Inibap Technical Guidelines 1

Screening of *Musa* Germplasm for resistance and tolerance to nematodes

Paul R. Speijer and Dirk De Waele

International Plant Genetic Resources Institute
IPGRI
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- to promote and strengthen regional efforts to address region-specific problems and to assist national programmes within the regions to contribute towards, and benefit from, the global research effort;
- to strengthen the ability of NARS to conduct research on bananas and plantains;
- to coordinate, facilitate and support the production, collection and exchange of information and documentation related to banana and plantain.

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1. Introduction

In most regions of the world, nematodes are recognized as important pests of bananas[1]. Crop losses caused by nematodes to bananas are very high, with average annual yield losses estimated at about 20 % worldwide (Sasser and Freckman 1987). Because banana nematodes attack the root and/or corm tissues, plant growth and yield are affected by the reduction in the mechanical (anchorage) and physiological (uptake and transportation of water and nutrients) function of the root system.

Where nematode attack cannot be prevented by using clean planting material in nematode-free soil and growing the plants under strict quarantine conditions, nematode management in bananas is mainly based on crop rotation and chemical control (Gowen and Quénéhervé 1990). However, in those areas where bananas are grown continuously, crop rotation cannot be practised, while at the same time, the price of chemical nematicides is often prohibitive for small farmers. It is also important to note that most nematicides are extremely toxic for the environment. 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 objective of these technical guidelines is to stimulate interest in nematode resistance and tolerance screening in bananas, and to provide a tried and tested methodology for carrying out such screening. Because research programmes often have limited access to trained nematologists, the technical guidelines are written for nematologists with little or no experience in the area of plant resistance and tolerance screening and for agricultural scientists with limited experience in nematology. Although, researchers may have to choose or modify the methods somewhat, according to their local conditions, it is also hoped that these technical guidelines will promote a level of standardization for future efforts in screening bananas for nematode resistance and tolerance.

[1] bananas = bananas and plantains throughout the text.

2. Bananas, banana nematodes and damage caused by banana nematodes

2.1. BANANAS

The banana plant is a herbaceous perennial. It is herbaceous because it has no woody parts: after ripening of the fruits, the aerial parts die down to the ground; it is perennial because new plants grow up from the base of the mature plant to replace the aerial parts which will die.

A mature banana plant or mother plant consists of:

- a corm with roots and suckers,
- a pseudostem with leaves,
- a bunch with fruits.

The corm is the underground part of the banana plant. It is in fact the true stem of the plant because at its tip it bears a growing point or apical meristem from which leaves develop. The corm consists of a central cylinder surrounded by a cortex (Figure 1).

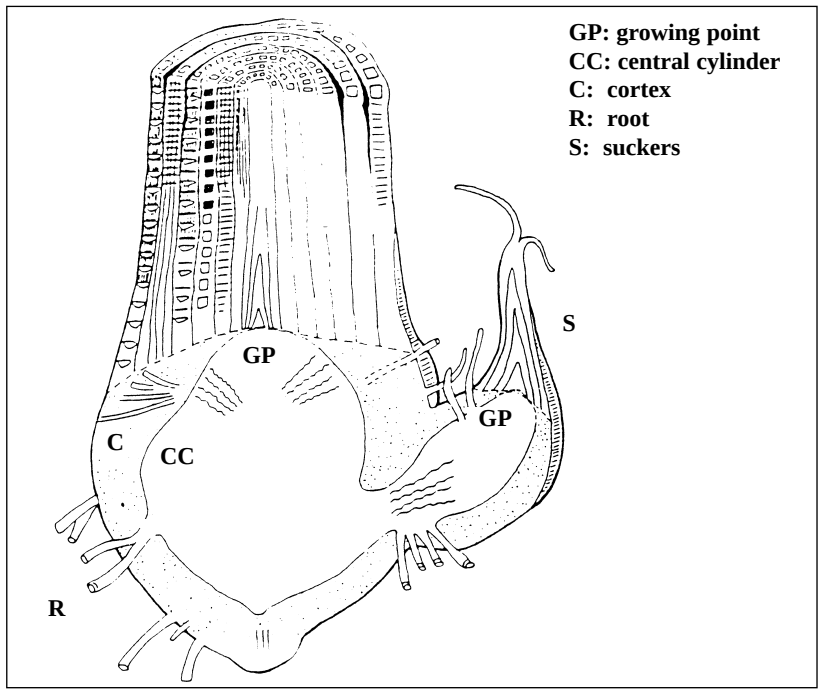


Figure 1. Section through the corm of an East African Highland banana (modified after Stover & Simmonds, 1987).

Primary roots develop, usually in groups of three to four, from the layer which separates the central cylinder and the cortex. Most are formed within 4 months after planting, in 3 to 4 waves. They grow horizontally through the cortex and remain in the top 50 cm of soil. They grow to a maximum thickness of 1 cm and may become 3 to 4 m long. From the primary roots, a system of secondary and tertiary roots and root hairs develop (Figure 2).

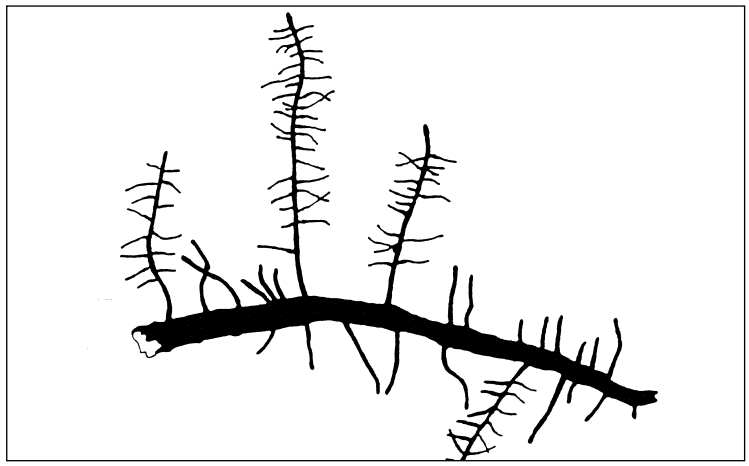


Figure 2. A primary banana root with secondary and tertiary roots.

Suckers develop, initially as swollen buds, from lateral meristems at the leaf bases on the corm. A sucker has different developmental stages:

- ❒ "peeper" : a large green bud that has just emerged above the ground,
- ❒ "sword sucker" : a sucker which is 50 to 150 cm high and has lanceolate leaves,
- ❒ "maiden sucker" : a sucker which is taller than 100 cm and has large leaves,
- ❒ "water sucker" : a small sucker with large leaves and a small corm due to poor underground connections with the mother plant.

Among the followers or daughter plants, a sucker or ratoon is selected to succeed the mother plant. A mother plant surrounded by a cluster of suckers and small plants is referred to as a mat or stool. A mat can consist of plants with bunches close to maturity (ready for harvest), recently flowered plants and pre-flowering plants (Figure 3).

The pseudostem or false stem is the cylindrical structure which rises from the soil and carries the leaves. It is not a true stem because it consists merely of the tightly compacted elongated leaf stalks of the leaves which develop, in almost concentric circles, from the apical meristem of the corm. New leaves are usually produced every 7 to 10 days. Most banana plants produce between 35 to 40 leaves.

The bunch develops from an inflorescence that also originates from the apical meristem of the corm. Flower initiation cannot be observed from the outside because it takes place inside the pseudostem. The developing flower stalk forces the inflorescence upwards until it emerges through the top of the pseudostem. This plant developmental stage, whereby the inflorescence is first visible from the outside, is called recently flowered. Subsequent growth of the flower stalk pushes the inflorescence clear of the pseudostem. The developing bunch bends under its own weight. The ovaries of the female flowers each develop into a fruit or finger. The

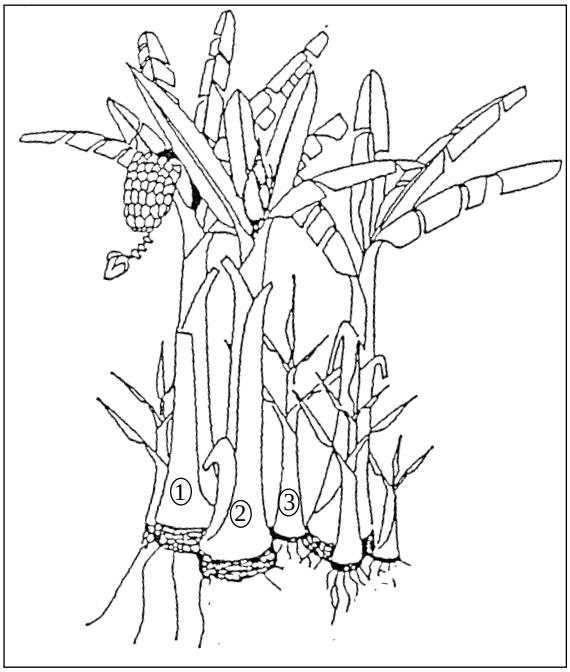


Figure 3.
A mat of an East African Highland banana consisting of a mother plant close to maturity (1), a recently flowered plant (2) and a pre-flowering plant (3).

fruits which occur together on a protuberance make up a hand. Fruit length and weight vary from 10 to 30 cm and 100 to 500 g, respectively. The number of hands per bunch varies from 1 to 15. A bunch can weigh from 5 to 70 kg. A crop cycle is the time between harvest of the fruits on the same mat. The second harvest from the mat is called the first ratoon crop.

2.2. BANANA NEMATODES

In general, based on their biology, three types of plant-parasitic root nematodes can be distinguished:

- ectoparasitic nematodes: remain outside the plant; pierce the outermost plant cell layers with a stylet in order to feed,
- migratory endoparasitic nematodes: invade the plant tissues; remain migratory (mobile); feed on numerous normal cells inside the plant; eggs are laid individually either inside or outside the plant,
- sedentary endoparasitic nematodes: invade the plant tissues; adult females become sedentary (immobile); feed on a few special cells inside the plant; eggs are laid together (for instance in a single egg sac) outside the plant.

The most damaging and widespread nematodes attacking bananas are migratory endoparasites:

- the burrowing nematode *Radopholus similis*,
- the root-lesion nematodes *Pratylenchus coffeae* and *Pratylenchus goodeyi*,
- the spiral nematode *Helicotylenchus multicinctus*.

Some sedentary endoparasitic root-knot (*Meloidogyne* spp.) nematodes are also often present in banana roots but their status as pathogens is unclear. The most frequently found species are:

- *Meloidogyne incognita*,

- *Meloidogyne javanica*,
- *Meloidogyne arenaria*,
- *Meloidogyne hapla*.

In addition to these nematode species, more than 150 other nematode species have occasionally been found associated with banana roots, and some of these may cause damage to banana roots.

Differential descriptions of banana nematodes

The migratory endoparasitic nematode species can be identified on the basis of a combination of morphological characters. The most important distinguishing characteristics are described in Table 1 (see also photographs p.10 and 11).

Protocol: Preparation of nematodes for identification using light microscope

It is essential that the nematode populations used in the screening are identified accurately at the species level.

This protocol describes a routine method by which whole nematodes are prepared for light microscope observation. Good results may be obtained when the nematodes are killed quickly and fixed in one process with hot formaldehyde (after Seinhorst 1966), transferred to glycerol by the ethanol-glycerol method (after Seinhorst 1959) and mounted on glass slides with the wax-ring method (after de Maeseneer and d'Herde 1963). These glass slides can be stored permanently and the preserved nematodes can be used as reference specimens.

This routine method is not suitable for the preparation of female root-knot (Meloidogyne spp.) nematodes. A method for the preparation of the perineal patterns of female root-knot nematodes for light microscope observation can be found in Hartman and Sasser (1985).

1. Killing and fixing the nematodes

- concentrate the nematodes as much as possible in a very small drop of water in a glass cavity vessel (for instance a glass staining block of 4 x 4 x 1,5 cm),
- boil the same volume of 8 % formaldehyde,
- add the hot 8 % formaldehyde as fast as possible to the drop of water containing the nematodes (resulting formaldehyde concentration: 4%).

2. Transfer of the nematodes from formaldehyde to ethanol

- prepare solution I (4 % formaldehyde + 1 drop glycerol/100 ml),
- fill a glass staining block with solution I,
- transfer the nematodes with a needle from the 4 % formaldehyde to solution I in the glass staining block,
- fill a closed glass vessel (for instance a desiccator) to approximately 1 cm depth with 95 % ethanol,
- place the glass staining block on a support in the desiccator so that it stands above the layer of ethanol,
- close the desiccator tightly,
- place the desiccator for 1 night in an incubator at 35 °C.

3. Transfer of the nematodes from ethanol to glycerol

- ❒ take the glass staining block out of the desiccator (ethanol will have replaced the 4 % formaldehyde),
- ❒ cover the glass staining block partially with a cover glass,
- ❒ place the glass staining block in an incubator at 35 °C,
- ❒ check after 15-20 minutes if the ethanol has evaporated. When it has, add a few drops of solution II (95 % ethanol + 2 drops glycerol/100 ml),
- ❒ repeat this process several times until the ethanol has evaporated,
- ❒ add a few drops of glycerol (just enough to immerse the nematodes).

4. Preparation of glass slides

- ❒ heat the 1.5 cm diameter tip of a copper tube in a flame,
- ❒ dip the hot tip in paraffin wax,
- ❒ when the paraffin wax has melted, press the tip on a glass slide making a thin wax ring which will soon solidify,
- ❒ put a small drop of glycerol in the middle of the wax ring,
- ❒ transfer the nematodes with a needle and place them in the centre of the glycerol drop (10 nematodes/glycerol drop),
- ❒ cover with a cover glass,
- ❒ place the glass slide on a hot plate for a few seconds (the wax ring will melt allowing the cover glass to settle down thus confining the glycerol to the centre of the ring),
- ❒ place the glass slide on a cool surface (the wax ring will soon solidify),
- ❒ seal the cover glass (for instance with nail varnish).

Sexual dimorphism in form of the anterior region is only present in *Radopholus similis*: in females the head region is low, hemispherical, continuous or slightly offset with strong cephalic sclerotization and stylet; in males the head region is high, often knob-like, more offset with weak cephalic sclerotization and degenerated stylet.

Helicotylenchus multicinctus can be further recognised as the bodies of both females and males have distinct annules and when killed and fixed they arcuate to a C-shape.

Full descriptions of *Radopholus similis* can be found in Orton Williams and Siddiqi (1973), of *Pratylenchus coffeae* in Siddiqi (1972), of *Pratylenchus goodeyi* in Machon and Hunt (1985) and of *Helicotylenchus multicinctus* in Siddiqi (1973).

Females of *Meloidogyne* spp. are sedentary (spherical body with a slender neck) while the males and juveniles are vermiform. Males are rare; the head region is high, cone shaped, not offset, clearly annulated; cephalic sclerotization and stylet strong; tail short, hemispherical, bursa absent. Shape of head of juveniles similar to males; cephalic sclerotization weak; tail tapering.

Because of the extensive morphological variation among and within *Meloidogyne* spp. and the existence of host plant races, accurate identification of these nematodes is difficult. Guidelines for the preparation of the perineal patterns of females for light microscope observation and for conducting the differential plant host test can be found in Hartman and Sasser (1985). A pictorial key and complete characterization of *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria* and *Meloidogyne hapla* are presented by Eisenback *et al*. (1981). Finally, electrophoresis

Characteristics	*R. similis*	*P. coffeae*	*P. goodeyi*	*H. multicinctus*
Occurrence of males	rather rare	common	common	common
Position of vulva	median, at 50 to 60 % of body length	well posterior, at 70 to 80% of body length	well posterior, at 70 to 80% of body length	posterior, at 60 to 70% of body length
Number of genital branches in females	2 equally developed	only the anterior branch is developed	only the anterior branch is developed	2 equally developed short, somewhat cylindrical,
Shape of the tail in females	somewhat elongate-conoid with a rounded or indented terminus	conoid, ventrally concave, terminus broadly rounded, truncate or irregularly crenate	conoid, ventrally concave, dorsal contour sinuate just prior to tail tip	hemispherical annulated terminus usually with greater curvature dorsally than ventrally
Shape of the tail in males	elongate, conoid, ventrally arcuate with bursa extending over 2/3 of tail length	convex, conoid with bursa extending until tail tip	convex, conoid with bursa extending until tail tip	short, with ventral vinger-like projection, bursa extending until tail tip

Table 1. Morphological characters of banana migratory endoparasitic nematodes

based on the thin-slab technique for polyacrylamide gels and isozyme (esterase and malate dehydrogenase) staining can be used to identify the most common *Meloidogyne* spp. (Esbenshade and Triantaphyllou 1985).

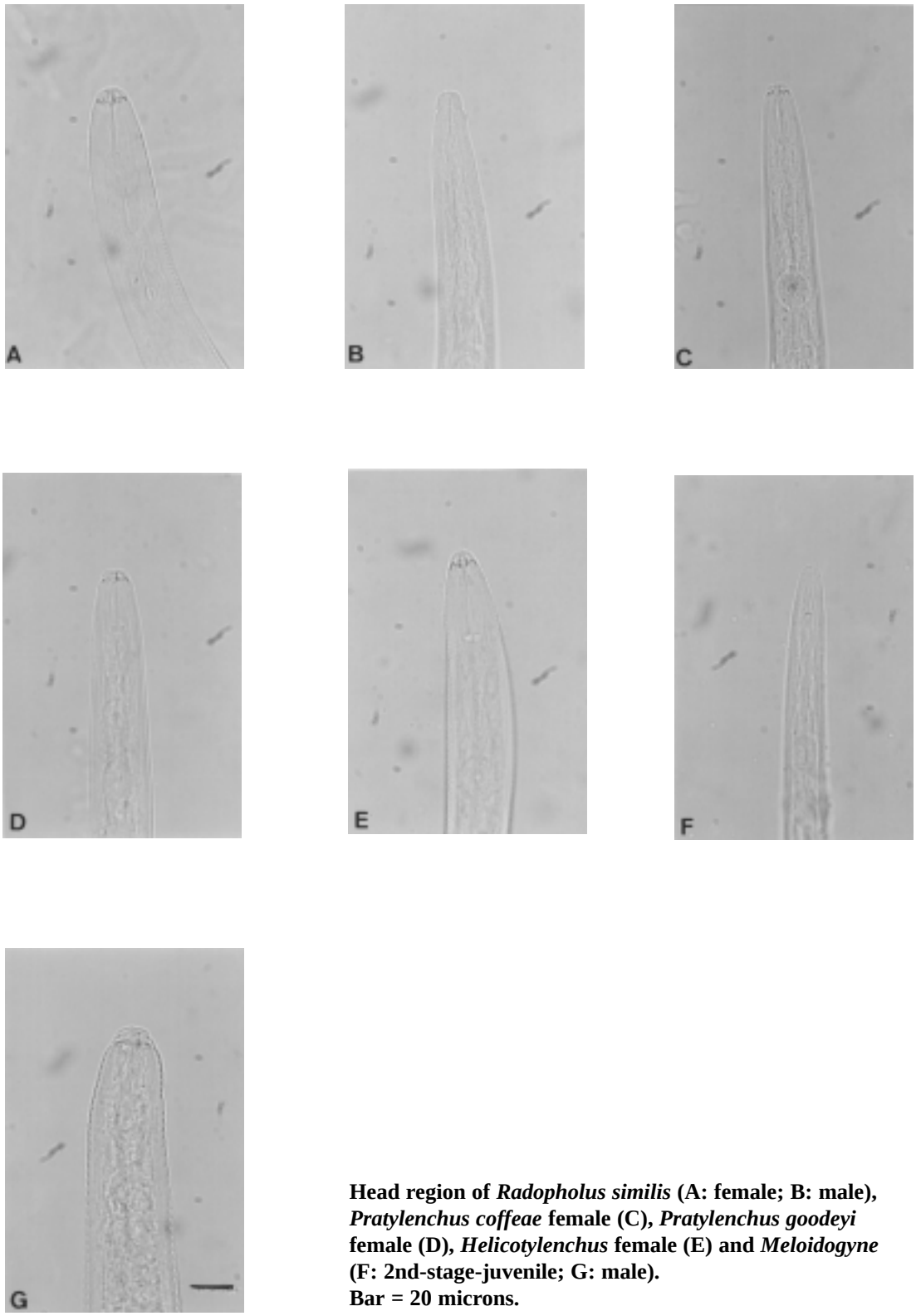
2.3. DAMAGE CAUSED BY BANANA NEMATODES

2.3.1. Root and corm damage

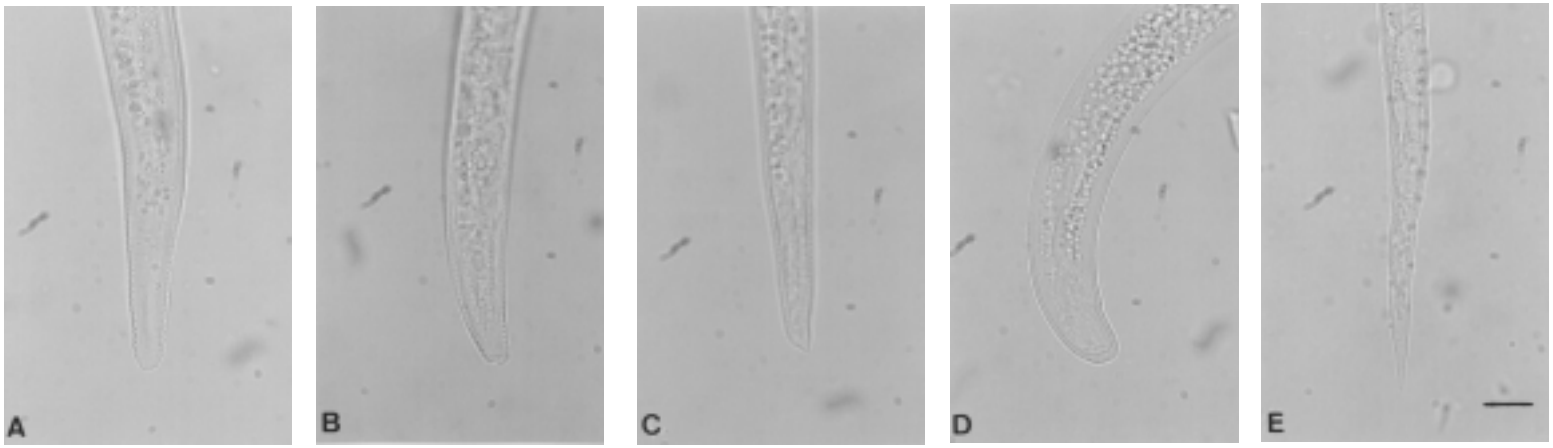
The migratory endoparasites (*Radopholus similis*, *Pratylenchus coffeae*, *Pratylenchus goodeyi* and *Helicotylenchus multicinctus*) cause similar root and corm damage. The migratory feeding behaviour of these nematodes in the root and corm tissues causes the formation of lesions, which may enlarge and coalesce, resulting in large necrotic areas.

On roots, initial damage can be seen as small dark purplish-red lesions on the outer part of the roots (Figure 4, see p. 24). These lesions may enlarge into purplish-black necrotic areas which usually extend throughout the cortex but not into the stele (Figure 5, see p. 24). Primary, secondary and tertiary roots can be affected. In addition to the damage directly caused by nematodes, nematode-induced root lesions and necrosis create a food base for weak, unspecialized fungal parasites of banana (*Fusarium* spp., *Cylindrocladium* spp., *Rhizoctonia* spp.), which enables these fungi to invade the stele and to extend the necrosis to this part of the root. As a result, the affected section of the root dies.

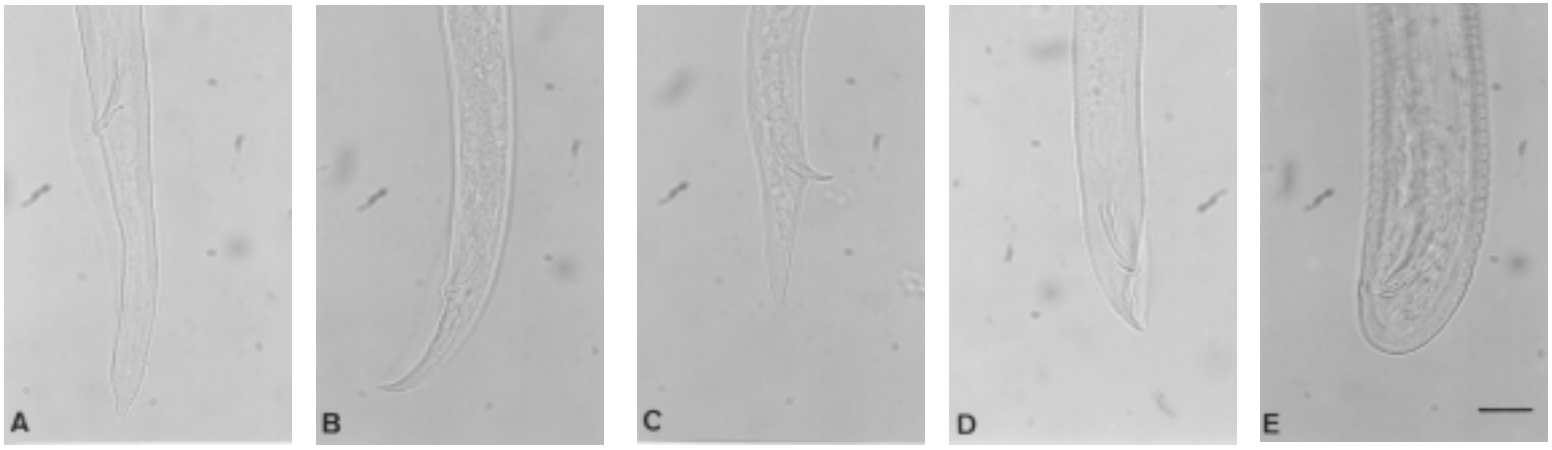
Because *Helicotylenchus multicinctus* often feeds on the cortical cells close to the epidermis, root lesions caused by this nematode are often shallow and superficial, reddish brown to black in colour.



Head region of *Radopholus similis* (A: female; B: male), *Pratylenchus coffeae* female (C), *Pratylenchus goodeyi* female (D), *Helicotylenchus* female (E) and *Meloidogyne* (F: 2nd-stage-juvenile; G: male).
Bar = 20 microns.



Female tail region of *Radopholus similis* (A), *Pratylenchus coffeae* female (B), *Pratylenchus goodeyi* (C), *Helicotylenchus multicintus* (D) and 2nd-stage-juvenile tail region of *Meloidogyne incognita* (E). Bar = 20 microns.



Male tail region of *Radopholus similis* (A), *Pratylenchus coffeae* (B), *Pratylenchus goodeyi* (C), *Helicotylenchus multicintus* (D) *Meloidogyne incognita* (E). Bar = 20 microns.

On corms, blackish-purple lesions can be seen around the root bases and/or other parts of the corm (Figure 6, see p. 24). Lesions around the root bases may enlarge and destroy the root bases killing the entire root. Lesions on the corm can be several centimetres deep.

The sedentary endoparasites (*Meloidogyne* spp.) cause galling of the primary and secondary roots. These symptoms can be seen as irregular swellings, often of the root tips (Figure 7, see p. 24). In thick, fleshy primary roots, swellings may not be obvious, however, length-wise slicing of the roots may reveal white and dark brown pit-like structures inside the root cortex which contain the swollen females (Figure 8, see p. 24). Roots infected with *Meloidogyne* spp. often show stunted growth.

2.3.2. Above ground symptoms

Root damage by nematodes affects the function of the root system at two levels: its anchorage ability and its ability to take up and transport water and nutrients. The decrease in these abilities leads to:

- toppling over or uprooting of plants (Figure 9, see p. 24),
- stunting,
- chlorosis,
- lengthening of the crop cycle,
- reduction in size and numbers of leaves,
- reduction in bunch weight,
- reduction of the production life of the plantation.

Severe root damage caused by nematodes will result in toppling over or uprooting of plants. In comparison, damage caused by banana weevils is more likely to result in snapping; breakage or doubling of the pseudostem may also occur (Figures 10a-c).

2.4. Damage caused by other constraints

Several symptoms other than those directly related to nematode infection, can be observed on roots and corms.

Cracks on the surface of primary roots may be a response to nematode attack but may also be the result of abiotic stress such as temperature and moisture fluctuations. Primary roots can be very soft with a bluish-black appearance due to waterlogging of the soil, whereby roots die because of lack of oxygen. The stele of the primary root can show a blackish-red discoloration that may be caused by fungal growth in the stele as a result of a dead root tip, root rot or mechanical damage resulting, for instance from weeding.

On corms, rough handling during transport may cause black blotches. Large, pale white to yellow brown lesions can be caused by *Marasmius* rot, an opportunistic fungus associated with decaying wood, for instance cut down coffee trees. The walls of old weevil tunnels can decay resulting in blackish-purple discoloration of the corm.

3. SCREENING FOR NEMATODE RESISTANCE AND TOLERANCE

3.1. Definitions and concepts

For the description of the host-plant nematode relationships, the terminology of Bos and Parlevliet (1995) is used.

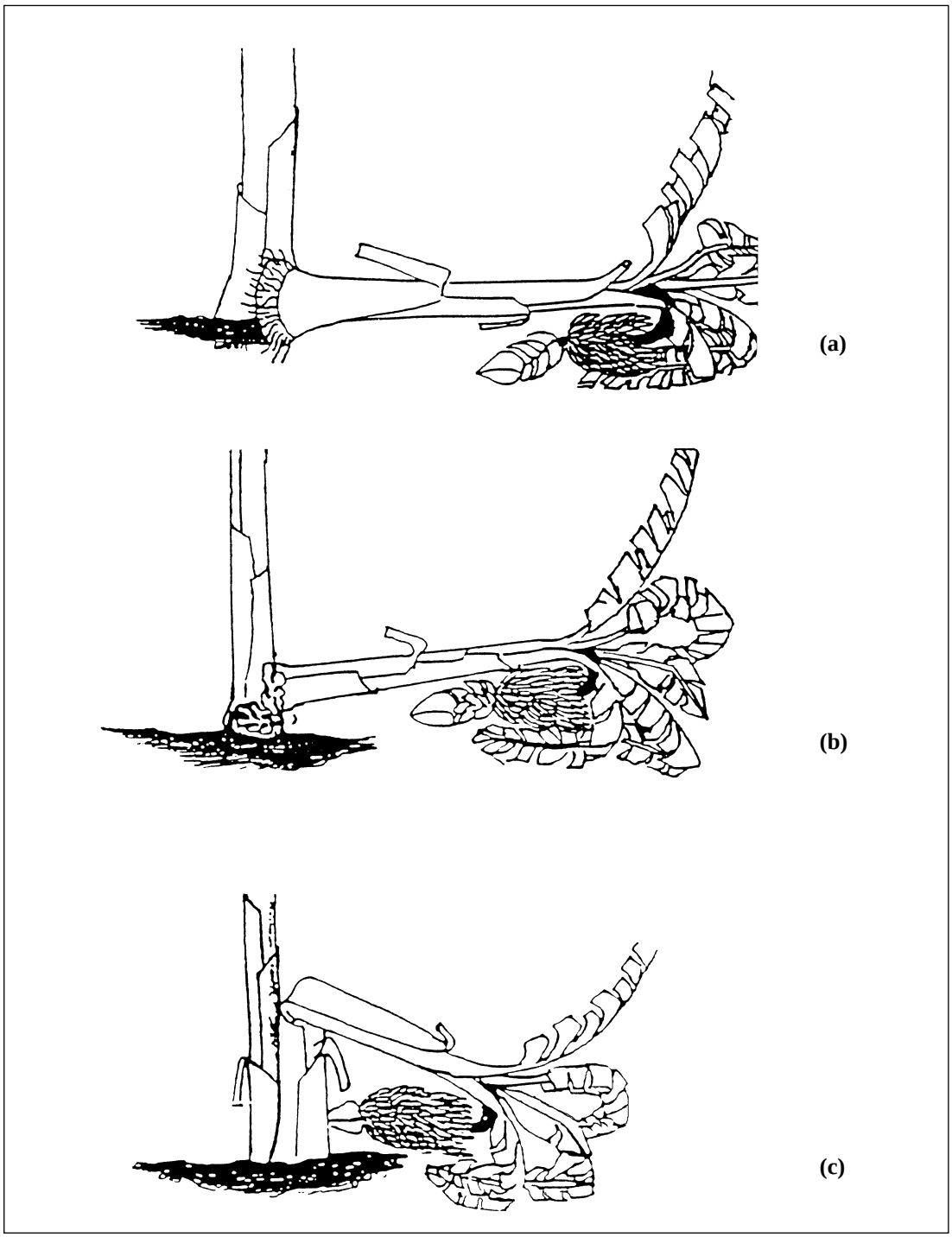


Figure 10. Toppling over or uprooting of bananas caused by root nematodes (a), snapping of bananas caused by banana weevils (b) and breakage or doubling of bananas (c).

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 (tolerance), even when quite heavily infected with nematodes, or much injury (sensitivity), even when relatively lightly infected with nematodes. The comparison between genotypes results in such indications as completely, highly, and moderately resistant genotypes describing, respectively, genotypes supporting no, little or an intermediate level of nematode reproduction. A non-resistant or susceptible genotype will allow nematodes to reproduce freely.

The evaluation/interpretation of the data obtained during the nematode resistance/tolerance screening should be based on a combination of nematode reproduction data and host plant response data including at least:

- ❒ number of nematodes in the roots
- ❒ percentage of dead roots and root necrosis index (eventually yield)

by preference taken at different (for instance three) moments during plant growth (for instance early plant growth, e.g. 2 months after planting, at flowering and at harvesting). The combination of the data will give a reliable indication that the genotype is resistant or susceptible, tolerant or sensitive.

3.2. Screening: plant material

Screening can be performed either with *in vitro* tissue cultured plants or suckers.

In vitro tissue cultured plants can be initiated from a sucker or obtained from an *in vitro* laboratory. Before they can be used for screening, the tissue culture plants must go through three *in vitro* stages: proliferation (= multiplication), regeneration (= shoot formation) and rooting (= root formation) (Vuylsteke and De Langhe 1985).

Suckers can be obtained from mother plants in the field. The suckers can be freed from nematode infection by peeling off the roots and the outer corm layers followed by immersion in a water tank at 53 to 55 °C for 20 minutes.

3.3. Screening: nematode inoculum

Plants can be infected with nematodes either by inoculation or by planting the plants in nematode-infested soil.

Protocol: *in vitro* tissue culturing of bananas

1. Initiation of an* in vitro *tissue culture from a sucker

Step A: Selection of the explants (buds or meristem)

- ❒ excise the adventitious buds (if present),
- ❒ reduce the size of the sucker to the meristem and some surrounding tissue by cutting (final size should be approximately 2 x 2 x 2 cm),
- ❒ place each bud or meristem in a container such as a 100 ml Erlenmeyer flask or small bottle.

Step B: Sterilization of the explants

Under laminar flow:

- close the container (a small sterile Petri dish can be used to close the Erlenmeyer),
- add 80 ml 95 % ethanol to the container, shake and pour the ethanol out,
- add either 60 ml 2 % NaOCl (containing some drops of Tween20) or 60 ml 0.2 % $HgCl_2$ to the container, ($HgCl_2$ is very toxic and should be used with care and only if the NaOCl proves not to be strong enough),
- during 20 or 30 minutes, respectively, shake a few times and then pour the NaOCl or $HgCl_2$ out ($HgCl_2$ should be disposed of with care in a separate container),
- rinse 3 times with sterile water for 10 minutes.

Step C: Transfer of the explants to proliferation medium in a culture vessel

Under laminar flow:

- flame the opening of the container,
- transfer to and blot the explant on sterile paper,
- remove the outer tissues of the explant (this will remove the remaining NaOCl or $HgCl_2$),
- cut the base of the leaves off and remove the leaves,
- flame the opening of the culture vessel (for instance a glass tube) which contains the proliferation medium (see next section),
- transfer the explant to the culture vessel,
- close the culture vessel with a cap and incubate at 28 °C with 24 hour light.

2. Proliferation of the explants (= multiplication)

Under laminar flow:

- after about 4 weeks, remove the cap and flame the opening of the culture vessel,
- transfer the explant to sterile paper (***use only one explant for the multiplication of a genotype***),
- excise 1 cm-pieces of each shoot tip and remove the leaves,
- excise also each bud or group of small buds,
- flame the opening of a new culture vessel which again contains proliferation medium,
- transfer one shoot tip, bud or group of buds to a new culture vessel,
- close the culture vessel with a cap and incubate at 28 °C with 24 hour light.

3. Regeneration of the explants (= shoot formation)

Under laminar flow:

- after about 4 weeks, remove the cap and flame the opening of the culture vessel,
- transfer the explant to sterile paper,
- excise 1cm-pieces of each shoot tip (without root primordia) and remove the leaves,
- excise also each bud or group of small buds,
- flame the opening of a new culture vessel which contains regeneration medium (see next section),
- transfer one shoot tip, bud or group of buds to a new culture vessel,
- close the culture vessel with a cap and incubate at 28 °C with 24 hour light.

4. Rooting of the explants (= root formation)

Under laminar flow:

- ❒ after about 4 weeks, remove the cap and flame the opening of the culture vessel,
- ❒ transfer the explant to sterile paper,
- ❒ excise 1 cm-pieces of shoot tip (leave at least one root primordium intact): remove the leaves,
- ❒ flame the opening of a new culture vessel which contains rooting medium (see next section),
- ❒ transfer one shoot tip to a new culture vessel,
- ❒ close the culture vessel with a cap and incubate at 28 °C with 24 hour light,
- ❒ transfer the explant after 8-10 weeks to soil in pots in the greenhouse using the protocol described in Section 3.4.1.

Protocol: Murashige and Skoog (MS) medium for *in vitro* tissue culturing of bananas

- ❒ fill a beaker with distilled water,
- ❒ start heating and stirring,
- ❒ add MS powder,
- ❒ for proliferation medium: use 4.594 g/l MS powder (including micro- and macro elements, and vitamins),
- ❒ for regeneration medium: use 4.594 g/l MS powder (including micro- and macro elements, and vitamins),
- ❒ for rooting medium: use 2.262 g/l modified MS powder (basal salt mixture, with FeNaEDTA at 50 % concentration) and add 1 ml/l MS vitamins,
  MS vitamins: glycine 0.20 g/100 ml
  thiamine hydrochloride 0.01 g/100 ml
  pyridoxine hydrochloride 0.05 g/100 ml
  nicotinic acid 0.05 g/100ml,
- ❒ add plant growth regulators:
  - for proliferation medium: 1 ml/l IAA 10^{-3}M + 1 ml/l BAP 10^{-2}M
  - for regeneration medium: 1 ml/l BAP 10^{-3}M + 1 ml/l IAA 10^{-3}M
  - for rooting medium: no plant growth regulators,
- ❒ add 1ml/l ascorbic acid (500 mg/50 ml),
- ❒ add distilled water to a volume of 1 l,
- ❒ add a mixture of 30 g/l sugar (or sucrose) and 2 g/l gelrite,
- ❒ adjust the pH to 6.12 at 60 °C (or to 5.8 at 60 °C following boiling),
- ❒ boil the medium,
- ❒ add 15-20 ml medium to each culture vessel,
- ❒ autoclave the culture vessels with the medium.

Inoculation is generally used in pot and plastic bag experiments. The following sources of nematodes can be used as nematode inoculum:

- nematode-infected roots,
- nematodes extracted either from roots collected from a field infested with nematodes or from nematode cultures.

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 (root-knot nematodes; Verdejo *et al*. 1988). Carrot discs allow the rearing of high numbers of *Radopholus 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 on carrot discs, as well as on transformed tomato roots, requires three steps: 1) extraction of the nematodes from infected roots, 2) sterilization of the nematodes, 3) transfer of the nematodes to the *in vitro* plant tissues.

Radopholus similis and *Pratylenchus* spp. can be extracted from the carrot discs for use either as inoculum or to start new *in vitro* cultures on carrot discs following sterilization.

Root-knot nematodes from transformed tomato roots can be used to start new *in vitro* cultures on transformed tomato roots or for the rearing of high numbers of nematodes on tomato plants in the soil.

Nematodes are generally between 0.5 and 1 cm length. The extraction and separation of nematodes from soil and root tissue relies on the use of different size of sieves (maceration-sieving) and/or on the mobility of the nematodes (maceration-Baermann funnel/dish). Oxygenation of the water in which the extracted nematodes are kept will keep the nematodes alive for a longer time and in better condition. Oxygenation can be obtained either by blowing air with a pipette/aquarium pup or by adding H_2O_2 (10 ml of 30 % per l water).

Protocol: Culturing of *Radopholus similis* and *Pratylenchus* spp. on carrot discs

1. Extraction of* Radopholus similis *and* Pratylenchus spp. *from infected roots

- wash the roots with tap water,
- cut the roots in 1 cm-pieces,
- put the root pieces in a kitchen blender with distilled water,
- macerate 3 times for 10 sec with a short rest period (duration of blending depends on the root type),
- pour the macerate suspension through a 40 µm sieve,
- rinse the residue in the sieve with tap water,
- collect the mixture of blended roots and nematodes which are retained in the sieve with distilled water in a beaker,

- ❒ place the mixture of macerated roots and nematodes in a 1 mm sieve covered with tissue paper; place the sieve in a funnel or in a dish with distilled water so that the distilled water just covers the nematode mixture (this is the so-called Baermann funnel/dish),
- ❒ wait 1 night (during this time the water will evaporate so that the mixture becomes somewhat dry; the nematodes will migrate downwards towards the remaining moisture, through the tissue paper and sieve openings, and as they are heavier than water, they will sink to the bottom of the funnel or dish),
- ❒ collect the nematodes from the base of the funnel or on the bottom of the dish in a beaker,
- ❒ pour the suspension with the nematodes through a 25 µm sieve,
- ❒ rinse the residue in the sieve with tap water (to eliminate bacteria etc.),
- ❒ collect the nematodes, which are retained in the sieve, with distilled water in a beaker.

clean the kitchen blender, sieves and Baermann funnel/dish first with ethanol (to kill remaining nematodes) and afterwards with soap and water

Under laminar flow:

- ❒ pour the nematodes into a counting dish,
- ❒ select the more movable nematodes.

a) for a "dirty" nematode solution:

- ❒ touch the selected nematode so that it starts floating (use a very thin needle),
- ❒ pick the nematode up with the needle, move it slowly to the water surface (focus the microscope continuously): on the water surface, the nematode will curl around the needle,
- ❒ transfer the nematode to sterile water in a sterile (Petri) dish.

b) for a "clean" nematode solution:

- ❒ heat a sterile micropipette* (with a rubber top) in a flame so that it can suck nematodes,
- ❒ suck the selected nematode(s) with the micropipette,
- ❒ transfer the nematode(s) to sterile water in a sterile (Petri) dish.

[*preparation of the micropipette, also under laminar flow:
press the tips of two sterile Pasteur pipettes against each other in a flame,
when the glass tips melt, pull the pipettes quickly away from each other,
cut the end of the pipettes with a sterile scalpel to obtain a tiny (micro) opening].

2. Sterilization of* Radopholus similis *and* Pratylenchus *spp.

It is necessary to sterilize the nematodes because they will be used as inoculum for the *in vitro* (sterile) carrot disc cultures. Although bacteria are present, even in the *in vitro* carrot disc cultures, sterilizing the nematodes, including during subculturing, aids in the management of bacterial infection.

Under laminar flow:

Step A: sterilization with $HgCl_2$

- ❒ start with a sterile (Petri) dish filled with nematodes in sterile water,

- ❒ transfer the nematodes with a sterile pipette to a small sterile 5, 10 or 20 µm sieve,
- ❒ put the sieve with the nematodes in 0.01 % $HgCl_2$ for 2 minutes,
- ❒ rinse the sieve with the nematodes 2 times with sterile water,
- ❒ place the sieve with nematodes in sterile water and transfer the nematodes with a sterile pipette to sterile water in a sterile test tube.

*Step B: sterilization with streptomycin sulphate**

- ❒ start with a sterile test tube filled with (already $HgCl_2$ sterilized) nematodes in sterile water (2 ml),
- ❒ add 1 ml 6000 ppm streptomycin sulphate with a sterile pipette (thus obtaining a final concentration of 2000 ppm streptomycin sulphate),
- ❒ wait 1 night,
- ❒ remove the streptomycin sulphate solution from above the nematodes (which remain in a white pellet at the bottom of the test tube) with a sterile pipette,
- ❒ add fresh sterile water,
- ❒ wait until the nematodes have settled to the bottom of the tube (***wait long enough***),
- ❒ repeat 2 to 3 times,
- ❒ use the nematodes for inoculation.

[*preparation of streptomycin sulphate 6000 ppm (= 6 mg/ml), also under laminar flow:
prepare a 6000 ppm solution with distilled water,
suck the streptomycin sulphate solution with a sterile syringe,
place a bacterial filter on the syringe,
add the streptomycin sulphate solution to the nematode suspension].
prepare new streptomycin sulphate each time

3. Culturing of Radopholus similis *and* Pratylenchus *spp. on carrot discs*

Step A: preparation of the carrot discs

use thick carrots (for instance carrot cultivar Nantes)
use fresh carrots with foliage
use Petri dishes suitable for* in vitro *tissue culturing - (the nematodes don't stick to the walls)

- ❒ remove the foliage,
- ❒ clean the carrots with paper,

Under laminar flow:

- ❒ spray ethanol on the carrot,
- ❒ keep the carrot in the flame until the ethanol is burned off (peel becomes black and dry),
- ❒ cut the tip of the carrot off,
- ❒ peel the carrot deeply (many layers) with a potato peeler,
  flame the peeler after each carrot layer is peeled off
- ❒ cut the carrot in discs and put 1 to 2 discs in each Petri dish,
  close the Petri dishes very well with parafilm
- ❒ place the Petri dishes in a plastic box (against mites) in the incubator at 28 °C.

Step B: inoculation of the carrot discs with nematodes

Under laminar flow:

use the carrot discs immediately after preparation (before bacteria and fungi can develop)

use discs of different carrots for inoculation with a given nematode population (this spreads the risk of contamination due to bacteria in the carrots)

use high inoculation levels (> 25 nematodes) to obtain many nematodes (for instance for experiments)

use low inoculation levels (by preference a series of 15-40 nematodes) to obtain nematodes for culture maintenance

- ❒ transfer with a sterile pipette 1 drop of the sterilized nematodes from the sterilized test tube to a sterile Petri dish and add distilled water,

  use Petri dishes in which the nematodes don't stick to the walls
- ❒ heat a sterile micropipette (with a rubber top) in a flame so that it can suck nematodes,
- ❒ suck females (especially thick, gravid females) with the micropipette from the drop on the sterile Petri dish,

  inoculate the nematodes on the margin of the carrot disc, not in the middle
- ❒ incubate at 28 oC in the dark.

4. Extraction of Radopholus similis *and* Pratylenchus *spp. from carrot discs (= maceration-sieving method)*

use only carrot disc cultures where you can see many nematodes on the Petri dish around the carrot disc

Fraction I: nematodes on the Petri dish

- ❒ rinse the Petri dish with distilled water and pour the water through a 25 µm sieve,
- ❒ collect the nematodes, which are retained in the sieve, with distilled water in a beaker.

Fraction II: nematodes on/in the carrot disc

- ❒ put the carrot disc in a kitchen blender with distilled water,
- ❒ blend 3 times for 10 sec with a short rest period (duration of maceration depends on the size of the carrot disc),
- ❒ pour the maceration solution through a 100 µm sieve placed in a bowl (to separate the nematodes, which will pass through the sieve, from the carrot tissue),
- ❒ rinse the residue in the sieve with tap water and collect the water with the nematodes in the bowl,
- ❒ pour the content of the bowl through a 25 µm sieve,
- ❒ collect the nematodes, which are retained in the sieve, with distilled water in a beaker,
- ❒ pour fractions I and II in a 1 mm sieve covered with tissue paper placed in a funnel or in a dish with distilled water,
- ❒ wait 1 night,
- ❒ collect the nematodes from the base of the funnel or on the bottom of the dish in a beaker,
- ❒ pour the nematodes quickly through a 25 µm sieve,
- ❒ collect the nematodes from the sieve with distilled water in a beaker,

Under laminar flow:

- ❒ pour the nematode suspension into a sterile tube test.

  clean the kitchen blender, sieves and Baermann funnel/dish first with ethanol (to kill

remaining nematodes) and afterwards with soap and water.

5. Sterilization of* Radopholus similis *and* Pratylenchus *spp.

Under laminar flow:

start with a sterile test tube filled with nematodes in sterile water (2 ml)

- ❒ add 1 ml 6000 ppm streptomycin sulphate with a sterile pipette (thus obtaining a final dilution of 2000 ppm streptomycin sulphate),
- ❒ wait 1 night,
- ❒ remove the streptomycin sulphate solution from above the nematodes (which remain in a white pellet at the bottom of the test tube) with a sterile pipette,
- ❒ add fresh sterile water,
- ❒ wait until the nematodes have settled to the bottom of the tube (***wait long enough***),
- ❒ repeat 2 to 3 times,
- ❒ use the nematodes for inoculation.

prepare new streptomycin sulphate each time

Protocol: Culturing of root-knot nematodes on transformed tomato roots

1. Extraction of root-knot nematodes from infected roots (= maceration-sieving method)

Juveniles of *Meloidogyne* are between 0.25 and 0.50 cm in length; the eggs are oval shaped and very much smaller: 50-100 x 25-50 µm.

A. Infected roots with visible egg masses

- ❒ remove the egg masses from the roots (for instance with a scalpel),
- ❒ transfer the egg masses to 0.25 % NaOCl (to disperse the egg masses) and shake for 5 minutes,
- ❒ pour the solution through a 100 µm sieve placed in a bowl (to separate eggs and juveniles, which will pass through the sieve, from the root tissue),
- ❒ rinse the sieve very well with tap water and collect the water with eggs and juveniles in the bowl,
- ❒ pour the contents of the bowl through a 25 µm sieve,
- ❒ collect eggs and juveniles, which are retained in the sieve, with distilled water in a beaker.

clean sieves first with ethanol (to kill remaining nematodes) and afterwards with soap and water

B. Infected roots without visible egg masses

- ❒ wash the roots with tap water,
- ❒ cut the roots into 1 cm-pieces,
- ❒ put the root pieces into a kitchen blender in 0.12 % NaOCL,

- ❒ blend for 10 sec,
- ❒ pour the macerated suspension t♒❒☐◆♑♒ ♋ 🗁🗀🗀 ✪❍ ♦ieve placed in bowl (to separate eggs and juveniles, which will pass through the sieve, from the root tissue),
- ❒ rinse the sieve very well with tap water and collect the water with eggs and juveniles in the bowl,
- ❒ pour the content of the bowl through a 25 µm sieve,
- ❒ collect eggs and juveniles, which are retained in the sieve, with distilled water in a beaker.

clean the kitchen blender and sieves first with ethanol (to kill remaining nematodes) and afterwards with soap and water

2. Sterilization of root-knot nematodes

A. Infected roots with visible egg masses

under laminar flow

- ❒ start with a sterile test tube filled with eggs and juveniles in 2 ml sterile water,
- ❒ add 1 ml 6000 ppm streptomycin sulphate with a sterile pipette (thus obtaining a final concentration of 2000 ppm streptomycin sulphate),
- ❒ wait 1 night,
- ❒ remove the streptomycin sulphate solution from above the nematodes (which remain in a white pellet at the bottom of the test tube) with a sterile pipette,
- ❒ add fresh sterile water,
- ❒ wait until the eggs and juveniles have settled to the bottom of the tube (***wait long enough***),
- ❒ repeat 2 to 3 times,
- ❒ use the eggs and juveniles for inoculation.

prepare new streptomycin sulphate each time

B. Infected roots without visible egg masses

a) in the case of a "clean" nematode solution:

under laminar flow

- ❒ pour the nematode suspension into a counting dish,
- ❒ heat a sterile micropipette (with a rubber top) in the flame so that it can suck eggs and juveniles,
- ❒ suck the eggs and juveniles with the micropipette,
- ❒ transfer the eggs and juveniles into sterile water in a sterile (Petri) dish,
- ❒ transfer the selected eggs and juveniles with a sterile pipette into a sterile test tube,
- ❒ sterilise eggs and juveniles with streptomycin sulphate for 1 night (as described above).

b) in the case of a "dirty" nematode solution:

- ❒ pour the water with eggs and juveniles into a Baermann funnel/dish a 1 mm sieve covered with tissue paper placed in a funnel or in a dish with distilled water,
- ❒ wait until the juveniles hatch from the eggs (may take a few days),

- ❒ collect the juveniles from the base of the funnel or on the bottom of the dish in a beaker,

- ❒ pour the suspension with the juveniles through a 25 µm sieve (to eliminate bacteria etc.),
- ❒ collect the juveniles in the sieve with distilled water in a beaker.

clean the kitchen blender, sieves and Baermann funnel/dish first with ethanol (to kill remaining nematodes) and afterwards with soap and water

under laminar flow

- ❒ pour the nematodes into a sterile (Petri) dish,
- ❒ heat a sterile micropipette (with a rubber top) in the flame so that it can suck juveniles,
- ❒ suck the selected juveniles with the micropipette,
- ❒ transfer the juveniles to sterile water in a sterile (Petri) dish,
- ❒ transfer the selected juveniles with a sterile pipette to a small sterile 20 µm sieve,
- ❒ put the sieve with the juveniles in 0.01 % $HgCl_2$ for 2 minutes,
- ❒ rinse the sieve with the juveniles 2 times with sterile water,
- ❒ place the sieve with the juveniles in sterile water and transfer the juveniles with a sterile pipette to sterile water in a sterile test tube,
- ❒ sterilize the juveniles with streptomycin sulphate for 1 night (as described above).

3. Culturing of root-knot nematodes on transformed tomato roots ("hairy" root cultures)

Step A: preparation of transformed tomato roots

- ❒ prepare Gamborg B5 (GB5) medium with pH 6.2 (see below),
- ❒ autoclave the medium,

under laminar flow

- ❒ fill Petri dishes with medium and wait until the water has evaporated,
- ❒ select some growing root tips from stock culture(s),
- ❒ transfer the growing root tips to the new medium (***place the root tips in the middle of the Petri dish***),
- ❒ close the Petri dishes with parafilm and store in the dark at 28 °C.

Step B: inoculation with nematodes

under laminar flow

a) inoculation with juveniles

- ❒ pour the sterilized juveniles into a sterile Petri dish,
- ❒ heat a sterile micropipette (with a rubber top) in the flame so that it can suck juveniles,
- ❒ suck the juveniles with the micropipette,
- ❒ transfer the juveniles to a growing root tip.

b) inoculation with egg masses

- ❒ remove an egg mass containing eggs and/or juveniles from the roots (for instance with

Figure 4. Initial root damage caused by migratory endoparasitic nematodes.

Figure 5. Root lesions caused by migratory endoparasitic nematodes.

Figure 6. Corm damage caused by migratory endoparasitic nematodes.

Figure 7. Galling of banana roots caused by root-knot nematodes.

Figure 9. Toppling over or uprooting of bananas caused by root nematodes.

Figure 8. Banana roots infected with swollen root-knot nematode females.

Protocol: Gamborg B5 medium for transformed tomato roots

- ❒ fill a beaker with distilled water,
- ❒ add GB5 powder including vitamins: 3.292 g/l,
- ❒ add 20 g/l sugar (sucrose),
- ❒ add distilled water to a volume of 1 l,
- ❒ adjust the pH to 6.2,
- ❒ add 15 g/l agar and stir very well,
- ❒ autoclave the medium,
- ❒ fill the Petri dishes with 20 ml medium.

Protocol: Culturing of root-knot nematodes on tomato plants in the soil

1. Preparation of the tomato plants

- ❒ sieve the soil through a 2 mm sieve,
- ❒ sterilize the soil,
- ❒ fill 12-cm-diameter plastic pots with the sterilized soil,
- ❒ water the soil up to field capacity,
- ❒ make a hole in the middle of each pot,
- ❒ place 1 seed in the hole and refill the hole with soil.

2. Extraction of root-knot nematodes from transformed tomato roots

- ❒ transfer the galls and egg masses to a beaker,
- ❒ add 0.12 % NaOCl,
- ❒ macerate the roots in a kitchen blender for 10 sec,
- ❒ pour the macerated suspension through a 100 µm sieve placed in a bowl (to separate eggs and juveniles, which pass through the sieve, from the root tissue),
- ❒ rinse the sieve very well with tap water and collect the water with eggs and juveniles in the bowl,
- ❒ pour the content of the bowl through a 25 µm sieve,
- ❒ collect eggs and juveniles from the sieve with distilled water in a beaker.

3. Inoculation with root-knot nematodes (2-3 weeks after sowing)

- ❒ bring the nematode suspension to a known volume with distilled water (***not too much*****),**
- ❒ blow air through the nematode suspension with a pipette (to homogenise the suspension),
- ❒ take a subsample,
- ❒ count the nematodes (eggs and vermiform nematodes = juveniles) in the subsample,
- ❒ calculate the total number of nematodes in the suspension,
- ❒ prepare a suspension which contains about 4000 eggs and juveniles in a few ml of distilled water,
- ❒ make 3-4 holes, about 3 cm deep, in the soil adjacent to the base of the tomato stem,

- blow air through the nematode suspension with a pipette (to homogenize the suspension),
- inject about 4000 eggs and juveniles in a few ml of distilled water into the holes,
- fill the holes with soil.

If nematode-infected roots are used for inoculation (for instance roots collected from a field infested with nematodes), the number of nematodes in a subsample of the roots (probably a mixture of two or more nematode species) should be determined in order to calculate the inoculum level. Extraction of the nematodes from the roots can be done either by the maceration-sieving method alone or in combination with the maceration Baermann funnel/dish method (see above).

3.4. Screening experiments

The screening experiments can be undertaken using either pots/plastic bags or in the field.

Screening experiments in pots or plastic bags will only allow observations to be made for a relatively short period (2 to 3 months) of the crop cycle. During this period, the susceptibility of the genotypes can be determined by assessing the nematode reproduction rate and, if uninfected plants are included in the screening experiments, some observations can be made on the sensitivity of the genotypes: root necrosis (migratory endoparasitic nematodes), root galling (root-knot nematodes) and plant growth (root weight, shoot weight, plant height).

Screening experiments in the field 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 and, if uninfected plants are included in the screening experiment, observations can be made on the sensitivity of the genotypes, including at the level of yield.

For screening experiments in pots or plastic bags, the plants should be harvested and observations on reproduction and damage assessment should be made at least 2 months after nematode inoculation.

For screening experiments in the field, a specific plant development stage should be selected as a standard stage to make observations on reproduction and damage assessment (see Section 3.4.2).

3.4.1. Screening experiments in pots/plastic bags

The size of the pots/plastic bags will depend upon the type of planting material used (*in vitro* tissue cultured plants or suckers) and the objective of the screening experiment. In general, the objective of screening experiments in pots/plastic bags is to evaluate the susceptibility of genotypes by maintaining the plants through one or two generations of the nematodes, i.e. about two months at 25-30 °C for all banana nematodes. Therefore, for such a period, 12 cm-diameter pots, containing about 1000 cm^3 soil, are sufficient. If suckers are used, larger pots, or if preferred, plastic bags, are needed. A soil type should be used which is representative of the soil type

in which bananas are cultivated in the region and in which nematode problems occur. This soil should first be sterilized. If possible the ambient temperature should be controlled. Watering and fertilization of the pots/plastic bags should be optimal.

This protocol describes a method which is modified after Pinochet (1988) and Sarah *et al.* (1992).

3.4.2. Screening experiments in the field

For field screening, a potential site should be sampled to determine the spectrum of nematodes present. Ideally, a site should be infested with a single plant-parasitic nematode species but this will seldom be the case. A site should be selected where the species composition is representative of the species composition occurring in the region.

Protocol: Pot/plastic bag screening

1. Preparation of the pots/plastic bags

- ❒ sieve the soil through a 2 mm sieve,
- ❒ sterilize the soil,
- ❒ fill 12 cm-diameter plastic pots/plastic bags with the sterilized soil,
- ❒ water the soil up to field capacity,
- ❒ make a hole in the middle of each pot/plastic bag,
- ❒ take the explant* out the test tube,
- ❒ remove the rooting medium with water,
- ❒ place the explant in the hole and fill the hole with soil,
- ❒ place the pots under a plastic or styrofoam cover (to maintain a high humidity),
- ❒ after 2 weeks: open the cover slightly,
- ❒ after 3 weeks: remove the cover,
- ❒ after 4 weeks: inoculate with nematodes.

[* when suckers are used, the plastic or styrofoam cover(s) are not necessary]

2. Inoculation with Radopholus similis and Pratylenchus spp.

- ❒ extract the nematodes from the carrot discs: macerate the carrot discs in a kitchen blender in distilled water 3 times for 10 sec with a short rest period (duration of maceration depends on the size of the carrot disc),
- ❒ pour the macerated suspension through a 100 µm sieve placed in a bowl (to separate the nematodes, which pass through the sieve, from the carrot tissue),
- ❒ rinse the residue on the sieve with tap water and collect the water with the nematodes in the bowl,
- ❒ pour the contents of the bowl through a 25 µm sieve,
- ❒ collect the nematodes from the sieve with distilled water in a beaker,
- ❒ bring the suspension with the nematodes to a known volume with distilled water,
- ❒ blow air through the nematode suspension with a pipette (to homogenize the suspension),
- ❒ take a subsample,

- count the nematodes (eggs and vermiform nematodes = juveniles and adults) in the subsample,
- calculate the total number of nematodes in the suspension,
- prepare a suspension which contains about 1000 vermiform nematodes in a few ml of distilled water,
- make 3-4 holes, about 3 cm deep, into the soil adjacent to the base of the stem,
- blow air through the nematode suspension with a pipette (to homogenize the suspension),
- inject about 1000 eggs and vermiform nematodes in a few ml of distilled water into the holes,
- fill the holes with soil.

3. Inoculation with root-knot nematodes

- transfert galls and egg masses to a beaker,
- add 0.12 % NaOCl,
- macerate the roots in a kitchen blencer for 10 sec.,
- pour the macerated suspension through a 100 µm sieve placed in a blowl to separate eggs and juveniles, which pass through the sieve, from the root tissue),
- rinse the sieve very well with tap water and collect the water with eggs and juveniles in the bowl,
- pour the content of the bowl through a 25 µm sieve,
- collect eggs and juveniles from the sieve with distilled water in a beaker,
- bring the suspension with the nematodes to a known volume with distilled water,
- blow air through the nematode suspension with a pipette (to homogenize the suspension),
- take a subsample,
- count the nematodes (eggs and vermiform nematodes = juveniles) in the subsample,
- calculate the total number of nematodes in the suspension,
- prepare a suspension which contains about 5000 eggs and vermiform nematodes in a few ml of distilled water,
- make 3-4 holes, about 3 cm deep, into the soil adjacent to the base of the stem,
- blow air through the nematode suspension with a pipette (to homogenize the suspension),
- inject about 5000 eggs and vermiform nematodes in a few ml of distilled water into the holes,
- fill the holes with soil.

The infestation level at the site should be investigated by examining roots of bananas growing at the site (Figure 11), not by taking soil samples. If the nematode population present is large enough, (at least 100 nematodes/g fresh roots) the infested field can be used immediately.

If the nematode population present is too small, the site can be either planted with a banana genotype which is a good host in order to increase the nematode population or nematode-infected roots can be added when the plants are planted (using macerated infected roots).

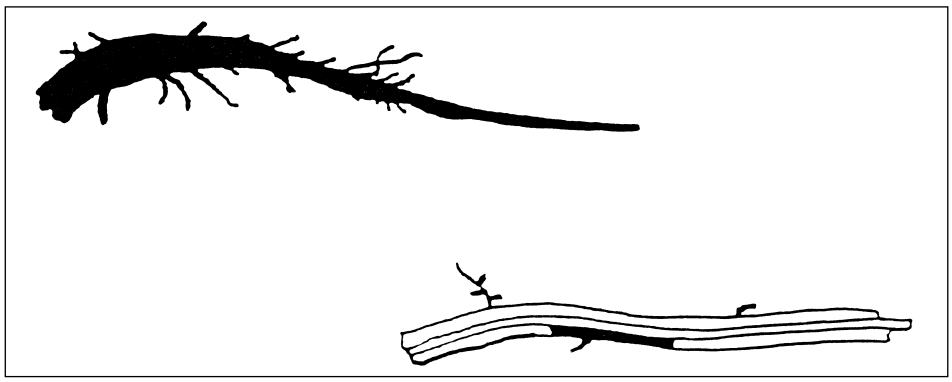


Figure 11. A dead root (top) and a functional root (bottom).

If resistance/susceptibility is being investigated and only the reproduction of the nematodes will be estimated, no uninfected plants are necessary.

If tolerance/sensitivity is being investigated in addition to the reproduction of the nematodes, information on the reaction of infected plants is also needed. In this case, uninfected plants are needed. Existing nematode populations can be eradicated by fumigation or with nematicides.

Experimental design

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

Reference genotypes

In order to be able to compare results between screening experiments undertaken either at the same locality or at different localities, at least one (susceptible) reference genotype or the complete set of reference genotypes should be included in the screening experiments:

Grand Naine	ITC 1256	AAA	susceptible to all nematodes
Gros Michel	ITC 1122	AAA	moderately resistant to *R. similis*
Yangambi Km5	ITC 1123	AAA	highly resistant to *R. similis*
Pisang Jari Buaya	ITC 0312	AA	completely resistant to *R. similis*

The complete set of reference genotypes can be obtained from INIBAP.

3.5. Evaluation of resistance/susceptibility = nematode reproduction assessment

Juveniles and adults of migratory endoparasitic nematodes (including *Helicotylenchus multicinctus*) and juveniles and males of root-knot nematodes can be extracted from banana roots by several methods. Two of these methods, the maceration-Baermann funnel/dish method and the maceration-sieving method have

been described above. In contrast with the centrifugal-flotation method, these two methods require little equipment. A description of the maceration-sieving method can also be found in Coolen and D'Herde (1972). In pot/plastic bag screening experiments, the number of egg-laying females (ELF; Hadisoeganda and Sasser, 1982) can be used to assess the reproduction rate of root-knot nematodes while in field screening experiments, the number of juveniles can be used, in combination with root-knot gall rating (Kinloch, 1990).

In pots/plastic bags, the total root fresh weight can be determined so that the nematode population can be expressed per root unit as well as per root system. In the field, the nematode population can only be expressed per root unit.

Protocol: Estimation of the reproduction of migratory endoparasitic nematodes

1. Determination of root fresh weight

When collecting roots from the field, collect all roots from a standard-size excavation of 20 x 20 x 20 cm extending outward from the corm of the plant (Figure 12). Take only roots from the plant selected; do not include roots from adjacent plants. In case of a young plant, it is often easier to remove the complete sucker from the mat (Figure 13). Roots should be removed at a specific plant stage (STG). Specific plant stages are:

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

In plants which have flowered for less than 14 days (RF), the white or pink bracts on the fingers of the bunch are not yet dry.

- ❒ remove the plant (pots/plastic bags) or the roots (field) from the soil,
- ❒ carefully wash the soil from the roots with tap water,
- ❒ cut the roots into pieces of 10 cm long and dry with paper tissue,
- ❒ if the total root system has been removed: determine the total root fresh weight,
- ❒ cut the roots in pieces of 1 cm,
- ❒ take a subsample of 15 g,
- ❒ add 100 ml distilled water and store the roots in the refrigerator at 4 °C.

2. Nematode extraction

- ❒ put the roots in 100 ml distilled water in the kitchen blender,
- ❒ macerate the roots 3 times for 10 sec (separated by 5 sec intervals),
- ❒ pour the macerated suspension through 250-106-40 mm sieves and rinse the sieves with tap water,
- ❒ collect the nematodes from the 40 mm sieve with distilled water in a beaker.

3. Assessment of the nematode population

- ❒ dilute the nematode suspension with distilled water in a graduated cylinder to 200 ml,
- ❒ blow air through the nematode suspension with a pipette (to homogenize the suspension),
- ❒ take a subsample of 6 ml (counting dish) or 2 ml (counting slide),
- ❒ count the nematodes in the counting dish (stereo microscope) or in the counting slide (light microscope),
- ❒ calculate the final nematode population per root unit and per root system.

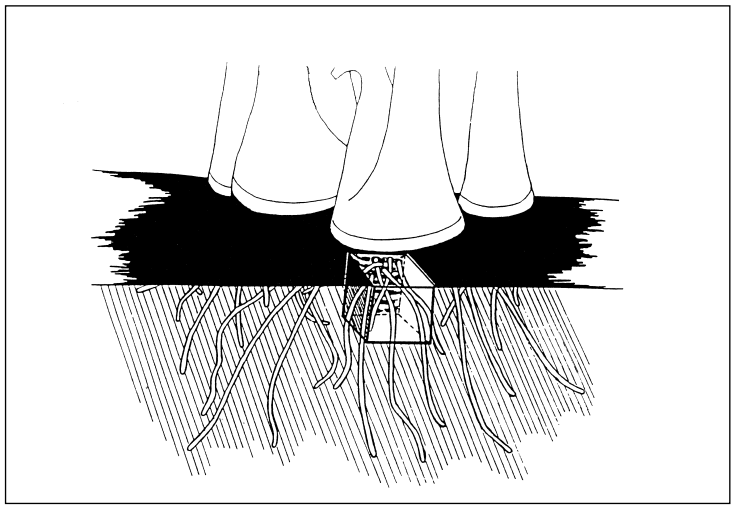


Figure 12.
Collection of roots from a standard-size excavation of 20 x 20 x 20 cm extending outward from the corm of the banana plant.

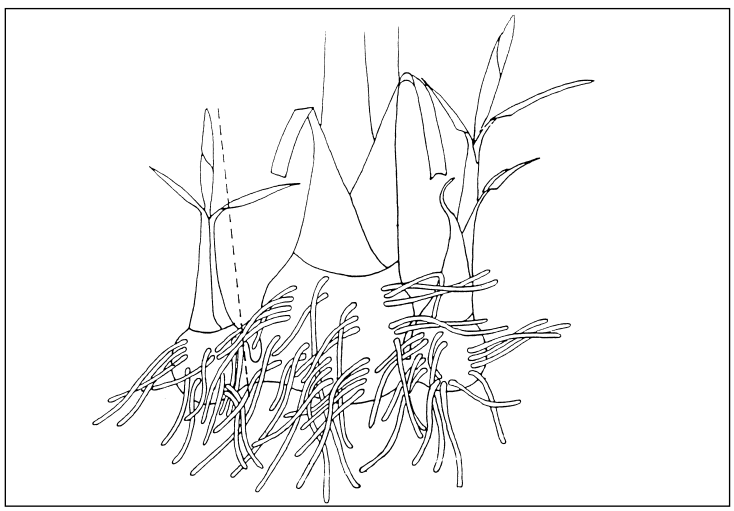


Figure 13.
Removal of a sucker from a mat.

Protocol: Estimation of the reproduction of root-knot nematodes

1. Determination of root fresh weight

- ❒ remove the plant (pots/plastic bags) or the roots (field) from the soil,
- ❒ carefully wash the soil from the roots with tap water,
- ❒ cut the roots into pieces of 10 cm long and dry with paper tissue,
- ❒ if the total root system has been removed: determine the total root fresh weight,
- ❒ cut the roots in pieces of 1 cm,
- ❒ take a subsample of 5 g,
- ❒ add 100 ml distilled water and store the roots in the refrigerator at 4 °C .

2. Assessment of the number of egg-laying females (ELF)

- ❒ stain the egg masses by immersing the roots in 0.15 g/l phloxine B for 15 minutes,
- ❒ count the number of egg-laying females (stereo microscope),

rating: 0: no egg masses
1: 1-2 egg masses
2: 3-10 egg masses
3: 11-30 egg masses
4: 31-100 egg masses
5: > 100 egg masses.

3. Assessment of root-knot galling

- ❒ estimate the root-knot galling

rating: 0: no galling
1: trace infections with a few small galls
2: < 25 % roots galled
3: 25-50 % roots galled
4: 50 -75 % roots galled
5: > 75 % roots galled.

3.6. Evaluation of tolerance/sensitivity = damage assessment

Damage can be assessed at the plantation and/or at the individual mat level.

3.6.1. Damage assessment at the plantation level

The condition of a plantation as a whole can be described by categorizing and quantifying the different types of damage:

- ❒ plant toppling: complete uprooting of the plant (including the corm), usually associated with root damage caused by nematodes (Figure 10a),
- ❒ plant snapping: breakage of the corm (the corm remains in the soil), usually associated with corm damage caused by weevils (weevil tunnels often present) (Figure 10b),
- ❒ plant breakage: breakage of the pseudostem of a plant with healthy roots and corm (Figure 10c): usually caused by strong winds when plants carrying a bunch are not additionally supported. May also be the result of severe water stress during periods of drought.

Protocol: Damage assessment at the plantation level

Use Form 1 (p. 43) for recording.

- ❒ SITE locality (for instance village)
- ❒ FARM name of the farmer
- ❒ DA, MTH, YEAR date (day, month, year) of the damage assessment
- ❒ MAT TOT total number of mats.

Flowering plants are plants being at any stage between flower emergence and close to/ready for harvest.

Pre-flowering plants are plants being at any stage before flowering but which are at least 1.5 m high.

- ❒ ST standing, not damaged plants
- ❒ TO toppled plants
- ❒ SN snapped plants
- ❒ BR broken plants.

3.6.2. Damage assessment at the individual mat level

The damage of individual plants of a mat can be assessed by measuring the root and corm damage, and the plant growth and yield.

Because root development and root decay are dynamic processes, which are genotype-dependent, influenced by plant growth and natural senescence on the one hand, and decay caused by abiotic and biotic factors (including nematode damage) on the other hand, a specific plant development stage should be selected as a standard stage for comparison of the sensitivity of different genotypes or one genotype grown under different conditions. Selecting the recently flowered plant stage as the standard stage has the advantage that, at this stage, the plant has produced its maximum number of roots and no new roots are being formed. Also, from this stage onwards, plant height will remain constant until harvest. Plants can also be selected on the basis of age, height and position in the mat.

Because root and corm damage of a sucker can be related to the height and circumference of the sucker, as well as to the height and bunch weight of the recently harvested mother plant, damage assessment of suckers can be undertaken. This is especially relevant in regions where high nematode populations are associated with banana roots and insufficient roots remain on harvested plants to be able to assess root damage. Observing damage on suckers has the additional advantage that an estimation can be made of the number of roots once produced, but already completely decayed, by relating the number of root bases to the number of dead and functional roots. Also observations on suckers allow an estimation of the corm damage for a complete mat.

The root and corm damage assessment presented has been developed at the International Institute for Tropical Agriculture (IITA; Speijer and Gold, 1996).

Root and corm damage

The extent of damage to the roots and corm of a plant are a measurement of the health of a root system and corm; the lower the damage, the more healthy the root system and corm.

In principle, root and corm damage consists of the following components:

- dead roots
- root necrosis of functional roots
- necrosis on the corm.

Dead roots are completely rotten or shriveled; functional roots show at least some healthy tissue.

The extent of root damage can be expressed as percentage dead roots, percentage root necrosis and percentage of root bases with lesions.

Protocol: Root damage assessment

Use Form 2 (p. 44) for recording.

Assess root damage by preference for a specific plant stage (STG). Specific plant stages are:

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

In plants which have flowered for less than 14 days (RF), the white or pink bracts on the fingers of the bunch are not yet dry.

Record the exact mat (MAT) and plant number (PLT) of the sampled plant. In addition to the plant stage (STG), also note the genotype (GENOTYPE), the circumference or girth of the pseudostem at 1 m (GR), the plant height (HT) and the number of standing leaves (SL). Plant height is the distance from the pseudostem base to the youngest leaf axil. At harvest, record the bunch weight (BW).

Step 1. Collect all roots from a standard-size excavation of 20 x 20 x 20 cm extending outward from the corm of the plant (Figure 12). Take only roots from the plant selected; do not include roots from adjacent plants. In case of a young plant, it is often easier to remove the complete sucker from the mat (Figure 13).

Step 2. Divide the collected roots into two categories:

- dead roots (DE)
- functional roots (OK)

and count the number of roots in each category.

Step 3. Select at random five functional primary roots, at least 10 cm long. Roots may vary in length; very short segments, which may have been cut during digging, can be discarded.

First, observe the general health condition of the secondary and tertiary roots attached. These roots are referred to as feeder roots (FR). All feeder roots of one sample can be scored as:

- ❒ all healthy = score 1
- ❒ mostly healthy = score 2
- ❒ mostly dead = score 3
- ❒ all dead = score 4.

Then, reduce the length of the five selected functional roots to 10 cm and slice the roots length-wise (Figure 14).

Migratory endoparasitic nematodes: Score one half of each of the five roots for the % of 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. Record the necrosis of the individual roots (RN1 to RN5). The sum is the total root necrosis of the sample (TOT RN).

Root-knot nematodes: The presence of root-knot nematodes (RK) can be observed either externally as galls (Figure 7) or internally as pit-like structures (Figure 8) on the roots.

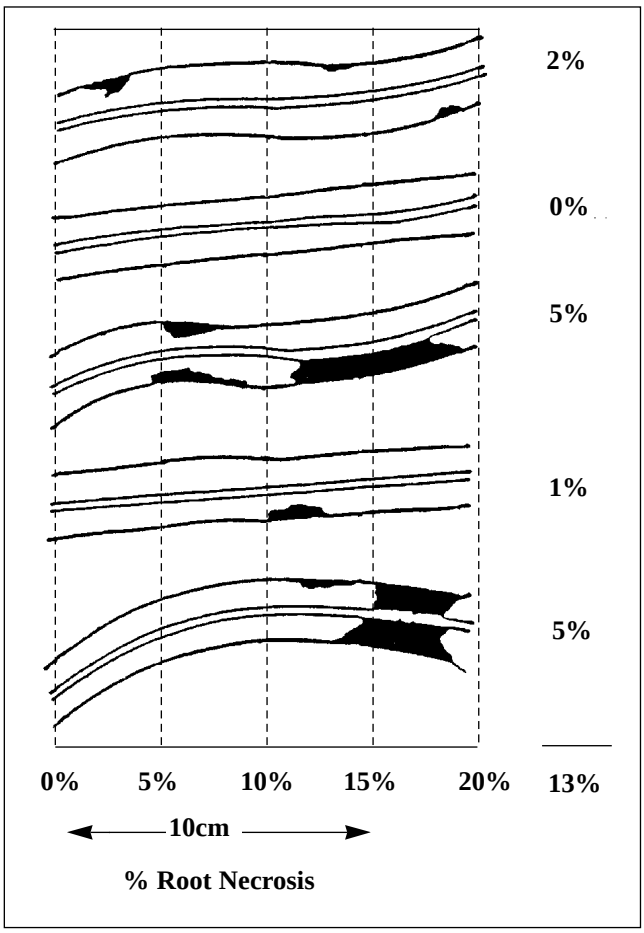


Figure 14.
Example of scoring five length-wise sliced banana roots for root necrosis (% of root cortex surface showing necrosis) caused by migratory endoparasites.

Protocol: Corm damage assessment

Use Form 2 for recording.
Assess corm damage by preference for a specific plant stage (STG; see above). Corms of young plants, especially from plantains, can often easily be removed from the mother plant with a spade or spear.
Record the exact mat (MAT) and plant number (PLT) of the sampled plant. In addition to the plant stage (STG), also note the genotype (GENOTYPE), the circumference or girth of the corm (GR), the plant height (HT) and the number of standing leaves (SL). Plant height is the distance from the ground level to the youngest leaf axil. At harvest, record the bunch weight (BWm) of the mother plant.

Step 1. Clean the corm thoroughly by shaking off the soil or washing with water. Because the inward half of the corm is connected with the mother plant, the roots at this side of the corm are not evenly distributed. Therefore, select the outward half of the corm for damage assessment (Figure 15). For ease of examination, the roots should be peeled off (and root damage assessment made as decribed above).

Step 2. Count the number of root basis (RB) on the selected outward half of the corm. These can be clearly seen on the peeled corm surface as circles. Root bases are often in groups of three to four. Nematode-related damage appears as blackish-purple lesions around the root bases.

Step 3. Count the number of small lesions (SL) and large lesions (LL) (Figure 16):
- ❒ small lesions (SL): lesions smaller in diameter than the root bases
- ❒ large lesions (LL): lesions equal or larger in diameter than the root bases.

Some of the large lesions can be very large and coalesce with lesions associated with adjacent root bases. In this case, a large lesion is counted for each of the root bases in the coalesced lesions.
Sometimes lesions are observed without association with a root base. These lesions should be excluded from the counting because these lesions may have been caused by nematode-infected roots from adjacent plants growing close to the corm examined.
Alternatively, suckers can be categorized into five groups according to lesion presence and lesion size on the selected out-ward corm surface. In this case the columns for root bases (RB), small lesions (SL) and large lesions (LL) are replaced by one column for the categories (SCORE):

- ❒ no lesions = score 0
- ❒ one small lesion = score 1
- ❒ several small lesions = score 2
- ❒ one large lesion = score 3
- ❒ several large lesions = score 4.

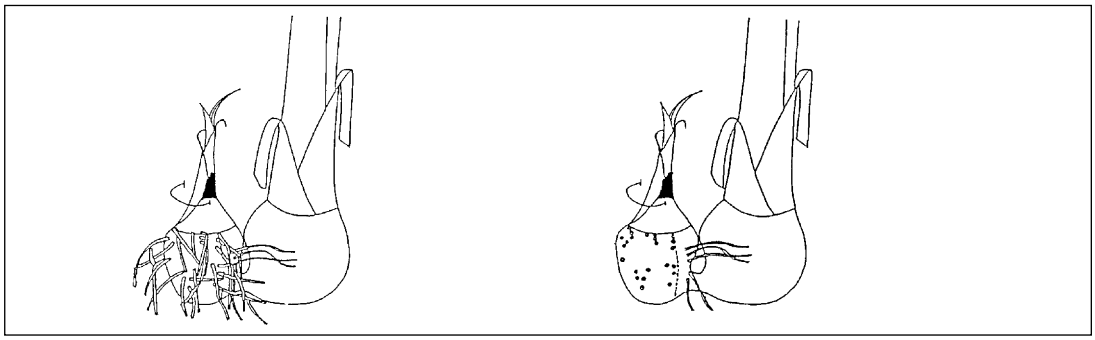


Figure 15. Selection and preparation for damage assessment of the outward half of the corm.

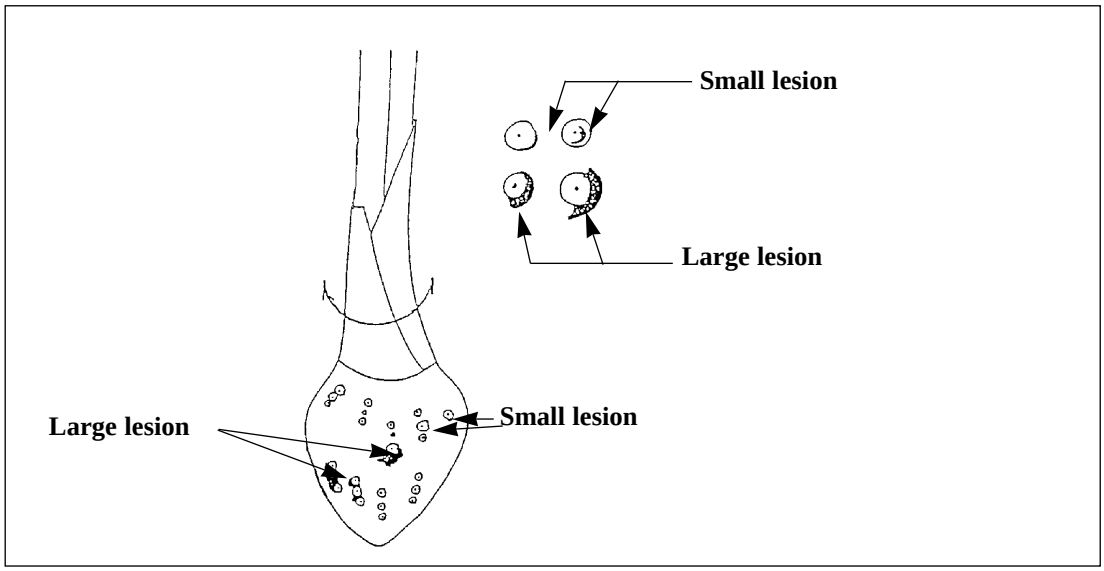


Figure 16. Small and large lesions and root bases on a corm.

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Appendix

Plantation Assessement

Form 1

Observer:....................................

Plant toppling (TO): complete up-rooting of the plant (dead root stumps can often be seen on the rhizome).

Plant snapping (SN): breakage of the corm; the rhizome remains in the soil and generally weevil tunnels are evident.

Plant breakage (BR): breakage of the pseudostem of the plant with a healthy root system and rhizome.

Pre-flowering: any stage prior to flower emerge, but a minimum height of 1.5m.

Flowering: any stage between flower emerge and ready for harvest.

Not damaged plants are standing (ST)

Site	Farm	Day	Mth	Year	Mat Tot	Pre-flowering				Flowering			
						ST	TO	SN	BR	ST	TO	SN	BR

ROOT AND CORM DAMAGE ASSESSEMENT

Form 2

Date: ..../..../.... **Observer**: .....

Trial: ..... **Plot**: .....

Plant stage (STG)
S: sucker <1.5m
PF: pre-fowering > 1.5m
RF: recently flowered
FL: flowering
CH: close to harvest
H: harvest

Count all dead roots (DE)
Count all functional roots (OK)

GR: girth (cm)
HT: height (cm)
SL: standing leaves (cm)
BW: bunch weight (kg)

RB: root bases
SL: small lesions (< diameter of associated root basis)
LL: large lesions (> diameter of associated root basis)

Randomly selected 5 functional primary roots. Score secondary and tertiary feeder roots (FR):
1 = all healthy 2 = most healthy
3 = most dead 4 = all dead

Slice primary roots length-wise
Score % root necrosis (RN)
Score root-knot nematodes (RK):
0 = absent 1 = present

Mat	PLT	STG	Genotype	GR	HT	SL	BW m	Roots			RN					Tot RN	RK	Corm		
								DE	OK	FR	1	2	3	4	5			RB	SL	LL

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