$ for 2 minutes and afterwards in 2000 ppm streptomycin sulphate overnight. The next morning, the nematodes are rinsed several times with sterile water.

Inoculation of transformed tomato roots with juveniles

It is recommended to use Petri dishes of different root stocks for inoculation with a given population to spread the risk of contamination due to bacteria or fungi.

The nematodes are transferred to the growing tips of the transformed tomato roots with a sterile micropipet.
The Petri dishes are sealed well with parafilm, labeled (date, nematode species, origin of population) and placed in a plastic box in an incubator at 28°C.

Subculture

Depending on the initial inoculum, the nutrients will become depleted after some weeks and subculturing will be necessary.
New cultures of transformed tomato roots are prepared from stock cultures as described before. Instead of extracting the juveniles from the tomato roots, egg masses are removed from the old roots with a scalpel and transferred to a new growing root tip on a new Petri dish.

Mass propagation of *Meloidogyne spp.* on tomato plants

Preparation of tomato plants

Pots are filled with sieved, sterilized soil and placed on dishes. The soil is watered up to field capacity. In the middle of each pot, a tomato seed is planted and allowed to grow for 2-3 weeks before inoculation.

Extraction of nematodes from transformed tomato roots

Galls and egg masses are macerated in a kitchen blender in 0.12% NaOCl and passed through a 100 µm sieve placed in bowl, to separate the eggs and juveniles that will pass through the sieve from the root tissue. The content of the bowl is poured through a 25 µm sieve. The eggs and juveniles which are retained on the sieve are rinsed with tap water and collected from the sieve with distilled water.

Inoculation of the nematodes on the tomato plants

The suspension with the nematodes is brought to a known volume with distilled water. The number of eggs and juveniles in a subsample is determined under the microscope and the nematode population in the total suspension is calculated. The suspension is diluted to about 5000 eggs and juveniles in a few ml of distilled water.
About 5000 eggs and juveniles in a few ml of water are inoculated in 3-4 holes adjacent to the base of each plant. After inoculation, the soil is lightly watered.

Subculture

Every 8 weeks, the roots of the inoculated tomato plants are cut into small pieces and placed around the roots of newly grown tomato plants.

Note: For all the procedures described above, all the equipment should be cleaned after use, first with ethanol (to kill remaining nematodes) and afterwards with soap and water.

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Tissue culture of banana

Olivia P. Damasco*

Introduction

Plant-tissue culture is a generic description of various techniques encompassing the *in-vitro* culture of embryos, organs, tissues, cells and protoplasts. These techniques involve the culture of isolated plant parts in artificial medium under sterile growing conditions.

The tissue-culture techniques established for banana include shoot and meristem culture, callus culture, somatic embryogenesis and cell- suspension culture and protoplast culture.

Establishment of banana shoot cultures

Collection of suckers

The micro-propagation work can be facilitated with the strict selection of planting materials at the onset the production cycle. Suckers can be collected from field genebanks, farmers' fields or isolated nursery areas.

1) Prepare the materials needed for collection, pack the following things:
   a) net bags
   b) marker pen
   c) record book
   d) plastic labels, string twine, pencil
   e) knife
   f) desuckering bar
2) Check for appropriate source mats. Check if there are symptoms of virus infections.
3) Check which sucker to collect. The suckers may range from peepers to sword suckers to maiden suckers.
4) Dig around the suckers to expose the point of connection to the main stem or the other suckers.
5) Use the desuckering bar to separate the sucker from the main stem. Make a clean excision. Avoid cracking the corm of the sucker during collection. For small suckers, particularly peepers, you may use a machete or appropriate knife. Make sure the cutting edge is sharp. Again, avoid making cracks during harvesting.
6) Collect at least two suckers from one plant source for both laboratory and nursery farm for future sucker needs.

Establishment of initial shoot culture

Preparation of shoot proliferation medium

The culture medium is based on Damasco and Barba (1984). You may compare this medium with other published culture media. The components of the medium are listed in Table 1.

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Table 1. Components of the banana shoot proliferation medium (at pH 5.7).

Stock solution/component	Concentration of stock solution	Volume weight for 1 L of culture medium
MS macronutrients (0.5x)	25x	20 ml
MS micronutrients (0.5x)	100x	5 ml
MS vitamins (1x)	100x	10 ml
Fe-EDTA (1x)	200x	5 ml
Sugar		30 g
BAP	100 mg/L	50 ml (5 mg)
Coconut water (optional)		100 ml
Agar		6 g

Disinfection

1) Prepare the planting material:
   a) Remove the suckers from the bags. Wash in running water to remove any adhering soil and dirt.
   b) Trim leaf bracts and corm tissues. The size would be dependent on the type (buttons, peeper, sword) collected for introduction to culture. The size should be sufficient to protect the growing point from bleaching.
2) Transfer the shoot into a container or plastic which is large enough to accommodate both shoot and disinfectant. When using a big container, place a wad of cotton to ensure complete wetting of the whole shoot.
3) Immerse the shoot in pure commercial bleach (5.25% sodium hypochlorite) for 30-45 minutes, depending on the size of plant material.
4) Decant the sterilant from the container taking care not to expose the shoots.
5) Bring the shoots (in the container) to the inoculation chamber.

Preparation of the sterile working area

1) Prepare the inoculation chamber (sterile air bench). Wipe the chamber thoroughly with 95% ethyl alcohol.
2) Arrange materials in the inoculation chamber.
3) Ensure that the inoculation chamber is not crowded with too many cultures and glasswares to facilitate movement during excision work.
4) Place instruments, including scalpels and forceps in a coupling jar or wide-mouth bottle with 95% ethyl alcohol. During the inoculation activity, always flame instruments before use.

Inoculation

1) Wash your hands with soap and water and wipe arms and hands with 70% ethyl alcohol.
2) Take out one shoot at a time from the container. Transfer to aseptic working surface (Petri dish, steel dish or sterile paper).
3) While holding the shoot with the forceps, carefully cut off the outer leaf sheath that comes in contact with the disinfectant.
4) Transfer to a fresh working surface and continue cutting until the shoot measures 1 x 1 cm with corm tissue as thin as possible.
5) Transfer the shoot to a fresh working surface.
6) Cut the shoot into quarters longitudinally, through the centre.
7) Inoculate each quarter onto a solid culture medium, which allows proliferation.

Maintenance of shoot cultures

1) Keep the shoot cultures in an air-conditioned room under a 16-hour photoperiod 40 $\mu E/m^2s^{-1}$ (provided by two 40-watt fluorescent tubes).
2) Observe the cultures for contamination. Discard contaminated cultures as soon as contamination is noted.
3) Observe for browning and bulging of corm tissue, greening of leaf tissues and growth of new shoots during the first month of culture.
4) Note when shoots that come out from the apex of the leaf axis are almost 2 cm in height. Your cultures are then ready for their first subculture.

Propagation by shoot proliferation

Propagation can be achieved in many ways. The simplest technique is to induce the established *in-vitro* plant to produce new shoots at a faster rate within the culture vessel. This technique consists of separating shoots, cutting them up if they have grown bigger than optimum, transferring the shoot or sections of the shoot to fresh culture medium and again going through the same cycle of activities for another subculture. This step is repeated for seven to eight cycles.

1) Cut the growing shoot from the initial explant before it extends beyond 2 cm in length. *Note: Overgrown shoots give less proliferation. When shoots have grown beyond 2 cm, make a longitudinal incision through the apex of the growing shoot.*
2) Subculture onto fresh half-strength Murashige and Skoog's medium supplemented with 5 mg/l BAP and 100 ml/l coconut water for multiplication or any recommended culture medium for shoot proliferation. We prefer medium without auxins because nubbins form earlier and at high frequencies when the proliferating medium contains both auxin and cytokinin.
3) After 1 month, divide the shoot cluster and transfer to fresh culture medium.
4) Subculture every 3-4 weeks until the desired number of shoots have been obtained.
5) Make an inventory of the number of proliferated shoots.
6) When sufficient shoots have proliferated as nuclear stock, proceed with rooting.

Rooting of in-vitro *derived shoots*

Preparation of culture medium for rooting

Prepare the rooting medium presented in Table 2. Use within a week of preparation for best results.

Table 2. Components of banana rooting medium.

Stock solution/component	Concentration of stock solution	Volume weight for 1 L of culture medium
MS macro nutrients (0.5x)	25x	20 ml
MS micronutrients (0.5x)	100x	5 ml
MS vitamins (1x)	100x	10 ml
Fe-EDTA (1x)	200x	5 ml
Sugar		30 g
Coconut water (optional)		100 ml
Agar		6 g

Rooting of shoots

1) Store shoots longer than the 3-4 week proliferation cycle until small plantlets are formed.
2) Separate individual shoots from a cluster of shoots and inoculate them onto rooting medium.
3) Expect shoots to form roots in 3-4 weeks.
4) Plant out bananas in soil when they have 3-4 expanded leaves and are well rooted.

Nursery establishment

The establishment of tissue-cultured plants in the nursery makes or breaks a micro-propagation system. Precaution should be taken to ensure maximum survival among potted-out tissue-cultured plants.

The tissue-cultured plants are exposed to high humidity, generally low light intensities or poor quality of light. Unless acclimatized to nursery conditions or cultured on medium which prepares the plants for more rigorous environments, the plantlets may become fragile, and survival may be poor.

Acclimatization

1) Select rooted plantlets which are about 4-7 cm high and with 3-4 expanded leaves among shoots cultured on rooting medium for 4-6 weeks.
   Note: Banana plantlets at this stage of growth and development have the highest chance of survival. Overgrown plants and unrooted shoots fare poorly.
2) Transfer selected cultures to the greenhouse or nursery 3 days before the scheduled potting out. Locate cultures in an area where there is no direct sunlight at midday.
   Note: This step preconditions cultures away from the laboratory conditions which are often characterized by even and cool temperatures.
3) Remove cotton plugs or vessel closure 1 day before potting out.
   Note: This step reduces the relative humidity within the culture vessel and acclimatizes plantlets to lower humidity.

Preparation of soil mixes

1) Prepare one of the following potting mixes:
   a) coir dust:garden soil (1:1, v:v)
   b) decomposed saw dust:garden soil (1:1)
   c) decomposed animal manure:garden soil (1:1)
   d) sand:garden soil (1:1)
2) Heat-sterilize the potting mix. Cool the mixture before use. (Heat sterilization has been disregarded by some nurseries by avoiding the use of contaminated soil or by drenching the soil with fungicide).
3) Moisten the potting mix.
4) Put sufficient quantity of potting mix into a suitable container with sufficient drainage holes.

Taking plants out from culture vessels

1) Add tap water to the culture, sufficient to cover the roots. Shake the vessel to loosen the agar medium.
2) Pull out the plants gently from the culture medium with a pair of forceps or with fingers.
3) Wash roots thoroughly with plain water to remove adhering culture medium.
4) Dip the roots in a fungicide solution before potting.

Potting to soil mix

1) Water the potting mix thoroughly. Allow water to drain, leaving the mixture moist but not sopping wet.
2) Make a hole in the potting mix with a clean stick or pointed tool. The hole should be large enough to accommodate the plantlet.
3) Position the plantlet into the hole. Press the soil around the base of the plant.

Nursery establishment

1) Protect the plants from excessive light during the first 2 weeks. Increase the light intensity when recovered from potting stresses.
2) To water, mist the plantlets during the 1st week and apply overhead watering done daily or as needed thereafter, until the 4th week of nursery cultivation. Adjust the watering scheme to ensure that the soil is not too dry or wet.
   Note: Control watering. The frequency of watering is dependent on the choice of potting mix, relative humidity, light, wind and temperature.
3) Fertilize the plants every 2 weeks using a foliar complete fertilizer. Use the recommended rate for seedlings.
4) Plants are ready for field planting about 2 to 3 months from potting.

References

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Assessment of the host-plant reaction of *Musa* to nematodes

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Introduction

Most of the research conducted worldwide to control nematodes on banana has been on chemicals, despite the high costs of nematicide applications and the negative consequences resulting from residue accumulation in the environment (Vilardebo and Guerot 1976; Buddenhagen 1987; Pinochet 1986). The use of resistant varieties is now recognized more and more as a valuable alternative.

For the description of the relationship between a host plant and nematodes, the terminology of Bos and Parlevliet (1995) is used. Resistance/susceptibility on the one hand and tolerance/sensitivity on the other hand are defined as independent, relative qualities of a host plant based on comparisons between genotypes. A host plant may either suppress (resistance) or allow (susceptibility) nematode development and reproduction. It may suffer either little injury, even when quite heavily infected with nematodes (tolerance), or much injury, even when relatively lightly infected with nematodes (sensitivity).

Although naturally occurring nematode resistance and tolerance has long been exploited for many agricultural crops (De Waele 1996), this method of nematode management has so far been neglected in bananas. This is despite the evidence, albeit limited, that nematode resistance and tolerance sources are present in the *Musa* genepool (Pinochet 1996). The development of nematode-resistant banana genotypes is extremely difficult because of the genetic complexity of the crop, its low fertility and the long period required for the nematological evaluation of crossings (Pinochet 1988a). There have been no reports to date of a transgenic banana that is either less susceptible or less sensitive to nematode pathogens (De Waele 2000).

It is evident that screening of all kinds of *Musa* genotypes will be an important activity in order to develop a more integrated pest management system that deals with the nematode problem in a more environmentally sustainable way.

Experimental design and reference genotypes

In screening experiments, replications should range between 8 and 15. To minimize variation in ambient conditions, the replications should be arranged in a completely randomized design, a randomized complete block design or a split-plot design.

In order to be able to compare results between screening experiments, it is recommended to include at least one, preferably more, reference genotype(s):

Grand naine (ITC.1256): highly susceptible to all nematodes
Gros Michel (ITC.1122): moderately resistant to *Radopholus similis*
Yangambi km 5 (ITC.1123): highly resistant to *R. similis*
Pisang jari buaya (ITC.0312): completely resistant to *R. similis*

The method described here is modified after Pinochet (1988b), Sarah *et al*. (1992) and Speijer and De Waele (1997).

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Screening under greenhouse conditions

Screening experiments in pots in the greenhouse will only allow observations to be made for a relatively short period (2-3 months) of the crop cycle. During this period, the susceptibility of genotypes can be determined by assessing the nematode reproduction rate. If uninoculated controls are included in the screening, some observations can be made on the sensitivity of the genotypes.

Preparation of plant material

Either rooted *in-vitro* plantlets, ready for transfer to *in-vivo* conditions, or suckers of uniform size, which are peeled immediately after detaching from the mother plant and immersed in water at 55°C for 15 minutes, can be used as starting material.

They are planted in well-draining pots filled with sieved, sterilized soil, placed on dishes. The soil is watered up to field capacity. The first weeks, the pots can be placed under a plastic or styrofoam cover for adaptation to the new environment (not necessary for suckers). The cover can be gradually opened after 2 weeks.

Four weeks after planting, 8-10 plants of each genotype are inoculated with nematodes (see next step), while another 8-10 plants are kept nematode-free as control plants.

The pots should be labeled (genotype, inoculated or control) and arranged in a completely randomized design or randomized complete block design.

Extraction and inoculation with nematodes

For *R. similis* and *Pratylenchus* spp., nematodes are extracted from carrot-discs cultures. It is best to use those carrot discs on which you can see many nematodes on the Petri dish around the carrot disc. For *Meloidogyne* spp., nematodes are extracted from the roots of tomato plants on which you can see many galls. In both cases, the maceration-sieving technique can be used.

The suspension with the nematodes is brought to a known volume with distilled water. The number of nematodes and eggs in a subsample is counted and the nematode population in the total suspension is calculated. The suspension is diluted to about 1000 vermiform nematodes (for *R. similis* and *Pratylenchus* spp.) or to about 5000 eggs and juveniles (for *Meloidogyne* spp.) in a few ml of distilled water.

This inoculum is inoculated in 3-4 holes, about 3 cm deep, into the soil adjacent to the base of each plant. After inoculation, the soil is lightly watered.

During the whole experiment, the plants should be watered as needed and fertilized with a nutrient solution every 2-4 weeks.

Data collection

The nematodes should be allowed to grow for one or two generations on the plants. For experiments with *R. similis* and *Meloidogyne* spp., collect data at 8 weeks after inoculation; for experiments with *P. coffeae*, at 10 weeks after inoculation.

First, general information about the experiment should be written down, such as soil type, date of transfer to the greenhouse, date of inoculation, initial inoculum, fertilizer application, climatic data, etc.

Then, the plant parameters are measured. Any visible disorders are written down. The plants are removed from the pots and the roots are washed with tap water. The roots are cut off from the corm. The plant height, girth of the pseudostem at the base, shoot weight, weight of the root system and number of healthy leaves (> 70% of the leaf surface healthy) are measured.

The root damage is assessed: number of dead and functional roots, the general health condition of the secondary and tertiary roots, the root necrosis (for root-lesion nematodes) and the root galling (for root-knot nematodes).

Finally, the nematode reproduction is measured. The egg masses of *Meloidogyne* spp. are stained with 0.15 g/L phloxine B for 15 minutes and counted under the stereo microscope. The vermiform nematodes are extracted from the roots using the maceration-sieving

technique. A subsample is counted under the microscope and the total nematode population per given amount of roots and for the whole root system is calculated.

Screening under field conditions

Screening experiments in the field have some advantages over screening experiments in the greenhouse. They will allow observations to be made during the whole crop cycle and subsequent ratoon crops. During this period, the susceptibility of the genotypes can be determined by assessing the nematode reproduction rate. If uninoculated controls are included in the screening, observations can be made on the sensitivity of the genotypes, including at the level of yield. On the other hand, screening experiments in the field are more time consuming and require more space and financial input.

Preparation of plant material

The same materials, either rooted *in-vitro* plantlets or suckers of uniform size, can be used as starting material. They are planted in well-draining polystyrene bags filled with sieved, sterilized soil. The soil is watered up to field capacity. The first weeks, the bags can be placed under a plastic or styrofoam cover for adaptation to the new environment (not necessary for suckers). The cover can be gradually opened after 2 weeks.

Site selection and preparation

A field with a good initial infestation should be selected. The infestation level of the field can be assessed by taking root samples (not soil samples) of the crop grown on the field. The site should preferably be infested with a single nematode species, though this will seldom be the case; the population should be at least 100 nematodes per gram of fresh roots.

The land should be cleared of any previously grown crops and prepared for the new planting. The planting holes should be made before the plantlets are transferred to the field. A split-plot design, with main-plot factor = infection (treated or not treated with nematicides) and sub-plot factor = genotype, can be followed. Nematicides can be applied to the planting holes of the control plants to eradicate the nematodes present in the soil.

Transfer to the field

It is best to transfer the plants in the early morning or late afternoon, to avoid the heat of the day. The plants should be watered just before planting. The bags should be removed carefully, in order not to disturb the ball of earth around the root system of the plants. Right after planting, the plants should be watered again. They should be watered and fertilized as needed during the whole experiment. The control plants should be treated with nematicides every few months to eradicate the present nematodes.

Data collection

General information about the experiment should be written down, such as soil type, date of transfer to the greenhouse and the field, initial infestation, irrigation, fertilizer application, climatic data, etc.

Data can be collected at several times: at a specific time after planting (e.g. at 6 months after planting), at shooting (emergence of flower) and at harvest; you can also collect data from the ratoon crops.

The plant parameters are measured. Any visible disorders are written down. The plant height, girth of the pseudostem at 50 cm above ground level and the number of healthy leaves (> 70% of the leaf surface healthy) are measured. The date of shooting and date of harvest is written down. At harvest, the bunch weight, number of hands and number of fingers are measured.

Samples are collected from an excavation of 20 x 20 x 20 cm^3 at the base of the mother plant. All banana roots in this soil volume are collected and placed with a handful of soil in a plastic bag. Do not forget to label the plastic bag.

The root damage is assessed: number of dead and functional roots, the general health condition of the secondary and tertiary roots, the root necrosis (for root-lesion nematodes) and the root galling (for root-knot nematodes).

Finally, the nematode reproduction is measured. The egg masses of *Meloidogyne* spp. are stained with 0.15 g/l phloxine B for 15 minutes and counted under the stereo microscope. The vermiform nematodes are extracted from the roots using the maceration-sieving technique. A subsample is counted under the microscope and the total nematode population per given amount of roots is calculated.

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Factsheets

NEMATODE SURVEY AND COLLECTION

EQUIPMENT AND MATERIALS TO BE TAKEN TO THE FIELD

- measuring tape
- ladder
- knife/machete
- spade
- hand trowel
- plastic bags for sampling
- permanent pen markers
- data collection forms + pencil
- camera
- Global Positioning System (GPS) (if available)

SURVEY AND SAMPLE COLLECTION

Site selection and general information

1. Select a site where you will collect samples.
2. Collect information on the history of the site, the age of the plantation, the size or scale of the plantation, the cropping system, irrigation, the application of fertilizers and pesticides and other management practices, as well as climatic data and other useful information.

Use Form 1 to write down any information about the site.

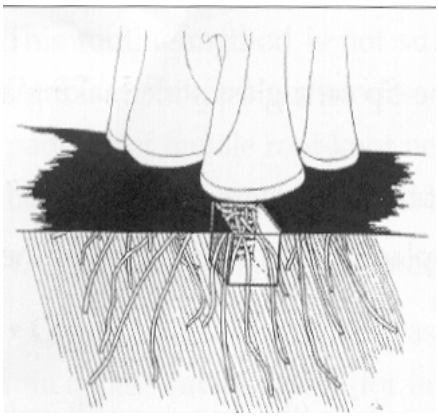
Plant parameters

1. Select 3-5 plants of the same cultivar at a growth stage somewhere between flowering and bunching.
2. Measure the plant height, the girth of the pseudostem at 50 cm above the ground, the number of healthy leaves (> 70% of leaf surface healthy), the number of hands and the number of fingers. If the number of fingers is difficult to count, just count the number of fingers on the third hand and the number of fingers on the second-to-last hand, and later calculate the total number of fingers using the formula of Turner *et al*. (1998).

Use Form 2 to write down the plant parameters.

Sample collection

1. Dig a hole of 20 x 20 x 20 cm^3 at the base of the mother plant.
2. Collect all banana roots from the selected, making sure not to collect the roots of the adjacent plants.
3. Place the roots together with a handful of soil in a plastic bag.
4. Label the plastic bag (date, site, sample number).



FORM 1. Site information

Date: _____

Place: Village: _____

Town: _____

Province: _____

Owner/Contact person: _____

History of site: _____

Age of plantation: _____

Size/scale of plantation: _____

Cropping system: _____

Intercropping with: _____

Irrigation: _____

Organic fertilizer: _____

Inorganic fertilizer: _____

Pesticides: _____

Mulching: _____

Weeding: _____

Desuckering: _____

Climatic data: _____

Other data: _____

_____

_____

_____

FORM 2. Plant parameters

No.	Cultivar	PS	PH	PG	HL	NH	NF	NF_3	NF_{n-1}
1									
2									
3									
4									
5									
6									
7									
8									

PS: Plant stage: S – small sucker less than 1.5 m high
PF – pre-flowering plant at least 1.5 m high
RF – recently flowered plant (emerging flowers or less than 14 days)
FL – flowered plant at any stage between RF and CH
CH – plant close to harvest
H – plant at harvest

PH: Plant height (cm)

PG: Pseudostem girth at 50 cm above the ground (cm)

HL: Number of healthy leaves

NH: Number of hands

NF: Total number of fingers, or estimation of total number of fingers, using the formula of Turner *et al.* (1998): $NF = 0.5\ NH\ (NF_3 + NF_{n-1})$

NF_3: Number of fingers on 3rd hand

NF_{n-1}: Number of fingers on 2nd-to-last hand

PROCESSING OF ROOT AND SOIL SAMPLES

EQUIPMENT AND MATERIALS

- sink with running water
- stereo microscope (and/or light microscope)
- kitchen blender
- scale (accuracy 0.1 g)
- scale (accuracy 100 g)
- camera
- tissue paper
- pair of scissors
- knife
- watch with seconds hand
- beaker
- distilled water
- phloxine B
- glass bottles with lid
- 5 ml or 10 ml pipet
- 2 mm, 250 μm, 100 μm, 40 μm, 25 μm sieve
- bowl on which the sieves can be placed
- counting dish (or counting slide)
- 2 pails or basins
- data collection forms + pencil

ROOT SAMPLES PROCESSING

Root-damage assessment

1. Wash the roots with tap water and pat them dry on tissue paper.
2. Divide the roots into dead roots and functional roots. Dead roots are shriveled and no healthy root tissue is left. Count the number of roots in each category and calculate the percentage of dead roots.
3. Score the general health condition of the secondary and tertiary roots.
4. Estimate the root galling.
5. Select at random five functional primary roots, at least 10 cm long. Cut the selected roots to 10-cm pieces and slice the roots lengthwise. Score one half of each of the roots for the percentage of the root cortex showing necrosis. The maximum root necrosis per root half can be 20%, giving a maximum root necrosis of 100% for the five root halves together (Figure 1).

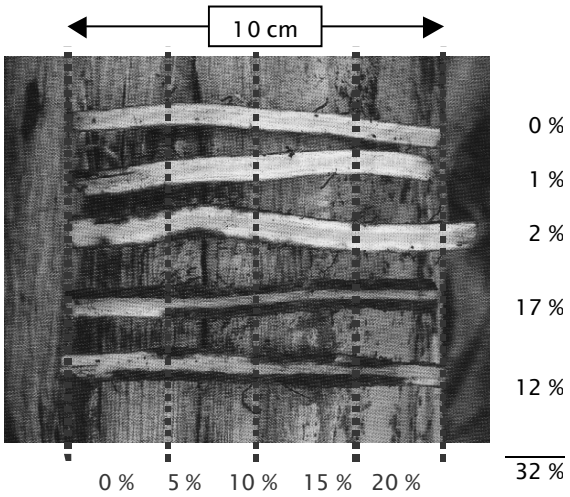


Figure 1: Root necrosis (%)

Use Form 1 to write down the root-damage data.

Staining and counting of the egg-laying females

1. Cut the roots in 1 cm pieces and take a subsample of 5 g.
2. Stain the egg masses by immersing the roots in 0.15 g/l phloxine B for 15 minutes.
3. Count the number of egg-laying females under the stereo microscope.

Use form 2 to write down the number of egg-laying females.

Note: The counting of the egg-laying females can also be done after the next step (extraction of nematodes). The stained roots can be stored in distilled water in the refrigerator at 4 °C.

Extraction and counting of the nematodes

1. Cut the roots in 1 cm pieces and take a subsample of 10 g.
2. Put the roots in a kitchen blender with tap water and macerate the roots three times for 10 sec at 5-sec interval.
3. Pour the macerate suspension through a 250 μm, 100 μm and 40 μm sieve.
4. Rinse the residue in the 250 μm and 100 μm sieves with tap water.
5. Collect the nematodes from the 40 μm sieve with distilled water in a beaker.
6. Dilute the suspension with distilled water to 200 ml and pour it in a labeled glass bottle.
7. Homogenize the nematode suspension by blowing air through it with a pipet and take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide).
8. Count the nematodes of each species in the counting dish under the stereo microscope (or in the counting slide under the light microscope).
9. Calculate the nematode population per 10 g of fresh roots.

Use Form 2 to write down the nematode count data.

FOR A CLEARER NEMATODE SUSPENSION

1. Cut tissue paper to fit the Baermann sieves and place it on the sieves.
2. Pour the mixture of nematodes with fine soil particles on the Baermann sieves (on the tissue paper) and place the Baermann sieves on dishes/funnels.
3. Add distilled water to the dishes/funnels until it just covers the mixture of roots and nematodes. Do not add too much water: it is the intention that the water will evaporate so that the mixture becomes somewhat dry, which will make the nematodes migrate downwards towards the remaining water (below the tissue paper and sieve openings).
4. Leave the mixture on the sieves for 1-2 nights.

Next morning:

5. Collect the nematodes from the bottom of the dishes or the base of the funnels in a beaker.
6. Pour the suspension with the nematodes through a 25 μm sieve.
7. Rinse the residue on the sieve with tap water (to eliminate bacteria, etc.).
8. Collect the nematodes from the sieve with distilled water in a beaker.

SOIL SAMPLES PROCESSING

Extraction of the nematodes from the soil

1. Mix thoroughly 300 g soil in a pail or basin half-filled with water. The nematodes become suspended in the water.
2. After about 20-30 sec (depending on the soil texture), pour the water through a 250 μm sieve into a second pail or basin. Heavy soil particles remain in the first pail and large particles of debris are caught by the sieve. The nematodes pass through the sieve.
3. Stir the muddy solution in the second pail and allow to stand for 20-30 sec.
4. Pour the waste with nematodes through a 100 μm sieve on top of a 40 μm sieve.
5. Collect the nematodes from the 40 μm sieve with distilled water into the beaker.

FORM 1. Root-damage assessment

No.	Cultivar	DR	FR	PDR	RH	RG	RN
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

DR: Number of dead roots
FR: Number of functional roots
PDR: Percentage of dead roots (%)
RH: Root health assessment of secondary and tertiary roots: 1 – all healthy
2 – most healthy
3 – most dead
4 – all dead
RG: Root galling: 0 – no galling
1 – trace infections with a few small galls
2 – less than 25% of the roots galled
3 – 25-50% of the roots galled
4 – 50-75% of the roots galled
5 – more than 75% of the roots galled
RN: Root necrosis (%) = percentage of necrosis observed on five 10 cm pieces of roots

FORM 2. Nematode counts

No.	Cultivar	ELF	Rs	Pc	M	Others (specify)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

ELF: Number of egg-laying females of *Meloidogyne* spp. per 5 g of fresh roots
Rs: Number of *Radopholus similis* per 10 g of fresh roots
Pc: Number of *Pratylenchus coffeae* per 10 g of fresh roots
M: Number of juveniles of *Meloidogyne* spp. per 10 g of fresh roots

PROCESSING, MOUNTING AND IDENTIFICATION OF NEMATODES

KILLING AND FIXATION OF NEMATODES

Method 1: heat killing before addition of fixative

1. Kill the nematodes by heating (55-60°C) over a flame, hot plate, in an oven or by partially immersing containers in a large volume of water at nearly boiling point (80-90 °C) for 2-3 min. Do not overheat!
2. Add an equal volume of double-strength (2x) cold fixative to the suspension.

Method 2: addition of hot fixative

1. Heat double-strength fixative to 80-90 °C.
2. Pour the hot double-strength fixative into a vessel containing an equal volume of nematode suspension.

Do not add cold fixative to a suspension containing live nematodes!
Caution: all fixatives have formalin as a component and when heated, fumes are emitted → work in a fume hood!

Fixatives

- Formalin:
  2-5% final concentration, buffered to pH > 7
  Caution: carcinogenic and highly denaturing → handle with due care and always work in a fume hood!

- Formalin-glycerol (FG):

Reagent	Normal strength (ml)	Double strength (ml)
formalin (40% formaldehyde)	10	10
glycerol	1	1
dH_2O	89	45

- Formalin-acetic acid (FAA):

Reagent	Normal strength (ml)	Double strength (ml)
formalin (40% formaldehyde)	6	6
ethanol (96%)	20	20
glacial acetic acid	1	1
dH_2O	40	20

- Triethanol-amine-formalin (TAF):

Reagent	Normal strength (ml)	Double strength (ml)
triethanolamine	2	2
formalin	7	7
dH_2O	91	45

MOUNTING

Materials needed

- glass slides (25 x 76 x 1 mm)
- cover slips (22 x 22 mm)
- picking implements: bamboo splinters or coconut midrib sharpened to have a fine point, eyelashes or cactus spines mounted on needles with water resistant glue, fine insect needle (#00) with hooked tip, etc.
- copper cylinder (1.5 cm diameter) and paraffin wax (melting point > 60 °C)
  OR nail polish and ringer (Fig. 1)
- alcohol lamp
- ethanol for soaking glass slides and cover slip
- lacto-glycerol (mounting medium)

Creating cover slip support

- Mounting of glass fibers with the same diameter as the specimen:
  Arrange three short pieces of glass fiber with thickness similar to that of the specimen radially and equidistantly from each other as a support for the nematodes.

- Using paraffin wax (after de Maeseneer and D' Herde, 1963):
  1. Soak the glass slide (and cover slip as well) in ethanol and wipe with clean tissue paper to get rid of the grease.
  2. Heat the copper cylinder, dip it in paraffin wax and introduce the melted wax onto the center of the glass slide.

- Using nail polish:
  1. Clip the glass slide on the rotating disc.
  2. Dip the brush in nail polish.
  3. Rest your hand on the stationary disc (hand rest) so that it will not move once the ring is being made.
  4. Touch the surface of the glass slide with the brush and rotate the main disc, so that the nail polish is spread over, creating a ring on the glass slide.

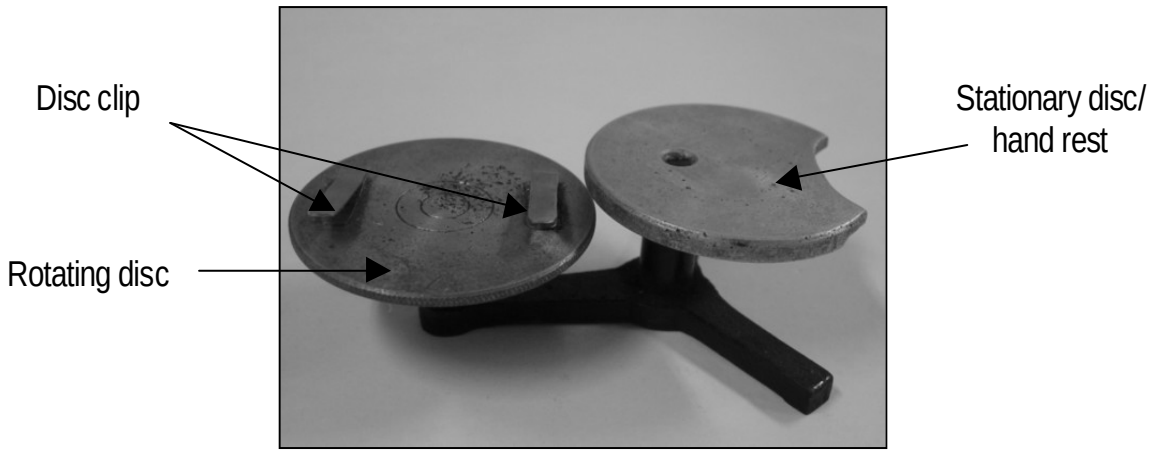


Fig 1. Ringer

Picking out nematodes

Pick the nematodes out from the suspension under the low magnification of a dissecting microscope:
1. Position the fine point of the needle under the nematode.
2. Gradually work the nematode upwards to the surface of the water.
3. Pick the nematode out of the water by draping it over the end of the point.

Tip: keep the level of the suspension in the dish shallow for easier picking.

Temporary mounts

1. Carefully pick selected (live) nematodes and transfer them to a drop of water on a glass slide.
2. Mount three glass fibers as described above.
3. Check the specimen and place the cover slip over the slide.

Semi-permanent mounts

- Lactophenol:
  Transfer fixed nematodes to a drop of stained or unstained lactophenol on a glass slide, which has been heated to 60 °C.
  Caution: hazardous fumes from lactophenol!

Formulation:		
	phenol crystals	1 part (caution! hazardous chemical)
	lactic acid	1 part
	glycerine	2 parts
	distilled water	1 part

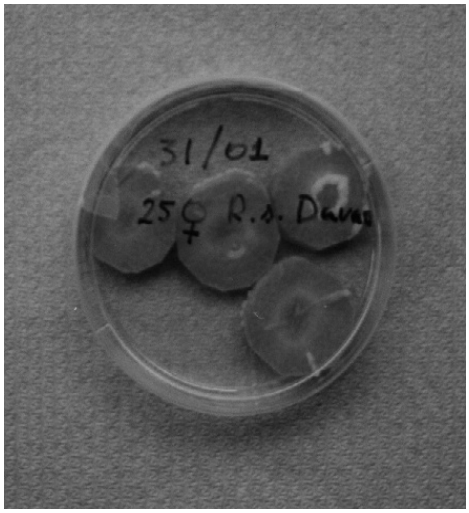
- Lacto-glycerol:
  Basically the same as above except for the exclusion of the hazardous chemical, phenol.
  Formulation: equal parts by volume of lactic acid, glycerol and distilled water.

- If a wax ring is used: arrange fixed nematodes in a small drop of mounting medium placed at the center of the wax ring, place a cover slip on top and put over a hot plate (65 °C) for a few seconds or until the paraffin wax melts. Melting of the wax causes the cover slip to settle down and confines the mounting medium at the center of the mount; aside from being the cover slip support, the paraffin wax, once solidified, serves also as the sealant.
- If a nail polish ring is used: introduce the mounting medium and the specimen in the same manner as above, place the cover slip and seal all sides and corners thoroughly with nail polish.

Labeling

1. Use a glass-slide sticker to provide a contrasting surface for writing information.
2. Indicate as much information on the labels as possible:
   - place and date of collection
   - identity of the specimen (once identified)
   - collector's name
   - host name and cultivar (if applicable)
   - number and life stages of the specimen mounted.

CULTURE AND MASS PROPAGATION OF *RADOPHOLUS SIMILIS* AND *PRATYLENCHUS* SPP. ON CARROT DISCS UNDER *IN-VITRO* CONDITIONS



EQUIPMENT AND MATERIALS

Equipment
- sink with running water
- autoclave
- laminar flow cabinet
- incubator (28 °C)
- stereo microscope
- kitchen blender
- scale (accuracy 0.1 mg)

Autoclaved materials
- sterile pair of tongs to hold the carrots
- sterile knife
- sterile potato peeler
- sterile pair of tweezers to take the carrot discs
- sterile scalpel
- glass plate
- small sterile Petri dishes, suitable for *in-vitro* tissue culturing (Ø 3-5 cm)
- three sterile Petri dishes
- sterile watch glass
- two sterile sealed calibrated test tubes
- ±10 sterile Pasteur pipets, with rubber aspirators
- a small sterile 5, 10 or 20 μm sieve
- sterile water (2 bottles)

Other materials
- pair of scissors
- watch with seconds hand
- 100 μm, 40 μm and 25 μm sieve
- bowl on which the sieves can be placed
- Baermann sieves + dishes/funnels
- strong tissue paper (preferably kitchen paper)
- three beakers
- counting dish
- ethanol lamp
- sprayer with ethanol
- beaker with ethanol
- fine needle with curved point
- Parafilm (cut in small strips)
- plastic box to store Petri dishes (against mites)
- 0.01% $HgCl_2$ (do not inhale: $HgCl_2$ is toxic)
- streptomycin sulphate
- distilled water
- thick, fresh carrots with foliage
- sample(s) of infected roots

FIRST CULTURE

Preparation of carrot discs

1. Remove the foliage of the carrots and clean the carrots with tissue paper.

Under the laminar flow:

2. Dip a pair of tongs in ethanol and flame them.
3. Hold the carrot with the tongs, spray ethanol on the carrot and flame it. Repeat this step three times.
4. Dip a knife in ethanol, flame it and cut off the tip of the carrot. Work on a glass plate.
5. Dip a potato peeler in ethanol, flame it and peel the carrot. Repeat this step three times.
6. Dip a knife in ethanol, flame it and cut the carrot in discs, 0.5 cm thick. Work on a glass plate.
7. Dip a pair of tweezers in ethanol, flame them and put 1-2 discs together in a Petri dish.
8. Seal the Petri dishes well with Parafilm.
9. Place the Petri dishes in a plastic box in an incubator at 28 °C.

Note: Use the carrot discs not too long after preparation, before bacteria and fungi can develop.

Extraction of nematodes from infected roots

1. Cut tissue paper to fit the Baermann sieves and place it on the sieves.
2. Wash the roots with tap water. It is important to wash off all the soil, to make the next steps (picking of the nematodes) easier.
3. Cut the roots in 1cm pieces
4. Place the roots in a kitchen blender with tap water and macerate the roots three times for 10 sec with a 5-sec interval.
5. Pour the macerate suspension through a 40 μm sieve.
6. Rinse the residue in the sieve with tap water.
7. Collect the mixture of blended roots and nematodes from the sieve with distilled water in a beaker.
8. Pour the mixture of blended roots and nematodes on the Baermann sieves (on the tissue paper) and place the Baermann sieves on the dishes/funnels.
9. Add distilled water to the dishes/funnels until it just covers the mixture of roots and nematodes. Do not add too much water: it is the intention that the water will evaporate so that the mixture becomes somewhat dry, which will make the nematodes migrate downwards towards the remaining water (below the tissue paper and sieve openings).
10. Leave the mixture on the sieves for 1 night.

Next morning:

11. Collect the nematodes from the bottom of the dishes or the base of the funnels in a beaker.
12. Pour the suspension with the nematodes through a 25-μm sieve.
13. Rinse the residue on the sieve with tap water (to eliminate bacteria, etc.).
14. Collect the nematodes from the sieve with distilled water in a beaker.

Selection and sterilization of the nematodes

Under the laminar flow and using the microscope:

1. Pour the nematodes into a counting dish.
2. Select female nematodes, especially gravid females that are actively moving. Touch the selected nematode with a needle so that it starts floating and move the nematode to the surface with the needle. At the surface, the nematode will curl around the needle so that you can pick it from the solution and transfer it to sterile water in a sterile watch glass.
3. Transfer the nematodes with a sterile pipet to a small sterile 5, 10 or 20 μm sieve.
4. Place the small sieve in a sterile Petri dish with 0.01% $HgCl_2$ for 2 minutes.
5. Rinse the nematodes in the small sieve two times with sterile water.
6. Place the sieve in a sterile Petri dish with sterile water and transfer the nematodes with a sterile pipet to sterile water in a sterile test tube.
7. Prepare streptomycin sulphate solution (6000 ppm or 6 mg streptomycin sulphate per ml sterile water) in a sterile test tube.
8. Add with a sterile pipet 1 ml 6000-ppm streptomycin sulphate solution per 2 ml nematode solution (thus obtaining a final concentration of 2000 ppm streptomycin sulphate).
9. Leave the solution in the sealed test tube for 1 night.

Next morning:

10. The nematodes will have settled on the bottom of the test tube, visible as a white pellet. With a sterile pipet, remove the streptomycin sulphate solution from above the nematodes.
11. Add fresh sterile water to the nematodes with a sterile pipet.
12. Wait until the nematodes have settled again to the bottom of the test tube (wait long enough), remove the water from above the nematodes and add again fresh sterile water with a sterile pipet. Repeat this two to three times.

Inoculation of the carrot discs

1. Prepare micropipets:
   Press the tips of two sterile Pasteur pipets against each other in a flame. When the glass tips melt, pull the pipets quickly away from each other, stretching the tips. Cut the ends of the pipets with a sterile scalpel to obtain a microopening.
2. Transfer with a sterile pipet a drop of the sterilized nematodes from the test tube to a sterile Petri dish and add sterile water.
3. Suck nematodes from the water with a sterile micropipet and inoculate them on the margin of a carrot disc: for culture maintenance, use low inoculum levels (= 25 nematodes)
   for mass multiplication (e.g. for use in experiments), use high inoculum levels (> 25 nematodes).
4. Seal the Petri dishes well with Parafilm.
5. Label the Petri dishes (date, nematode species, origin of population, number of females).
6. Place the Petri dishes back in the plastic box and place the box in the incubator at 28 °C.

Note: Use discs of different carrots for inoculation with a given nematode population, to spread the risk of contamination due to bacteria in the carrots

SUBCULTURE

Depending on the initial inoculum and on the nematode species, the nutrients will become depleted after some weeks and the nematodes will leave the carrot discs. Use these discs on which you can see many nematodes on the Petri dish around the carrot disc for subculturing.

Preparation of the carrot discs

As described for first culture.

Extraction of the nematodes from the old carrot discs

1. Cut tissue paper to fit a Baermann sieve and place it on the sieve.
2. Rinse the Petri dishes with distilled water and pour the water through a 25 μm sieve.
3. Collect the nematodes which are retained on the sieve with distilled water in a beaker (fraction 1).
4. Put the carrots in the kitchen blender with distilled water and macerate the carrots three times for 10 sec at 5 sec intervals.
5. Pour the macerate suspension through a 100-μm sieve placed in a bowl, to separate the nematodes that will pass through the sieve from the carrot tissue.
6. Rinse the residue in the sieve with tap water and collect the water with the nematodes in the bowl.
7. Pour the content of the bowl through a 25-μm sieve
8. Collect the nematodes which are retained on the sieve with distilled water in a beaker (fraction 2).
9. Pour fractions 1 and 2 on the Baermann sieve (on the tissue paper) and place the Baermann sieve on the dish/funnel.
10. Add distilled water to the dish/funnel until it just covers the tissue paper. Do not add too much water: it is the intention that the water will evaporate so that the tissue paper becomes somewhat dry, which will make the nematodes migrate downwards towards the remaining water (below the tissue paper and sieve openings).
11. Leave the nematodes on the sieve for 1 night.

Next morning:

12. Collect the nematodes from the bottom of the dish or the base of the funnel in a beaker with distilled water.
13. Pour the suspension with the nematodes through a 25-μm sieve.
14. Rinse the residue on the sieve with distilled water (to eliminate bacteria, etc.).
15. Collect the nematodes from the sieve with distilled water in a beaker.

Selection and sterilization of the nematodes

The picking of the nematodes is not necessary anymore since you have already a pure monoculture. The sterilization in $HgCl_2$ is also skipped to avoid a very high pressure on the nematodes.
Under the laminar flow and using the microscope:

1. Pour the nematodes into a counting dish.
2. Transfer the nematodes with a sterile pipet to sterile water in a sterile test tube.
3. Prepare streptomycin sulphate solution (6000 ppm or 6 mg streptomycin sulphate per ml sterile water) in a sterile test tube.
4. Add with a sterile pipet 1 ml 6000 ppm streptomycin sulphate solution per 2 ml nematode solution (thus obtaining a final concentration of 2000 ppm streptomycin sulphate)
5. Leave the solution in the sealed test tube for 1 night. The nematodes will settle on the bottom of the test tube (visible as a white pellet).

Next morning:

6. Remove the streptomycin sulphate solution from above the nematodes with a sterile pipet.
7. Add fresh sterile water to the nematodes with a sterile pipet.
8. Wait until the nematodes have settled again to the bottom of the test tube (wait long enough), remove the water from above the nematodes and add again fresh sterile water with a sterile pipet. Repeat this two to three times.

Inoculation of the carrot discs with the nematodes

As described for first culture.

Note: All the equipment should be cleaned after use, first with ethanol (to kill remaining nematodes) and afterwards with soap and water.

CULTURING OF *MELOIDOGYNE* SPP. ON TRANSFORMED TOMATO ROOTS UNDER *IN-VITRO* CONDITIONS AND MASS PROPAGATION ON TOMATO PLANTS IN THE SOIL

EQUIPMENT AND MATERIALS

Equipment:
- sink with running water
- autoclave
- laminar-flow cabinet
- incubator (28 °C)
- stereo microscope
- kitchen blender
- scale (accuracy 0.1 mg)
- pH meter

Autoclaved materials:
- sterile scalpel
- sterile Petri dishes, suitable for *in-vitro* tissue culturing
- three extra sterile Petri dishes
- sterile watch glass
- two sterile sealed calibrated test tubes
- ±10 sterile Pasteur pipets, with rubber aspirators
- a small sterile 5, 10 or 20 μm sieve
- sterile water (2 bottles)

Other materials:
- pair of scissors
- watch with seconds hand
- 100 μm, 40 μm and 25 μm sieve
- bowl on which the sieves can be placed
- Baermann sieves + dishes/funnels
- strong tissue paper (preferably kitchen paper)
- three beakers
- counting dish
- ethanol lamp
- Gamborg B5 powder
- sucrose
- agar
- 0.12% NaOCl
- 0.01% $HgCl_2$ (do not inhale: $HgCl_2$ is toxic)
- streptomycin sulphate
- distilled water
- Parafilm (cut in small strips)
- plastic box to store Petri dishes
- soil suitable for growing tomato plants
- 1L plastic pots with dishes
- stock cultures of transformed tomato roots
- sample(s) of infected roots
- tomato seeds

FIRST CULTURE

Preparation of the transformed tomato roots

1. Fill a beaker with distilled water.
2. Add Gamborg B5 powder including vitamins 3.292 g/L and 20 g/L sucrose.
3. Add distilled water to a volume of 1L.
4. Adjust the pH to 6.2.
5. Add 15 g/L agar and stir very well.
6. Autoclave the medium.

Under the laminar flow:

7. Fill the Petri dishes with 20 ml medium each.
8. Select some growing root tips from stock cultures and transfer them to the new medium. Place them in the middle of the dish.
9. Seal the Petri dishes well with Parafilm.
10. Place the Petri dishes in a plastic box in an incubator at 28 °C.
11. Let the roots grow for 2 weeks before inoculation with nematodes.

Extraction of the nematodes from infected roots

1. Cut tissue paper to fit Baermann sieves and place it on the sieves.
2. Wash the roots with tap water. It is important to wash off all the soil, to make the next steps (picking of the nematodes) easier.
3. Cut the roots in 1cm pieces.
4. Put the roots in a kitchen blender with 0.12% NaOCl and macerate them for 10 sec.
5. Pour the macerate suspension through a 40 μm sieve.
6. Rinse the residue in the sieve with tap water.
7. Collect the mixture of blended roots and nematodes from the sieve with distilled water in a beaker.
8. Pour the mixture of blended roots and nematodes on the Baermann sieves (on the tissue paper) and place the Baermann sieves on the dishes/funnels.
9. Add distilled water to the dishes/funnels until it just covers the mixture of roots and nematodes. Do not add too much water: it is the intention that the water will evaporate so that the mixture becomes somewhat dry, which will make the nematodes migrate downwards towards the remaining water (below the tissue paper and sieve openings).
10. Leave the mixture on the sieves for a few days (wait until the juveniles hatch from the eggs).

After a few days:

11. Collect the juveniles from the bottom of the dishes or the base of the funnels in a beaker.
12. Pour the suspension with the juveniles through a 25 μm sieve.
13. Rinse the residue on the sieve with tap water (to eliminate bacteria, etc.).
14. Collect the juveniles from the sieve with distilled water in a beaker.

Selection and sterilization of the juveniles

Under the laminar flow and using the microscope:

1. Prepare micropipets:
   Press the tips of two sterile Pasteur pipets against each other in a flame. When the glass tips melt, pull the pipets quickly away from each other, stretching the tips. Cut the ends of the pipets with the sterile scalpel to obtain a micro-opening.
2. Pour the nematodes into a counting dish.
3. Heat a sterile micropipet (with a rubber aspirator) in a flame, so that it can suck juveniles.
4. Suck the selected juveniles with the micropipet and transfer them to a small sterile 5, 10 or 20 μm sieve.
5. Place the small sieve in a sterile Petri dish with 0.01 % $HgCl_2$ for 2 minutes.
6. Rinse the juveniles in the small sieve 2 times with sterile water.
7. Place the sieve in a sterile Petri dish with sterile water and transfer the juveniles with a sterile pipet to sterile water in a sterile test tube.
8. Prepare streptomycin sulphate solution (6000 ppm or 6 mg streptomycin sulphate per ml sterile water) in a sterile test tube.

9. Add with a sterile pipet 1 ml 6000-ppm streptomycin sulphate solution per 2 ml nematode solution (thus obtaining a final concentration of 2000 ppm streptomycin sulphate).
10. Leave the solution in the sealed test tube for 1 night. The nematodes will settle on the bottom (visible as a white pellet)

Next morning:

11. Remove the streptomycin sulphate solution from above the juveniles with a sterile pipet.
12. Add fresh sterile water to the juveniles with a sterile pipet.
13. Wait until the juveniles have settled again to the bottom of the test tube (wait long enough), remove the water from above the juveniles and add again fresh sterile water with a sterile pipet. Repeat this two to three times.

Inoculation of the transformed tomato roots with the juveniles

1. Pour the sterilized juveniles into a sterile Petri dish.
2. Suck nematodes from the water with a sterile micropipet and inoculate them on the growing tips of the transformed tomato roots.
3. Seal the Petri dishes well with parafilm.
4. Label the Petri dishes (date, nematode species, origin of population).
5. Place the Petri dishes back in the plastic box and place the box in the incubator at 28 °C.

SUBCULTURE

Depending on the initial inoculum, the nutrients will become depleted after some weeks.

Preparation of the transformed tomato roots
As described for first culture.

Inoculation of the transformed tomato roots with egg masses
With a scalpel, remove an egg mass containing eggs and/or juveniles from an old root and transfer the egg mass to a new growing root tip on a new Petri dish.

MASS PROPAGATION ON TOMATO PLANTS

For mass propagation of *Meloidogyne* spp. for use in experiments, the nematodes must be multiplied on tomato plants in the soil.

Preparation of the tomato plants

1. Sieve the soil through a 2 mm sieve and sterilize it.
2. Fill pots with the sterilized soil and place them on dishes.
3. Water the soil up to field capacity.
4. Make a hole in the middle of each pot.
5. Place one seed in each hole and fill the hole with soil.
6. Grow the plants for 2-3 weeks before inoculation.

Extraction of nematodes from transformed tomato roots

1. Transfer galls and egg masses to a kitchen blender with 0.12% NaOCl and macerate them for 10 sec.
2. Pour the macerate suspension through a 100 μm sieve placed in a bowl, to separate the eggs and juveniles that will pass through the sieve from the root tissue.
3. Rinse the residue in the sieve with tap water and collect the water with the eggs and juveniles in the bowl.
4. Pour the content of the bowl through a 25 μm sieve.
5. Collect the eggs and juveniles which are retained on the sieve with distilled water in a beaker.

Inoculation of the nematodes on the tomato plants

1. Bring the suspension with the nematodes to a known volume with distilled water.
2. Homogenize the nematode suspension by blowing air through it with a pipet and take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide).
3. Count the eggs and juveniles in the counting dish under the stereo microscope (or in the counting slide under the light microscope).
4. Calculate the nematode population in the total suspension.
5. Dilute the suspension to about 5000 eggs and juveniles in a few ml of distilled water.
6. Make three to four holes, about 3 cm deep, into the soil adjacent to the base of each plant.
7. Homogenize the nematode suspension by blowing air through it with a pipet and inoculate about 5000 eggs and juveniles in a few ml of water into the holes
8. Fill the holes with soil.
9. Water the soil lightly.

Note: All the equipment should be cleaned after use, first with ethanol (to kill remaining nematodes) and afterwards with soap and water

TISSUE CULTURE OF BANANA

COLLECTION OF SUCKERS

Materials

- net bags
- marker pen
- record book
- plastic labels, string twine, pencil
- knife
- desuckering bar

Collection

1. Check for appropriate source mats: check if there are symptoms of virus infections and check which sucker to collect (peepers, sword suckers, maiden suckers).
2. Dig around the suckers to expose the point of connection to the main stem or the other suckers.
3. Use the desuckering bar to separate the sucker from the main stem. Make a clean excision. Avoid cracking the corm of the sucker during collection. For small suckers, particularly peepers, use a machete or appropriate knife. Make sure the cutting edge is sharp and again, avoid making cracks during harvesting.
4. Collect at least two suckers from one plant source for both laboratory and nursery for future sucker needs.

ESTABLISHMENT OF INITIAL SHOOT CULTURE

Preparation of shoot proliferation medium
Prepare the shoot proliferation medium presented in Table 1.

Stock solution/component	Concentration of stock solution	Volume (weight) for 1L culture medium
MS macronutrients (0.5x)	25x	20 ml
MS micronutrients (0.5x)	100x	5 ml
MS vitamins (1x)	100x	10 ml
Fe-EDTA (1x)	200x	5 ml
Sugar		30 g
BAP	100 mg/l	50 ml (5 mg)
Coconut water (optional)		100 ml
Agar		6 g
pH 5.7		

Disinfection

1. Wash the suckers in running water to remove any adhering soil and dirt.
2. Trim leaf bracts and corm tissues. The size is dependent on the type (buttons, peeper, sword) collected for introduction to culture, and should be sufficient to protect the growing point from bleaching.
3. Transfer the shoot into a container or plastic which is large enough to accommodate both shoot and disinfectant. When using a big container, place a wad of cotton to ensure complete wetting of the whole shoot.
4. Immerse the shoot in pure commercial bleach (5.25% sodium hypochlorite) for 30-45 minutes, depending on the size of plant material.
5. Decant the sterilant from the container taking care not to expose the shoots.
6. Bring the shoots (in the container) to the inoculation chamber.

Preparation of the sterile working area

1. Wipe the chamber (sterile air bench) thoroughly with 95% ethyl alcohol.
2. Arrange materials in the inoculation chamber. Ensure that the inoculation chamber is not crowded with too many cultures and glass wares to facilitate movement during excision work.
3. Place instruments, including scalpels and forceps in a coupling jar or wide-mouth bottle with 95% ethyl alcohol. During the inoculation activity, always flame instruments before use.

Inoculation

1. Wash your hands with soap and water and wipe your arms and hands with 70% ethyl alcohol.
2. Take out one shoot at a time from the container and transfer it to an aseptic working surface (Petridish, steel dish or sterile paper).
3. While holding the shoot with the forceps, carefully cut off the outer leaf sheath that comes in contact with the disinfectant.
4. Transfer the shoot to a fresh working surface and continue cutting until the shoot measures 1 cm x 1 cm with corm tissue as thin as possible.
5. Transfer the shoot to a fresh working surface and cut it into quarters longitudinally, through the center.
6. Inoculate each quarter onto a solid culture medium which allows proliferation.

Maintenance of shoot cultures

1. Keep the shoot cultures in an air-conditioned room under a 16-hour photoperiod 40 µE x m^2s^{-1} (provided by two 40 watt fluorescent tubes).
2. Observe the cultures for contamination: discard contaminated cultures as soon as contamination is noted.
3. Observe the cultures for browning and bulging of corm tissue, greening of leaf tissues and growth of new shoots during the 1st month of culture.
4. Note when shoots that come out from the apex of the leaf axis are almost 2 cm in height; your cultures are then ready for their first subculture.

PROPAGATION BY SHOOT PROLIFERATION

1. Cut the growing shoot from the initial explant before it extends beyond 2 cm in length. Overgrown shoots give less proliferation. When shoots have grown beyond 2 cm, make a longitudinal incision through the apex of the growing shoot.
2. Subculture onto fresh half-strength Murashige and Skoog's medium, supplemented with 5 mg/l BAP and 100 ml/L coconut water for multiplication, or any recommended culture medium for shoot proliferation. Preferably use medium without auxins because nubbins form earlier and at higher frequencies when the proliferating medium contains both auxin and cytokinin.
3. After 1 month, divide the shoot cluster and transfer it to fresh culture medium.
4. Subculture every 3-4 weeks until the desired number of shoots have been obtained.
5. Make an inventory of the number of proliferated shoots. When sufficient shoots have proliferated as nuclear stock, proceed with rooting.

ROOTING OF *IN-VITRO* DERIVED SHOOTS

Preparation of culture medium for rooting

Prepare the rooting medium presented in Table 2. Use within a week of preparation for best results.

Table 2. Components of banana rooting medium.

Stock solution/component	Concentration of stock solution	Volume (weight) for 1 L culture medium
MS macronutrients (0.5x)	25x	20 ml
MS micronutrients (0.5x)	100x	5 ml
MS vitamins (1x)	100x	10 ml
Fe-EDTA (1x)	200x	5 ml
Sugar		30 g
Coconut water (optional)		100 ml
Agar		6 g

Rooting of shoots

1. Store shoots longer than the 3-4 week proliferation cycle until small plantlets are formed.
2. Separate individual shoots from a cluster of shoots and inoculate them onto rooting medium.
3. Expect shoots to form roots in 3-4 weeks.
4. Plant out bananas in soil when they have 3-4 expanded leaves and are well rooted.

NURSERY ESTABLISHMENT

Acclimatization

1. Select rooted plantlets which are about 4-7 cm high and have 3-4 expanded leaves among shoots cultured on rooting medium for 4-6 weeks. Banana plantlets at this stage of growth and development have the highest chance of survival; overgrown plants and unrooted shoots fare poorly.
2. Transfer selected cultures to the greenhouse or nursery 3 days before the scheduled potting out; locate cultures in an area where there is no direct sunlight at midday, to precondition the cultures away from the laboratory conditions which are often characterized by even and cool temperatures.
3. Remove cotton plugs or vessel closure 1 day before potting out, to reduce the relative humidity within the culture vessel and acclimatize plantlets to lower humidity.

Preparation of soil mixes

1. Prepare one of the following potting mixes:
   coir dust:garden soil (1:1, v:v)
   decomposed saw dust:garden soil (1:1)
   decomposed animal manure:garden soil (1:1)
   sand:garden soil (1:1).
2. Heat-sterilize the potting mix and cool the mixture before use. Heat-sterilization has been disregarded by some nurseries by avoiding the use of contaminated soil or by drenching the soil with fungicide.
3. Moisten the potting mix.
4. Put sufficient quantity of potting mix into a suitable container with sufficient drainage holes.

Taking plants out from culture vessels

1. Add tap water to the culture, sufficient to cover the roots.
2. Shake the vessel to loosen the agar medium.
3. Gently pull out the plants from the culture medium with a pair of forceps or with fingers.
4. Wash roots thoroughly with plain water to remove adhering culture medium.
5. Dip the roots in a fungicide solution before potting.

Potting to soil mix

1. Water the potting mix thoroughly.
2. Allow water to drain, leaving the mixture moist but not sopping wet.
3. Make a hole, large enough to accommodate the plantlet, in the potting mix with a clean stick or pointed tool.
4. Position the plantlet into the hole and press the soil around the base of the plant.

Nursery establishment

1. Protect the plants from excessive light during the first 2 weeks. Increase the light intensity when the plants have recovered from potting stresses.
2. To water, mist the plantlets during the 1st week and apply overhead watering daily or as needed thereafter, until the 4th week of nursery cultivation. Adjust the watering scheme to ensure that the soil is not too dry or wet.
3. Fertilize the plants every 2 weeks using a foliar complete fertilizer at the recommended rate for seedlings.
4. Plants are ready for field planting about 2 to 3 months from potting.

ASSESSMENT OF THE HOST-PLANT REACTION OF *MUSA* TO MIGRATORY ENDOPARASITIC NEMATODES UNDER GREENHOUSE CONDITIONS

EQUIPMENT AND MATERIALS

Equipment:

- autoclave or other means to sterilize soil
- sink with running water
- stereo microscope (and/or light microscope)
- kitchen blender
- scale (accuracy 0.1 g)

Materials:

- soil, suitable for growing bananas
- 1L plastic pots, with drainage holes in the bottom (larger pots are needed when suckers are used instead of *in-vitro* plantlets)
- plastic dishes
- plastic or styrofoam cover
- pot labels
- measuring tape
- knife
- tissue paper
- pair of scissors
- watch with seconds hand
- 2-mm, 250 μm, 100 μm, 40 μm, 25 μm sieve
- bowl on which the sieves can be placed
- beaker
- distilled water
- glass bottles with lid
- 5 ml or 10 ml pipet
- counting dish (or counting slide)
- 20-25 *in-vitro* plantlets (or suckers) of each of the genotypes to be tested, as well as 20-25 *in-vitro* plantlets (or suckers) of each of the reference genotypes
- *in-vitro* carrot disc cultures, containing large numbers of nematodes
- data collection forms + pencil

SET-UP OF THE EXPERIMENT

Preparation of plant material

1. Start with rooted *in-vitro* plantlets, ready for transfer to *in-vivo* conditions. For suckers, use suckers of uniform size, which are peeled immediately after detaching from the motherplant and are immersed in water at 55 °C for 15 minutes.
2. Sieve the soil through a 2 mm sieve and sterilize it.
3. Fill pots with the sterilized soil and place them on dishes.
4. Water the soil up to field capacity.
5. Make a hole in the middle of each pot.
6. Place an *in-vitro* plantlet (or sucker) in each hole and fill the hole with soil.
7. Place the pots under a plastic or styrofoam cover for adaptation to the new environment (not necessary for suckers). Gradually open the cover after 2 weeks.
8. Four weeks after planting, inoculate 8-10 plants of each genotype with nematodes (see next step), while keeping another 8-10 plants nematode-free as control plants.
9. Label all the pots (genotype, inoculated or control) and arrange them in a randomized complete block design.

Extraction and inoculation with nematodes

1. Use carrot discs on which you can see many nematodes on the Petri dish around the carrot disc.
2. Rinse the Petri dishes with distilled water and pour the water through a 25 μm sieve.
3. Collect the nematodes which are retained on the sieve with distilled water in a beaker (fraction 1).
4. Put the carrots in the kitchen blender with distilled water and macerate the carrots three times for 10 sec at 5 sec intervals.
5. Pour the macerate suspension through a 100 μm sieve placed in a bowl, to separate the nematodes that will pass through the sieve from the carrot tissue.
6. Rinse the residue in the sieve with tap water and collect the water with the nematodes in the bowl.
7. Pour the content of the bowl through a 25 μm sieve.

8. Collect the nematodes which are retained on the sieve with distilled water in the beaker, together with fraction 1.
9. Bring the suspension with the nematodes to a known volume with distilled water.
10. Homogenize the nematode suspension by blowing air through it with a pipet and take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide).
11. Count the eggs and vermiform nematodes in the counting dish under a stereo microscope (or in the counting slide under a light microscope)
12. Calculate the nematode population in the total suspension.
13. Dilute the suspension to about 1000 vermiform nematodes in a few ml of distilled water.
14. Make 3-4 holes, about 3 cm deep, into the soil adjacent to the base of each plant.
15. Homogenize the nematode suspension by blowing air through it with a pipet and inoculate about 1,000 vermiform nematodes in a few ml of water into the holes
16. Fill the holes with soil.
17. Water the soil lightly.
18. During the whole experiment, water the plants as needed and fertilize them with a nutrient solution every 2-4 weeks.

DATA COLLECTION

For experiments with *R. similis*, collect data at 8 weeks after inoculation; for experiments with *P. coffeae*, collect data at 10 weeks after inoculation.

General information

Use Form 1 to write down any general information about the experiment.

Plant parameters

1. Write down any visible disorders.
2. Gently remove the plant from the pot and shake off the soil from the roots.
3. Wash the roots with tap water and pat them dry with tissue paper.
4. Cut off the roots from the corm.
5. Measure the plant height, the girth of the pseudostem at the base, the shoot weight, the weight of the

Root-damage assessment

1. Divide the roots into dead roots and functional roots and count the number of roots in each category. Dead roots are shriveled and no healthy root tissue is left.
2. Score the general health condition of the secondary and tertiary roots.
3. Select at random five functional primary roots, at least 10 cm long, cut them to 10 cm pieces and slice the roots lengthwise. Score one half of each of the five roots for the percentage of the root cortex showing necrosis. The maximum root necrosis per root half can be 20%, giving a maximum root necrosis of 100% for the five root halves together (Figure 1).

Use Form 3 to write down the root-damage data.

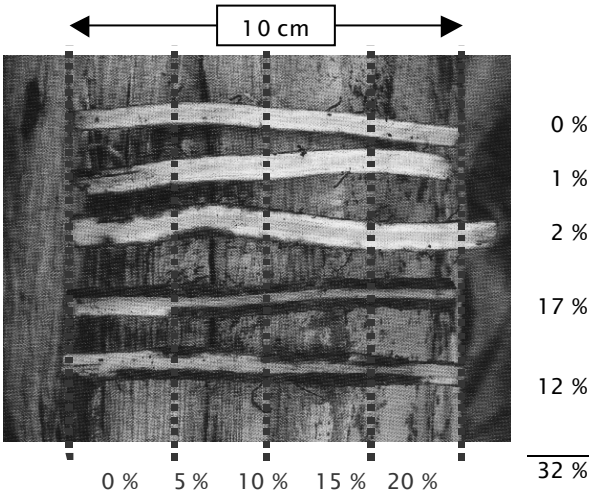


Figure 1: Root necrosis (%)

Extraction and counting of the nematodes from the roots of the inoculated plants

1. Cut the roots in 1 cm pieces and take a subsample of 10 g.
2. Put the roots in the kitchen blender with tap water and macerate them three times for 10 sec at 5 sec interval.
3. Pour the macerate suspension through a 250 μm, 100 μm and 40 μm sieve.

4. Rinse the residue in the 250 μm and 100 μm sieves with tap water.
5. Collect the nematodes from the 40 μm sieve with distilled water in a beaker.
6. Dilute the suspension with distilled water to 200 ml and pour it in a labeled glass bottle.
7. Homogenize the nematode suspension by blowing air through it with a pipet.
8. Take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide).
9. Count the nematodes (males, females, juveniles) and eggs in the counting dish under the stereo microscope (or in the counting slide under the light microscope).
10. Calculate the nematode population per 10 g of fresh roots.

Use Form 4 to write down the nematode count data.

FORM 1. General information

Date: _____
Place: _____
Soil type: _____
Date of transfer to greenhouse: _____
Date of inoculation: _____
Initial inoculum: _____
Irrigation: _____
Organic fertilizer: _____
Inorganic fertilizer: _____
Pesticides: _____
Climatic data: _____
_____
_____
Other data: _____
_____
_____

FORM 2. Plant parameters

Cultivar												
	Inoculated plants						Control plants					
	PH	PG	SW	RW	HL		PH	PG	SW	RW	HL	
1						1						
2						2						
3						3						
4						4						
5						5						
6						6						
7						7						
8						8						
9						9						
10						10						

PH: Plant height (cm)
PG: Pseudostem girth at the base (cm)
SW: Shoot weight (kg)
RW: Root weight (kg)
HL: Number of healthy leaves

FORM 3. Root-damage assessment

Cultivar											
	Inoculated plants						Control plants				
	DR	FR	PDR	RH	RN		DR	FR	PDR	RH	RN
1						1					
2						2					
3						3					
4						4					
5						5					
6						6					
7						7					
8						8					
9						9					
10						10					

DR: Number of dead roots
FR: Number of functional roots
PDR: Percentage of dead roots (%)
RH: Root health assessment of secondary and tertiary roots: 1 – all healthy
2 – most healthy
3 – most dead
4 – all dead
RN: Root necrosis (%) = percentage of necrosis observed on the section of five 10-cm pieces of roots

FORM 4. Nematode counts

Cultivar				
	F	M	J	E
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

F: Number of females per 10 g of fresh roots
M: Number of males per 10 g of fresh roots
J: Number of juveniles per 10 g of fresh roots
E: Number of eggs per 10 g of fresh roots

ASSESSMENT OF THE HOST-PLANT REACTION OF *MUSA* TO ROOT-KNOT NEMATODES UNDER GREENHOUSE CONDITIONS

EQUIPMENT AND MATERIALS

Equipment:
- autoclave or other means to sterilize soil
- sink with running water
- stereo microscope (and/or light microscope)
- kitchen blender
- scale (accuracy 0.1 g)

Materials:
- soil, suitable for growing bananas
- 1-l plastic pots, with drainage holes in the bottom (larger pots are needed when suckers are used instead of *in-vitro* plantlets)
- plastic dishes
- plastic or styrofoam cover
- pot labels
- measuring tape
- knife
- watch with seconds hand
- 2-mm, 250 μm, 100 μm, 40 μm and 25 μm sieve
- bowl on which the sieves can be placed
- beaker
- distilled water
- 0.12 % NaOCl
- phloxine B
- glass bottles with lid
- 5-ml or 10 ml pipet
- counting dish (or counting slide)
- 20-25 *in-vitro* plantlets (or suckers) of each of the genotypes to be tested, as well as 20-25 *in-vitro* plantlets (or suckers) of each of the reference genotypes
- infested tomato plants containing large numbers of galls on their roots
- data collection forms + pencil

SET-UP OF THE EXPERIMENT

Preparation of plant material

1. Start with rooted *in-vitro* plantlets, ready for transfer to *in-vivo* conditions. For suckers, use suckers of uniform size, which are peeled immediately after detaching from the motherplant and are immersed in water at 55 °C for 15 minutes.
2. Sieve the soil through a 2 mm sieve and sterilize it.
3. Fill pots with the sterilized soil and place them on dishes.
4. Water the soil up to field capacity.
5. Make a hole in the middle of each pot.
6. Place an *in-vitro* plantlet (or sucker) in each hole and fill the hole with soil.
7. Place the pots under a plastic or styrofoam cover for adaptation to the new environment (not necessary for suckers). Gradually open the cover after 2 weeks.
8. Four weeks after planting, inoculate 8-10 plants of each genotype with nematodes (see next step), while keeping another 8-10 plants nematode-free as control plants.
9. Label all the pots (genotype, inoculated or control) and arrange them in a randomized complete block design.

Extraction and inoculation with nematodes

1. Start with tomato plants on the roots of which you can see many galls.
2. Gently remove the plants from the pots and shake off the soil from the roots.
3. Wash the roots with tap water and pat them dry with tissue paper.
4. Transfer root pieces with many galls and eggs to the kitchen blender, add 0.12% NaOCl and macerate the roots for 10 sec.
5. Pour the macerate suspension through a 100 μm sieve placed in a bowl, to separate eggs and juveniles that will pass through the sieve from the root tissue.
6. Rinse the residue in the sieve with tap water and collect the water with the eggs and juveniles in the bowl.
7. Pour the content of the bowl through a 25 μm sieve.

8. Collect the nematodes which are retained on the sieve with distilled water in a beaker.
9. Bring the suspension with the nematodes to a known volume with distilled water.
10. Homogenize the nematode suspension by blowing air through it with a pipet, take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide).
11. Count the eggs and juveniles in the counting dish under the stereo microscope (or in the counting slide under the light microscope).
12. Calculate the nematode population in the total suspension.
13. Dilute the suspension to about 5000 eggs and juveniles in a few ml of distilled water.
14. Make 3-4 holes, about 3 cm deep, into the soil adjacent to the base of each plant.
15. Homogenise the nematode suspension by blowing air through it with a pipet and inoculate about 5,000 eggs and juveniles in a few ml of water into the holes.
16. Fill the holes with soil.
17. Water the soil lightly.

During the whole experiment, water the plants as needed and fertilize them with a nutrient solution every 2-4 weeks.

DATA COLLECTION

Collect data at 8 weeks after inoculation.

General information

Use Form 1 to write down any general information about the experiment.

Plant parameters

1. Write down any visible disorders.
2. Gently remove the plant from the pot and shake off the soil from the roots.
3. Wash the roots with tap water and pat them dry with tissue paper.
4. Cut off the roots from the corm.
5. Measure the plant height, the girth of the pseudostem at the base, the shoot weight, the weight of the root system and the number of healthy leaves (> 70% of the leaf surface healthy).

Use Form 2 to write down the plant parameters.

Root-damage assessment

1. Divide the roots into dead roots and functional roots and count the number of roots in each category. Dead roots are shriveled and no healthy root tissue is left.
2. Score the general health condition of the secondary and tertiary roots.
3. Estimate the root galling.

Use Form 3 to write down the root-damage data.

Staining and counting of the number of egg-laying females from the roots of the inoculated plants

1. Cut the roots in 1 cm pieces and take a subsample of 5 g.
2. Stain the egg masses by immersing the roots in 0.15 g/L phloxine B for 15 minutes.
3. Count the number of egg-laying females under the stereo microscope.

Note: The counting of the egg-laying females can also be done after the next step (extraction of nematodes).

The stained roots can be stored in distilled water in the refrigerator at 4 °C.

Use form 4 to write down the number of egg-laying females.

Extraction and counting of the number of juveniles from the roots of the inoculated plants

1. Cut the roots in 1 cm pieces and take a subsample of 10 g.
2. Put the roots in the kitchen blender with tap water and macerate them three times for 10 sec with a 5-sec interval.
3. Pour the macerate suspension through a 250 μm, 100 μm and 40 μm sieve.
4. Rinse the residue in the 250 μm and 100 μm sieves with tap water.
5. Collect the nematodes from the 40 μm sieve with distilled water in a beaker.
6. Dilute the suspension with distilled water to 200 ml and pour it in a labeled glass bottle.

7. Homogenize the nematode suspension by blowing air through it with a pipet and take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide),
8. Count the juveniles and eggs in the counting dish under the stereo microscope (or in the counting slide under the light microscope).
9. Calculate the nematode population per 10 g of fresh roots.

Use Form 4 to write down the nematode count data

FORM 1. General information

Date: _____
Place: _____
Soil type: _____
Date of transfer to greenhouse: _____
Date of inoculation: _____
Initial inoculum: _____
Irrigation: _____
Organic fertilizer: _____
Inorganic fertilizer: _____
Pesticides: _____
Climatic data: _____
_____
_____
Other data: _____
_____
_____

FORM 2. Plant parameters

Cultivar											
Inoculated plants						Control plants					
	PH	PG	SW	RW	HL		PH	PG	SW	RW	HL
1						1					
2						2					
3						3					
4						4					
5						5					
6						6					
7						7					
8						8					
9						9					
10						10					

PH: Plant height (cm)
PG: Pseudostem girth at the base (cm)
SW: Shoot weight (kg)
RW: Root weight (kg)
HL: Number of healthy leaves

FORM 3. Root-damage assessment

Cultivar											
	Inoculated plants						Control plants				
	DR	FR	PDR	RH	RG		DR	FR	PDR	RH	RG
1						1					
2						2					
3						3					
4						4					
5						5					
6						6					
7						7					
8						8					
9						9					
10						10					

DR: Number of dead roots
FR: Number of functional roots
PDR: Percentage of dead roots (%)
RH: Root health assessment of secondary and tertiary roots: 1 – all healthy
2 – most healthy
3 – most dead
4 – all dead

RG: Root galling: 0 – no galling
1 – trace infections with a few small galls
2 – less than 25% of the roots galled
3 – 25-50% of the roots galled

FORM 4. Nematode counts

Cultivar			
	ELF	J	E
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

ELF: Number of egg-laying females per 5 g of fresh roots
J: Number of juveniles per 10 g of fresh roots
E: Number of eggs per 10 g of fresh roots

ASSESSMENT OF THE HOST-PLANT REACTION OF *MUSA* TO NEMATODES UNDER FIELD CONDITIONS

EQUIPMENT AND MATERIALS

Equipment
- autoclave or other means to sterilize soil
- sink with running water
- stereo microscope (and/or light microscope)
- kitchen blender
- scale (accuracy 0.1 g)
- scale (accuracy 100 g)

Materials
- soil, suitable for growing bananas
- 1L plastic bags, with drainage holes (larger bags are needed when suckers are used instead of *in-vitro* plantlets)
- plastic or styrofoam cover
- bag labels
- nematicides
- measuring tape
- plastic bags for sampling
- spade and shovel
- knife
- tissue paper
- pair of scissors
- watch with seconds hand
- 2 mm, 250 μm, 100 μm, 40 μm, 25 μm sieve
- bowl on which the sieves can be placed
- beaker
- distilled water
- phloxine B
- glass bottles with lid
- 5 ml or 10 ml pipet
- counting dish (or counting slide)
- 20-25 *in-vitro* plantlets (or suckers) of each of the genotypes to be tested, as well as 20-25 *in-vitro* plantlets (or suckers) of each of the reference genotypes
- data collection forms + pencil

SET-UP OF THE EXPERIMENT

Preparation of plant material

1. Start with rooted *in-vitro* plantlets, ready for transfer to *in-vivo* conditions. For suckers, use suckers of uniform size, which are peeled immediately after detaching from the mother plant and are immersed in water at 55 °C for 15 minutes.
2. Sieve the soil through a 2 mm sieve and sterilize it.
3. Fill bags with the sterilized soil.
4. Water the soil up to field capacity.
5. Make a hole in the middle of each bag.
6. Place an *in-vitro* plantlet (or sucker) in each hole and fill the hole with soil.
7. Place the bags under a plastic or styrofoam cover for adaptation to the new environment (not necessary for suckers). Gradually open the cover after 2 weeks.
8. Label all the bags (genotype).

Site selection and preparation

1. Assess the infestation level of the field by taking root samples of the crop grown on the field. The site should preferably be infested with a single nematode species, though this will seldom be the case. The population should be at least 100 nematodes per gram of fresh roots.
2. Clear the land of any previously grown crops and prepare it for the new planting.
3. Dig the planting holes, large enough for the *in-vitro* plant (or sucker) + ball of earth, following a split-plot design, with main-plot factor = infection (treated or not with nematicides) and sub-plot factor = genotype.
4. Apply nematicides to the planting holes of control plants to eradicate the nematodes present in the soil.

Transfer to the field

1. Transfer the plants in the early morning or late afternoon and water the plants just before planting.
2. Strip off the bottom part of the plastic bag, place the plant in the planting hole, partly cover the plant with soil, remove the plastic bag by pulling it over the plant and add more soil until the plant is stable.
3. Water the plants right after planting.
4. During the whole experiment, water and fertilize the plants as needed. Treat the control plants with nematicides every few months to eradicate the present nematodes.

DATA COLLECTION

Collect data at a specific time after planting (e.g. at 6 months after planting), at shooting (emergence of flower) and at harvest. You can also collect data from the ratoon crops.

General information

Use Form 1 to write down any general information about the experiment.

Plant parameters

1. Write down any visible disorders.
2. Measure the plant height, the girth of the pseudostem at 50 cm above ground level and the number of healthy leaves (> 70 % of the leaf surface healthy).
3. Write down the date of shooting and the date of harvest.
4. At harvest, measure the bunch weight, the number of hands and the number of fingers.

Use Form 2 to write down the plant parameters.

Sample collection

1. Dig an excavation of 20 x 20 x 20 cm^3 at the base of the mother plant.
2. Collect all banana roots in this soil volume. Take only the roots from the selected plant; do not include roots from adjacent plants.
3. Place the roots with a handful of soil in a plastic bag.
4. Label the plastic bag.

Root-damage assessment

1. Shake off the soil from the roots.
2. Wash the roots with tap water and pat them dry with tissue paper.
3. Divide the roots into dead roots and functional roots and count the number of roots in each category. Dead roots are shriveled and no healthy root tissue is left.
4. Score the general health condition of the secondary and tertiary roots.
5. Estimate the root galling.
6. Select at random five functional primary roots, at least 10 cm long, cut the five selected roots to 10 cm pieces and slice the roots lengthwise. Score one half of each of the five roots for the percentage of the root cortex showing necrosis. The maximum root necrosis per root half can be 20%, giving a maximum root necrosis of 100% for the five root halves together (Figure 1).

Use Form 3 to write down the root-damage data.

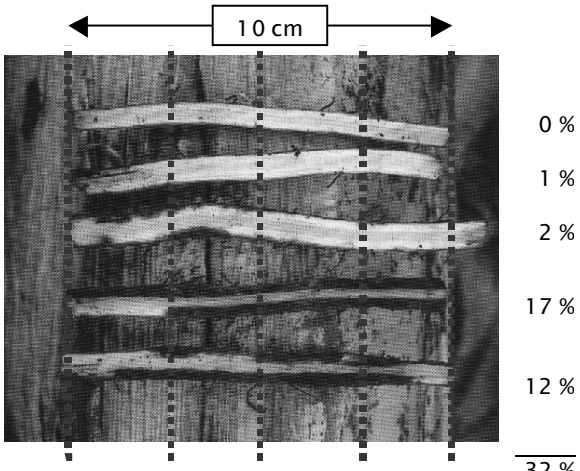


Figure 1: Root necrosis (%)

Staining and counting of the number of egg-laying females

1. Cut the roots in 1 cm pieces and take a subsample of 5 g.
2. Stain the egg masses by immersing the roots in 0.15 g/l phloxine B for 15 minutes.
3. Count the number of egg-laying females under the stereo microscope.

Note: The counting of the egg-laying females can also be done after the next step (extraction of nematodes). The stained roots can be stored in distilled water in the refrigerator at 4 °C.

Use Form 4 to write down the number of egg-laying females.

Extraction and counting of the nematodes

1. Cut the roots in 1-cm pieces and take a subsample of 10 g.
2. Put the roots in the kitchen blender with tap water and macerate them three times for 10 sec with a 5-sec interval.

3. Pour the macerate suspension through a 250 μm, 100 μm and 40 μm sieve.
4. Rinse the residue in the 250 μm and 100 μm sieves with tap water.
5. Collect the nematodes from the 40 μm sieve with distilled water in a beaker.
6. Dilute the suspension with distilled water to 200 ml and pour it in a labeled glass bottle.
7. Homogenize the nematode suspension by blowing air through it with a pipet and take a subsample of 5 ml and put it in a counting dish (or 1-2 ml in a counting slide).
8. Count the nematodes of each species in the counting dish under the stereo microscope (or in the counting slide under the light microscope).
9. Calculate the nematode population per 10 g of fresh roots.

Use Form 4 to write down the nematode count data.

FORM 1. General information

Date: _____
Place: _____
Soil type: _____
Date of transfer to field: _____
Initial infestation: _____
Irrigation: _____
Organic fertilizer: _____
Inorganic fertilizer: _____
Pesticides: _____
Mulching: _____
Weeding: _____
Desuckering: _____
Climatic data: _____
_____
Other data: _____
_____

FORM 2. Plant parameters

Cultivar											
Inoculated plants						Control plants					
	PH	PG	SW	RW	HL		PH	PG	SW	RW	HL
1						1					
2						2					
3						3					
4						4					
5						5					
6						6					
7						7					
8						8					
9						9					
10						10					

PS: Plant stage:
S – small sucker less than 1.5 m high
PF – pre-flowering plant at least 1.5 m high
RF – recently flowered plant (emerging flowers or less than 14 days)
FL – flowered plant at any stage between RF and CH
CH – plant close to harvest

PH: Plant height (cm)
PG: Pseudostem girth at 50 cm above the ground (cm)
HL: Number of healthy leaves
BW: Bunch weight (kg)
NH: Number of hands
NF: Number of fingers

FORM 3. Root-damage assessment

Cultivar													
Inoculated plants							Control plants						
	DR	FR	PDR	RH	RG	RN		DR	FR	PDR	RH	RG	RN
1							1						
2							2						
3							3						
4							4						
5							5						
6							6						
7							7						
8							8						
9							9						
10							10						

DR: Number of dead roots
FR: Number of functional roots
PDR: Percentage of dead roots (%)
RH: Root health assessment of secondary and tertiary roots: 1 – all healthy; 2 – most healthy; 3 – most dead; 4 – all dead

RG: Root galling: 0 – no galling; 1 – trace infections with a few small galls; 2 – less than 25% of the roots galled; 3 – 25-50% of the roots galled; 4 – 50-75% of the roots galled; 5 – more than 75% of the roots galled

RN: Root necrosis (%) = percentage of necrosis observed on the section of five 10-cm pieces of roots

FORM 4. Nematode counts

Cultivar				
	ELF	Rs	Pc	M
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

ELF: Number of egg-laying females of *Meloidogyne* spp. per 5 g of fresh roots
Rs: Number of *Radopholus similis* per 10 g of fresh roots
Pc: Number of *Pratylenchus coffeae* per 10 g of fresh roots
M: Number of juveniles of *Meloidogyne* spp. per 10 g of fresh roots

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(Note: For the ease of the use of the factsheets, no citations were made in the text. Below is the list of key references used by the authors in the compilation of the factsheets.)

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