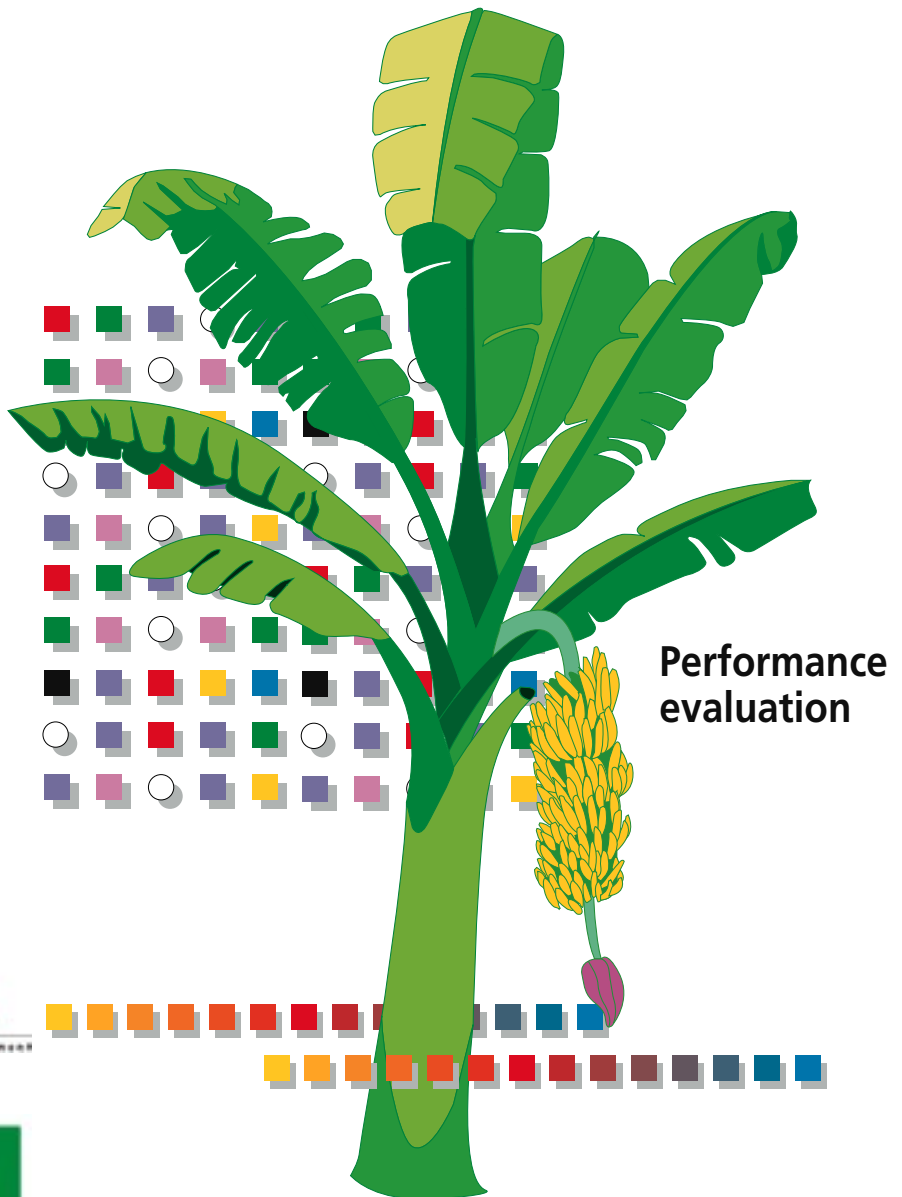


# Global evaluation of *Musa* germplasm for resistance to *Fusarium* wilt, *Mycosphaerella* leaf spot diseases and nematodes

Jean Carlier, Dirk De Waele and Jean-Vincent Escalant



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:

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To promote and strengthen collaboration and partnerships in banana-related research 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.

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# **Global evaluation of *Musa* germplasm for resistance to *Fusarium* wilt, *Mycosphaerella* leaf spot diseases and nematodes**

## **Performance evaluation**

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## Foreword

These Technical Guidelines replace the Technical Guidelines 1 (*Screening of Musa germplasm for resistance and tolerance to nematodes*) and 3 (*Evaluation of Musa germplasm for resistance to Sigatoka diseases and Fusarium wilt*) as far as performance evaluations are concerned. Technical Guidelines for in-depth evaluations, which were also part of Technical Guidelines 3, are published separately.

The Fusarium wilt and *Mycosphaerella* leaf spot sections have been updated following recommendations and comments made after analysing IMTP II results.

INIBAP and the authors wish to thank all the scientists who contributed to these guidelines and the Technical Center for Agricultural and Rural Cooperation (CTA) for its support to the publication and dissemination of these guidelines.

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# 1. Introduction

Fusarium wilt, *Mycosphaerella* leaf spot diseases and nematodes are recognised as the most important pests and diseases of bananas<sup>1</sup>. This document describes protocols for the performance evaluation of resistance to these pathogens and parasites. Contrary to in-depth evaluations, which are more complete disease resistance evaluations carried out on a smaller number of sites, (INIBAP Technical Guidelines No. 6) performance evaluations use a simplified protocol to obtain information on cultivar/hybrid performance under local conditions as well as basic information on disease resistance and tolerance.

The guidelines have been written with the collaboration of the Fusarium, Sigatoka and Nematology working groups of PROMUSA and INIBAP staff with the objective of helping researchers to:

- design their trial
- choose the appropriate location for the trial
- artificially infest the trial site in case it is not sufficiently infested with the pathogen (for Fusarium wilt and nematodes only)
- evaluate the genotypes for disease resistance and tolerance and agronomic traits.

## Notions of resistance and tolerance

Resistance/susceptibility and tolerance/sensitivity are defined as independent, relative qualities of a host plant based on comparisons between genotypes. Resistance/susceptibility refers to the plant's ability to prevent or limit pest development, whereas tolerance/sensitivity is the plant's ability to survive in the presence of a pest. A host plant may suppress (resistance) or allow (susceptibility) pathogen development and reproduction; it may suffer little injury (tolerance), even when heavily infected, or much injury (sensitivity), even when lightly infected.

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<sup>1</sup> Throughout the text, bananas is used for bananas and plantains.

## 2. Evaluation of resistance to Fusarium wilt

### Introduction

The pathogen responsible for Fusarium wilt (also known as Panama disease), the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* – *Foc*, was first recognized in 1874 in Australia. By the 1950s, the disease had reached such epidemic proportions that it was considered one of the most destructive plant diseases in recorded history. Infection occurs when the pathogen penetrates the roots of the banana plant. The fungus then invades the xylem vessels and, if not blocked by vascular occluding responses of the host, advances into the corm.

### Reference cultivars

Clones against which the new, improved hybrids are to be evaluated for their reaction to Fusarium wilt are:

Gros Michel (AAA)	Race 1 susceptible	ITC1122
Bluggoe (ABB) – Cachaco	Race 2 susceptible	ITC0643
Cavendish (AAA) cv. Williams	Race 4 susceptible	ITC0570
cv. Rose	Resistant	ITC0712
Local cultivar (if this is not Cavendish)	To be selected by collaborator at each test site as an appropriate local standard.	

INIBAP compiles a list of virus-indexed material from which reference genotypes can be selected. Please contact the IMTP coordinator at INIBAP. For information on how to handle plant material, see Appendix I.

### Establishment of plots

For a meaningful evaluation of germplasm resistance to Fusarium wilt, it is important that the evaluation site be located in an area of known occurrence of Fusarium wilt. If possible, the evaluation site should be located in a farmer's field and it should be managed according to local banana growing practices.

The site should be uniformly infested with only one strain of *Foc*. If possible, a clone of broad susceptibility (e.g. Gros Michel, Silk or the local susceptible variety) should be grown on the site for one season prior to establishing the trial. At harvest, these plants should be chopped and ploughed back into the soil. Before planting the experiment, the site should be deeply ploughed to evenly distribute the inoculum throughout the site. Care must be taken not to infest the site with more than one strain of *Foc*. To avoid contamination with other strains, it is recommended that infected plants and soil from other sites not be introduced into the evaluation site.



## Pathogen identification

Prior to establishing the evaluation trial, the identity of the particular strain of *Fusarium* at the site must be determined. If it is not known, samples should be collected from banana plants and sent to a recognised laboratory for analysis. Details on how to send samples for analysis are given in Appendix II.

## Experimental design

Once plants reach 0.3 to 0.5 m in height, they can be planted in the experimental field. The experimental design will depend on the particular site characteristics. Alternatives are described below. A randomized complete block design should be used whenever possible, but regardless of the design chosen, plant density should never exceed 2000 plants per hectare.

### Completely randomized design (CRD)

A completely randomized design (CRD) should be used when there is no identifiable source of variation at the evaluation site, i.e. the site is uniform for soil type, slope, planting date, etc. This is a very powerful design, which allows for statistical analysis even when there are different numbers of experimental units per treatment.

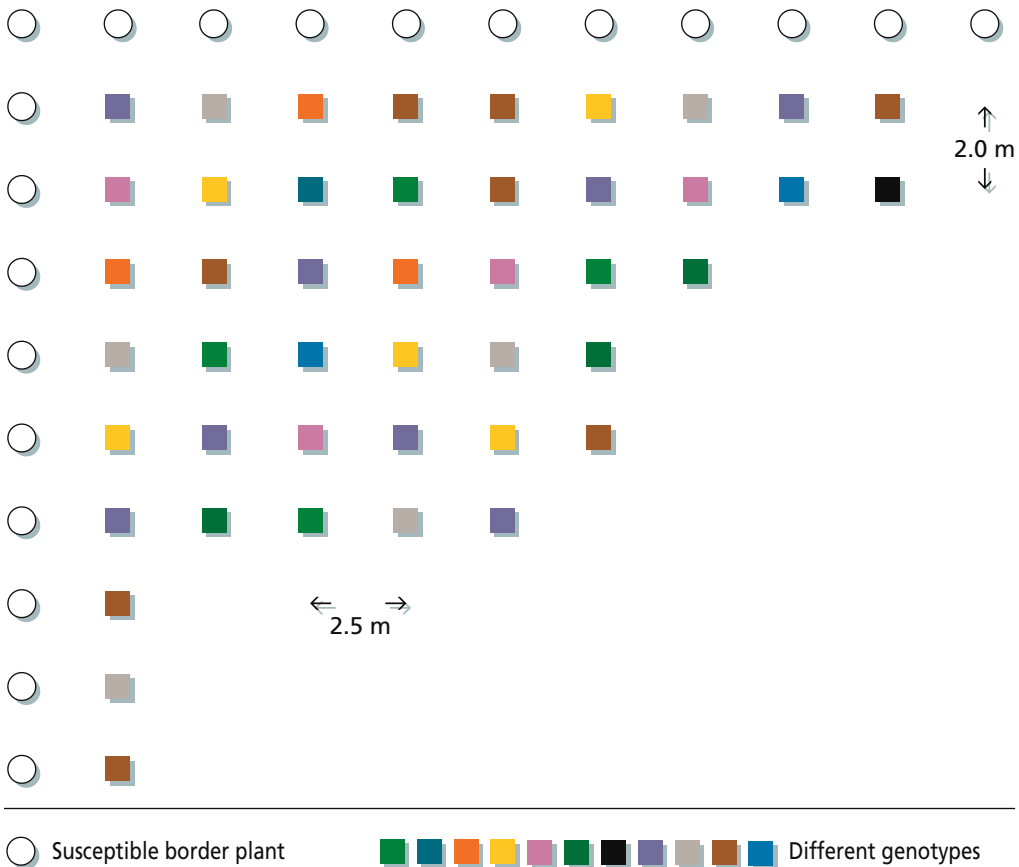
Plant spacing of 2 m x 2.5 m is recommended, but plants may be spaced differently in keeping with the institute or farmer's usual management practices.

There should be 20 replicates per treatment and the experimental site should be surrounded by a row of border plants. The layout is as shown in Figure 1.

### Randomized complete block design (RCBD)

A randomized complete block design (RCBD) may be used when there is an identifiable source of variation and the experimental units can be meaningfully grouped. The object of grouping is to have the units in a block as uniform as possible so that the observed differences will be largely due to the treatments (in this case the genotypes). The source of variation, like slope, pH gradient, planting date etc., should be identifiable and blocks should be arranged to account for this variation. Blocks, also called replications, should consist of a nearly square group of plots. Plots within blocks should be arranged in staggered double rows, as shown in Figure 2, in order to minimize the effect of competition between the plants. There should be at least six plants per plot but data will be taken only from the central plants; the two outer plants are considered to be border plants.

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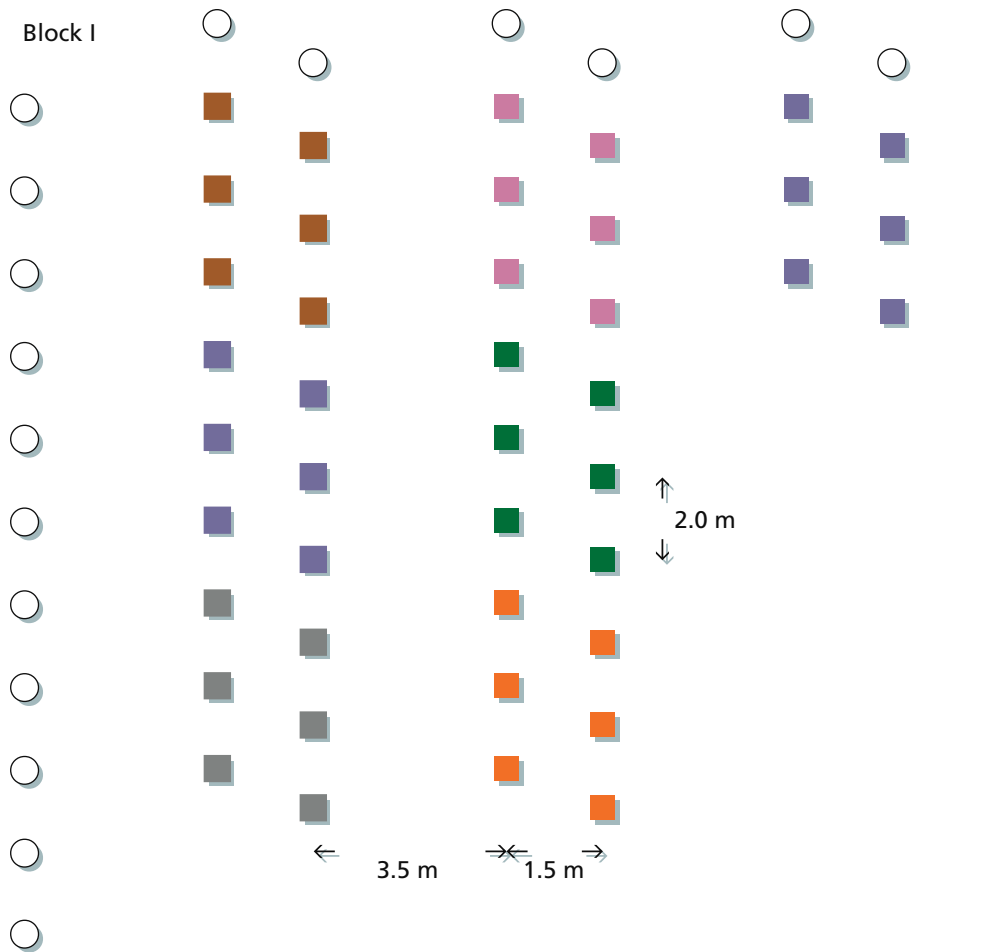
**Figure 1.** Layout of the completely randomized design for the evaluation of Fusarium wilt.

Five blocks or replications should be planted. Within a block, all genotypes should be randomly assigned to the plots. The layout is as shown in Figure 2.

*For more information and advice on experimental design, please contact Natalie Moore (e-mail: MooreN@prose.qld.gov.au).*

## Agronomic practices

Fertilizer application and irrigation should be carried out according to local practices. Fungicides must not be applied. In areas where it is normally practised, mats should be desuckered every three months using local methods leaving only the follower sucker. Likewise, deleafing should be practised if and when it is normally conducted in the test location. Experiments should be conducted for the mother plant crop and first ratoon.



○ Susceptible border plant

■ ■ ■ ■ ■ ■ Different genotypes

The block should be as square as possible to minimize variation.

**Figure 2.** Layout of the randomized complete block design for the evaluation of Fusarium wilt.

## Data to be collected

### Disease evolution data

On a monthly basis, beginning three months after planting, plants should be observed for symptom development. Plants that are clearly affected by Fusarium wilt should be recorded as diseased in Field form 1, Appendix III and marked for future reference. Thereafter, these plants need only be observed either when they die or produce fruits. Fields should be inspected more frequently (weekly) near to flowering time in order to accurately determine the flowering date. If plants are going to die before yielding fruit, they should

be examined internally to verify the presence of the disease. If plants ultimately yield fruit, internal ratings of disease severity should be taken at harvest.

### *Internal symptoms*

For the mother plant, internal symptoms in the pseudostem only should be collected. At harvest the plant should be cut at the base of the pseudostem. The extent to which vascular discoloration extends up the pseudostem should be determined by making transverse cuts, from the base of the pseudostem upwards, and examining the internal tissues following each cut. The point at which discoloration is no longer visible should be noted and the distance from this point to the pseudostem base recorded. Use Field form 1 in Appendix III to record the external and internal symptoms and Field form 2 to summarize the data.

For the first ratoon crop, symptoms within the pseudostem, and if practical, within the corm, should be assessed. The complete corm should be removed from the soil, the roots cut off and excess soil removed. Using a guillotine (Figure 3), or any other suitable device, transverse sections of the corm should be cut to obtain five slices of equal thickness per corm. The upper surface of each cut section should be examined and the extent of vascular discoloration noted on a scale of 1 to 6 (Plates 1-6 p. 12). An average grade for the five sections should be calculated and recorded.

Use Field form 3 in Appendix III if you decide to record data from the corm.

**Note:** Corm and pseudostem material must not be removed from the harvest site for, or following, assessment.

---





1. Corm completely clean, no vascular discoloration.



2. Isolated points of discoloration in vascular tissue.



3. Discoloration of up to one-third of vascular tissue.



4. Discoloration of between one-third and two-thirds of vascular tissue.



5. Discoloration of greater than two-thirds of vascular tissue.



6. Total discoloration of vascular tissue.

**Plates 1-6.** Scale for rating the internal symptoms caused by Fusarium wilt (Photographs courtesy of Dr Zilton Cordeiro, EMBRAPA-CNPMP).

**Agronomic data**

The following agronomic traits should be recorded. Table 1 details when data should be collected, and field form 6 in Appendix XI is provided to help you record the data.

**Name of surveyor****Planting date****Time from planting to shooting (*days*)**

Number of days between planting and bunch emergence.

**Height of pseudostem at shooting (bunch emergence) (*cm*)**

Distance in cm from the ground to the angle made between the bunch stalk and bunch cover leaf.

**Height of following sucker at shooting (*cm*)**

Distance in cm from the ground to the junction between the youngest and next youngest leaf of the following sucker at the time the bunch emerges from the mother plant. All other suckers except the following sucker should be rogued as they appear.

**Number of functional leaves**

Functional leaves are leaves that have photosynthetic activity. Consider that a leaf is functional if it has more than 50% of green area.

**Plant crop cycle (*days*)**

Number of days between the date of planting and harvest.

**Girth of pseudostem at harvest (*cm*)**

Measured at 1 m from the base of the pseudostem.

**Weight of bunch (*kg*)**

Cut the bunch stalk (peduncle) above the first hand at the level of the last scar and immediately below the last hand.

**Number of hands in bunch at harvest**

Cut the hands from each bunch following weighing and record the number of hands.

**Number of fruits at harvest****Weight of fruit (*g*)**

Weigh all the hands cut from the peduncle and divide by the number of fruits.

**Environmental data**

It is also necessary to monitor climatic conditions. Weekly readings should be obtained from a weather station situated as close as possible to the experimental site. For your convenience, field form 7 in Appendix XI is provided to record environmental data.

---

**Table 1.** Timetable for recording disease evolution and agronomic data

Type of data	Growing phase (from 3 months after planting)	Shooting phase	Harvest
<b>Disease evolution data</b>			
External symptoms	Once a month	Once a month	
Internal symptoms			X
<b>Agronomic data</b>			
Time from planting to shooting		X	
Height of pseudostem		X	
Height of following sucker		X	
Number of functional leaves		At flowering	X
Plant crop cycle			X
Girth of pseudostem			X
Weight of bunch			X
Number of hands in bunch			X
Number of fruits			X
Weight of fruit			X

### Management practices

Details of fertilizer application, nematode/weevil control measures and irrigation/drainage management should be recorded.



### 3. Evaluation of resistance to *Mycosphaerella* leaf spot diseases

#### Introduction

Leaf spot diseases of bananas include three related pathogenic ascomycete fungi: *Mycosphaerella fijiensis*, causing black leaf streak disease (also known as black Sigatoka), *M. musicola*, responsible for Sigatoka disease (also known as yellow Sigatoka) and the recently discovered *M. eumusae*, the causal agent of eumusae leaf spot disease (formerly known as Septoria leaf spot disease). Black leaf streak disease and Sigatoka disease can cause extensive defoliation, but *M. fijiensis* is characterized by its stronger pathogenicity on a broader range of hosts, making black leaf streak disease the most destructive leaf disease of bananas. In general, the fungi are disseminated locally by ascospores and conidia. Long distance spread of the diseases is believed to be by the movement of germplasm (infected suckers, diseased leaves) and wind-borne ascospores.

#### Reference cultivars

Clones against which the new, improved hybrids are to be evaluated for their reaction to *Mycosphaerella* leaf spot diseases are:

Yangambi km 5	Highly resistant	ITC1123
Calcutta 4	Highly resistant	ITC0249
Pisang lilin	Partially resistant (high)	ITC1400
Pisang Ceylan	Partially resistant	ITC1441
Pisang Berlin	Susceptible	ITC0611
Grande naine	Susceptible	ITC1256
Local cultivar	To be selected by collaborator at each site as an appropriate local standard to compare reactions	

INIBAP compiles a list of virus-indexed material from which reference genotypes can be selected. Please contact the IMTP coordinator INIBAP. For information on how to handle plant material, see Appendix I.

#### Establishment of plots

Fields must be established in areas where there is sufficient presence of the pathogen. The evaluation site should be located in a farmer's field and it should be managed according to local banana growing practices. Moreover, the field layout must include plots of susceptible clones interspersed among those being evaluated. Susceptible local clones can be used.

It is not always easy to differentiate between the symptoms of the various *Mycosphaerella* leaf spot diseases. When possible, it is preferable to choose sites where only one leaf spot disease is present. Having a mix of pathogens makes it difficult to compare the results with those from other evaluation sites.

## Pathogen identification

The most reported *Mycosphaerella* leaf spot diseases on *Musa* are caused by *M. fijiensis* and *M. musicola*. *M. eumusae* has been reported only recently. This pathogen was discovered in South and Southeast Asia (Carlier *et al.* 2000, Crous and Mourichon 2002). In 2001, the three species have been detected in a very small area around Kuala Lumpur, Malaysia (J. Carlier pers. comm.).

Before evaluating new hybrids or selected clones, it is very important to know exactly which *Mycosphaerella* sp. is present at the site and, if possible, in the country. Sampling and identification of the pathogen are described in Appendix IV.

## Experimental design

Once plants reach 0.3-0.5m in height, they can be planted in the experimental field. Experimental design will depend on the characteristics of the particular site and alternatives are described below. Where practical and possible, a randomized complete block design should be used. However, whichever the design chosen, plant density should not exceed 2000 plants per hectare.

### Completely randomized design (CRD)

A completely randomized design should be used when there is no identifiable source of variation at the evaluation site, i.e. the site is uniform for soil type, slope, planting date etc. This is a very powerful design, which allows statistical analysis even when there are different numbers of experimental units per treatment.

Plant spacing of 2m x 2.5m is recommended, but plants may be spaced differently if this is more appropriate for the normal management practices of the institute/farmer.

There should be 8-10 replicates per treatment. The experimental site should be surrounded by a row of border plants. Layout should be in quincunx (interspersing susceptible plants) as shown in Figure 4.

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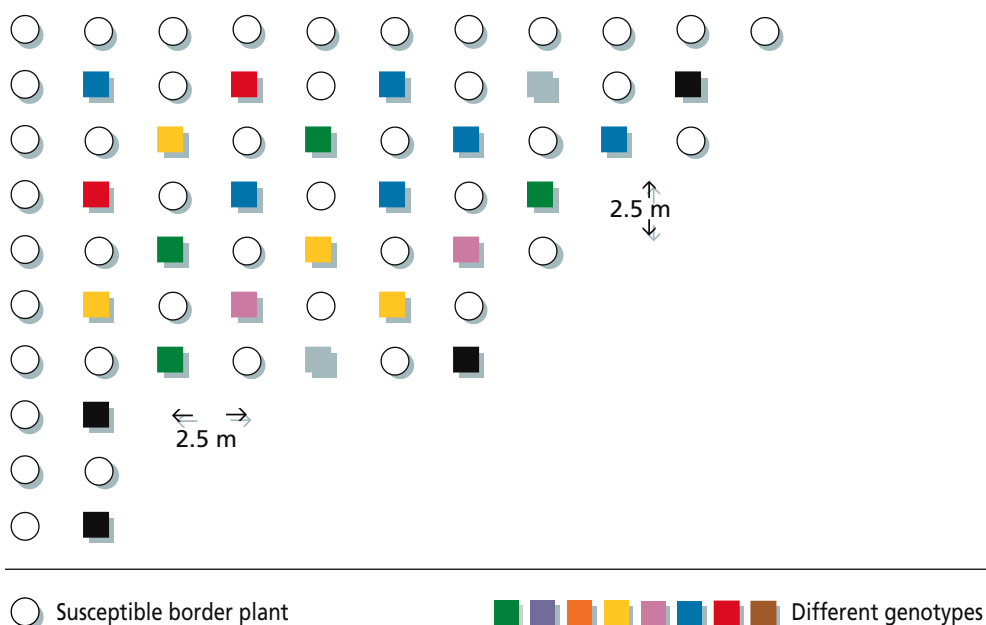


Figure 4. Completely randomized design in quincunx.

### Randomized complete block design (RCBD)

A RCBD may be used when there is an identifiable source of variation and the experimental units can be meaningfully grouped. The object of grouping is to have the units in a block as uniform as possible so that observed differences will be largely due to the treatments (in this case to the genotypes). The source of variation, such as slope, pH gradient, planting date etc. should be identifiable and blocks should be arranged to account for this variation. Blocks, also called replications, should consist of a nearly square group of plots.

Three to four blocks, or replications should be planted. All genotypes should be randomly assigned to the plots within blocks. Each plot should have three to six plants. The number of blocks and plants per plot will depend on land and human resources available. Layout as shown in Figure 5

*For more information and advice on experimental design, please contact Jean-Vincent Escalant (e-mail: [j.escalant@cgiar.org](mailto:j.escalant@cgiar.org)).*

### Agronomic practices

Fertilization and irrigation should be optimized to the greatest extent possible. Do not apply any kind of fungicides. In areas where it is normally practised, mats should be desuckered every three months using local methods, leaving only the following sucker. Experiments should be conducted for the mother plant crop and first ratoon.

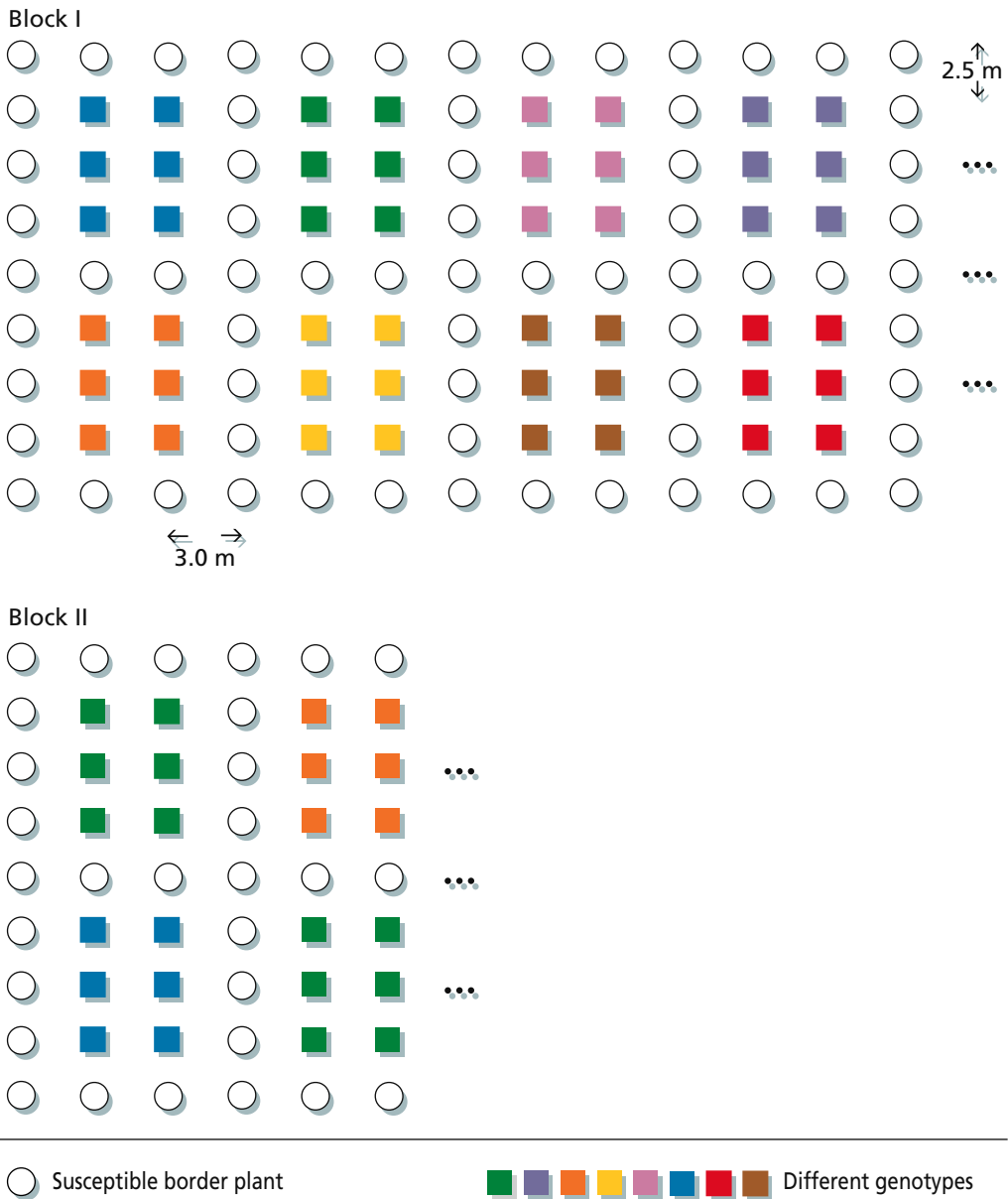


Figure 5. Layout of the randomized complete block design.

## Data to be collected

### Disease evolution data

Only two variables should be recorded: youngest leaf spotted at flowering (Vakili 1968) and number of standing leaves at flowering and harvest.

### *Youngest leaf spotted (YLS)*

Counting down from the top of the plant, the youngest leaf spotted (YLS) is the leaf number of the first fully unfurled leaf with at least 10 discrete, mature, necrotic lesions or one large necrotic area with 10 light-coloured dry centres (see Appendix V).

### *Number of standing leaves (NSL)*

The number of leaves counting from the youngest (i.e. the highest and unrolled) leaf downwards. Do not count those leaves with the petiole bent back, other leaves should be recorded regardless of their colour or the colour of their petiole.

These two variables should be recorded for every plant. Every plant in the trial should be assigned a unique identifier that will facilitate identification and follow-up. This identifier should be an integer between 1 and the total number of plants in the experiment assigned in a correlative way. Field form 4 in Appendix VI is provided as a model for the recordings. Please also record the block or replication number. This system enables data to be analysed using sub-sampling, which allows greater precision.

These variables will be used to calculate the index of non-spotted leaves (INSL), which is the proportion of standing leaves without the typical late-stage symptoms of black leaf streak disease, i.e. a black spot with a necrotic centre (Ortiz *et al.* 1997). This index provides an estimation of available photosynthetic leaf area prior to fruit filling and is a measure of resistance. It also corrects the difference in the number of leaves produced by different types of bananas and plantains.

It is calculated as follows:

$$INSL = \frac{100 * (YLS - 1)}{NSL}$$

### *Agronomic data*

Please refer to page 13 for a complete list of variables. Table 2 details when data should be collected, and field form 6 in Appendix XI is provided to help you record the data.

**Table 2.** Timetable for recording disease evolution and agronomic data.

Type of data	Growing phase (from 3 months after planting)	Flowering	Harvest
<b>Disease evolution data</b>			
Youngest leaf spotted	X	X	
Number of standing leaves		X	X
<b>Agronomic data</b>			
Time from planting to shooting		X	
Height of pseudostem		X	
Height of following sucker		X	
Number of functional leaves	X	X	X
Plant crop cycle			X
Girth of pseudostem			X
Weight of bunch			X
Number of hands in bunch			X
Number of fruits			X
Weight of fruit			X

### **Environmental data**

Environmental data should be collected from the weather station nearest the trial. Where trials are conducted in the grounds of collaborating institutes, this should not be a problem, as many centres have recording equipment.

Daily fluctuations in temperature and in humidity should be monitored. Readings should be taken at the same hour everyday and as early as possible. Rainfall can be calculated for the week if daily readings cannot be taken. Readings should begin at planting and continue until harvest. The soil of the test site should be analysed. When possible, a climatic map on the long-term climatic trend should be provided to give an overview of the annual fluctuations of temperature and rainfall.

For your convenience, a format for recording environmental data is provided on field form 7 in Appendix XI.

### **Management data**

Details of fertilizer application, nematode/weevil control measures and irrigation/drainage management should be recorded.

## 4. Evaluation of resistance to nematodes

### Introduction

In many banana growing regions, nematodes are responsible for high crop losses. The average annual yield loss is estimated at about 20% worldwide (Sasser and Freckman 1987). As they attack the root and/or corm tissues, nematodes affect plant growth and yield by reducing the mechanical (anchorage) and physiological (uptake and transportation of water and nutrients) functions of the root system.

Crop rotation and nematicides can be used to control nematodes (Gowen and Quénehervé 1990) but crop rotation is impracticable in areas where bananas are grown continuously, and the price of 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 been comparatively neglected in bananas. This is despite the evidence, albeit limited, that nematode resistance and tolerance sources are present in the *Musa* gene pool (Pinochet 1996, De Waele and Elsen 2002).

The objective of these technical guidelines is to stimulate interest in screening for resistance and tolerance to nematodes in bananas, and to provide a tried and tested methodology for carrying out such screenings. Comparing genotypes results in indications such as a completely, highly or moderately resistant genotype whether it supports no, little or an intermediate level of nematode reproduction, respectively.

Since 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 screening for resistance and tolerance, and for agricultural scientists with limited experience in nematology. Although researchers may have to choose or modify the methods somewhat, to fit local conditions, it is hoped that these technical guidelines will promote standardization of future efforts in screening for resistance and tolerance to nematodes.

---

## Reference cultivars

Clones against which the new, improved hybrids are to be evaluated for their reaction to nematodes are:

Pisang jari buaya	Resistant to <i>Radopholus similis</i>	ITC0312
Yangambi km 5	Resistant to <i>Radopholus similis</i>	ITC1123
Grande naine	Susceptible to <i>Radopholus similis</i>	ITC1256

## Genotypes to be evaluated

List of genotypes to be evaluated for their resistance and tolerance to various species of nematodes.

Pisang jari buaya*	AA	ITC0312
Calcutta 4	AA	ITC0249
Pisang mas	AA-Sucrier	ITC0653
Pisang Ceylan	AAB-Mysore	ITC1441
Cachaco	ABB	ITC0643
Saba	ABB	ITC1138
FHIA-03	FHIA hybrid	ITC0506
FHIA-18	FHIA hybrid	ITC1319
FHIA-23	FHIA hybrid	ITC1265
FHIA-25	FHIA hybrid	ITC1418
TMB2x9128-3	IITA hybrid	ITC1437
TM3x15108-6 (Pita 16)	IITA hybrid	ITC1417

Additional genotypes suggested by the PROMUSA Nematology working group are:

Grande naine*	AAA	ITC1256
Yangambi km 5*	AAA	ITC1123
Paka	AA	ITC0320
Selangor	AA	ITC1060
Kunnan	AB (Ney poovan)	ITC1034
<i>Musa balbisiana</i>	BB (Honduras)	ITC0247
<i>Musa balbisiana</i>	BB (Tani)	ITC1120
Foconah	AAB (Pome-Prata)	ITC0649
Pisang lemak manis	ABB	ITC1183
FHIA-01	FHIA hybrid	ITC0504

\* Does not apply when evaluating resistance to *R. similis*

INIBAP compiles a list of virus-indexed material from which reference genotypes can be selected. Please contact the IMTP coordinator at INIBAP. For information on how to handle plant material, see Appendix I.



## Establishment of plots

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. The species composition at the selected site should be representative of the species composition of the region.

The infestation level at the site should be investigated by examining roots (Figure 6), not by taking soil samples. If the nematode population present is large enough, at least 100 nematodes/g of fresh roots, the infested field can be used immediately.

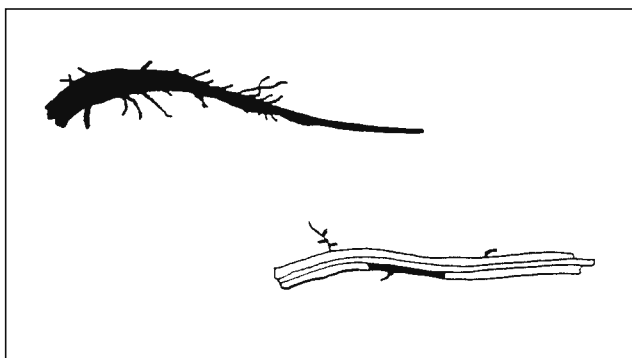


Figure 6. A dead root (top) and a functional root (bottom).

If the initial nematode population is too small, a susceptible genotype can be planted in order to increase the nematode population, or nematode-infected roots can be added when the plants are planted (using macerated infected roots).

## Nematode identification

In general, three types of root nematodes are distinguished.

- Ectoparasitic nematodes remain outside the plant and pierce the outermost plant cell layers with a stylet in order to feed.
- Migratory endoparasitic nematodes invade plant tissues, remain mobile and feed on numerous normal cells inside the plant. The eggs are laid individually either inside or outside the plant. Migratory endoparasites, such as the burrowing nematode *Radopholus similis*, the root-lesion nematodes *Pratylenchus coffeae* and *P. goodeyi*, and the spiral nematode *Helicotylenchus multicinctus* are the most damaging and widespread nematodes attacking bananas.
- Sedentary endoparasitic nematodes also invade plant tissues but adult females become sedentary and feed on a few special cells inside the plant. The eggs are laid together (for instance in a single egg sac) outside the plant. Some sedentary endoparasitic root-knot nematodes (*Meloidogyne* spp.) are also often present in banana roots but their status as pathogens is unclear. The most frequently found species are *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*.

It is very important that prior to the evaluation of new hybrids or selected clones to know exactly the type of nematode present in the site. The identification and preparation of nematodes for microscopy are described in Appendix VII.

## Experimental design

The number of replications should range between 8 and 15. Depending on the site characteristics, the replications should be arranged in a completely randomized design, a randomized complete block design or a split-plot design.

*For more information and advice on experimental design, please contact Dirk De Waele (e-mail: Dirk.DeWaele@agr.kuleuven.ac.be).*

## Agronomic practices

The trial should be managed according to the local agronomic practices of the collaborating organization. All management practices should be applied uniformly over the whole trial site. The nematodes should not be controlled and the data should be collected on the mother plant and first sucker.

## Data to be collected

The interpretation of the data are based on a combination of nematode reproduction data and host plant response data which include the number of nematodes in the roots, the percentage of dead roots and the root necrosis index.

We suggest recording data at the following stages of the:

### *Mother plant*

- EG early growth: 10 to 12 weeks after planting
- RF recently flowered plant (less than 14 days) or plant with emerging flowers
- H harvest

### *Sucker*

- RFMP at the recently flowered stage of the mother plant
- HMP at the harvest of the mother plant

## Disease evolution data

### *Nematode reproduction assessment*

Juveniles and adults of migratory endoparasitic nematodes (including *H. 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 are described in Appendix VIII. The advantage of these methods is that they require little equipment. In field screening experiments, the number of juve-

niles can be used in combination with root-knot gall rating (Kinloch 1990). In the field, the nematode population can only be expressed per root unit.

### *Damage assessment*

The damage of individual plants in a mat can be assessed by measuring root damage, plant growth and yield. Because root development and root decay are genotype-dependent dynamic processes — and are influenced by plant growth and natural senescence, as well as decay caused by abiotic and biotic factors (including nematode damage) — a specific plant development stage should be selected for comparing the sensitivity of different genotypes or of one genotype grown under different conditions. Selecting the recently flowered plant 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 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 the number of completely decayed roots can be estimated by relating the number of root bases to the number of dead and functional roots.

The extent of damage to the roots of a plant are a measure of their health; the lower the damage, the healthier the root system is.

In principle, root damage consists of the following components:

- dead roots, and
- root necrosis of functional roots.

Dead roots are completely rotten or shrivelled whereas functional roots show at least some healthy tissue. The extent of root damage can be expressed as percentage of dead roots, percentage of root necrosis and percentage of root bases with lesions. Appendix IX contains the protocol to assess the damage caused by the nematodes. Table 3 details when data should be collected, and field form 5 in Appendix X is provided to record the data.

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## Agronomic data

Please refer to page 13 for a complete list of variables. Table 3 details when data should be collected, and field form 6 in Appendix XI are provided to help you record the data.

**Table 3.** Timetable for recording disease evolution and agronomic data

Type of data	Mother plant			Sucker	
	EG	RF	H	RFMP	HMP
<b>Disease evolution data</b>					
Nematode reproduction	X	X	X	X	X
Damage assessment	X	X	X	X	X
<b>Agronomic data</b>					
Time from planting to shooting	At flowering				
Height of pseudostem	At flowering				
Height of following sucker	At flowering				
Number of functional leaves	At flowering		X	X	X
Plant crop cycle				X	
Girth of pseudostem				X	
Weight of bunch				X	
Number of hands in bunch				X	
Fruit characteristics				X	
Number of fruits				X	
Weight of fruit				X	
<b>Mother plant</b>					
<b>EG</b>	early growth: 10 to 12 weeks after planting				
<b>RF</b>	recently flowered plant (less than 14 days) or plant with emerging flowers				
<b>H</b>	harvest				
<b>Sucker</b>					
<b>RFMP</b>	at the recently flowered stage of the mother plant				
<b>HMP</b>	at the harvest of the mother plant				

## Environmental data

It is also necessary to monitor the climatic conditions in a weather station as close as possible to the experimental site. Daily readings should be taken as early as possible at the same time every day. For your convenience, field form 7 in Appendix XI is provided to record environmental data.

## Management practices

Details of fertilizer application, nematode/weevil control measures and irrigation/drainage management should be recorded.

# Appendices



## Appendix I. Handling plant material

Clones will be supplied by the INIBAP Transit Centre (ITC), preferably as proliferating tissue cultures, to laboratories with tissue culture facilities. Collaborators at these sites will be responsible for producing plantlets for use in the experiments. To collaborators who do not have tissue culture facilities, clones can be supplied as rooted plantlets derived from tissue culture. In both cases plantlets should be grown in plastic bags/pots filled with pasteurized soil or potting mix.

### Proliferating tissue cultures

Proliferating shoot-tip cultures are distributed by ITC in sterile single use transparent polystyrene vials, sealed with a screw cap. The cultures are grown in semi-solid multiplication medium. They should be sub-cultured onto fresh medium as soon as possible upon arrival.

Proliferating shoot-tip cultures can be used for further multiplication and regeneration of rooted plantlets (suitable for transfer to soil) through sub-culturing on an appropriate growth medium. Relatively high levels of cytokinin in the medium tend to stimulate the formation of multiple shoots and buds whereas the development of individual plants is induced when propagules are transferred to a medium without or with low cytokinin concentration.

The composition of media used for *Musa* micropropagation at the INIBAP Transit Centre is described in Table 1.

**Note:** Aseptic conditions must be maintained throughout the manipulations.

**Table 1.** Composition of the modified Murashige and Skoog nutrient medium, used for *in vitro* propagation of bananas and plantains at INIBAP Transit Centre

		mg/L
Macronutrients	NH <sub>4</sub> NO <sub>3</sub>	1650
	KNO <sub>3</sub>	1900
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	440
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	370
	KH <sub>2</sub> HPO <sub>4</sub>	400
Micronutrients	H <sub>3</sub> BO <sub>3</sub>	6.18
	MnSO <sub>4</sub> ·H <sub>2</sub> O	16.90
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.60
	KI	0.83
	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.24
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.024
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025

**Table 1. (cont.)**

		mg/L
Iron	FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.80
	Na <sub>2</sub> -EDTA·2H <sub>2</sub> O	37.22
Vitamins		
	Glycine	2.00
	Thiamine hydrochloride	0.10
	Nicotin acid	0.50
	Pyridoxine hydrochloride	0.50
Antioxidant	Ascorbic acid	10.00
Carbon source	Sucrose	30000
Gelling agent	Gelrite®	2000
	or Agar	8000
Growth regulators	N <sup>6</sup> -benzylaminopurine	2.25*
		0.225**
	Indole-3-acetic acid	0.175

Adjust the pH of the medium to 5.8 before autoclaving.

\* for multiplication

\*\* for regeneration.

## Multiplication

The desired numbers of proliferating propagules can be obtained through repeated sub-culturing the cluster as follows:

- Unscrew the culture vial and flame the brim for a few seconds.
- Gently remove the cluster of shoots and buds out of the vial.
- Separate a cluster into smaller groups of 2 to 3 micro-shoots and/or buds.
- Trim away superfluous corm tissue and blackened tissue. Shorten shoots to a size of 5 to 7 mm in height.
- Transfer each excised group of shoots/buds to fresh pre-sterilized multiplication medium.
- Incubate the cultures at a temperature of  $28 \pm 2^\circ\text{C}$  with a light intensity of 1000-3000 lux, and a 16-hour photoperiod.
- After 4 to 6 weeks, when new lateral shoots and/or buds are produced, the procedure can be repeated.

**Note:** The multiplication rate depends on the genotype of the cultivar and is influenced by the composition of the medium (particularly cytokinins), the explant size and age of culture.



## Regeneration

The aim of this step is to produce individual rooted plantlets for establishment in soil.

- Unscrew the culture vial and flame the brim for a few seconds.
- Gently remove the cluster of shoots and buds from the vial.
- Subdivide proliferating clusters into individual shoots.
- Remove superfluous corm tissue and blackened tissue. Trim away the upper part of the shoots to a height of 10 mm.
- Place each trimmed shoot onto fresh pre-sterilized regeneration medium, inducing shoot elongation and promoting root growth.
- Grow cultures at an ambient temperature of  $28 \pm 2^\circ\text{C}$  and in a 12 to 16-hour light cycle. High light intensity between 5000 and 10000 lux is recommended.
- If new lateral shoots or buds arise, repeat subculturing until individual rooted shoots are obtained.
- Keep rooting shoots in culture for 4 to 6 weeks.

*In vitro* plants that are 5 to 10 cm tall, with at least four leaves and with a well-developed root system are ready for soil planting.

## Handling of *in vitro* rooted plantlets

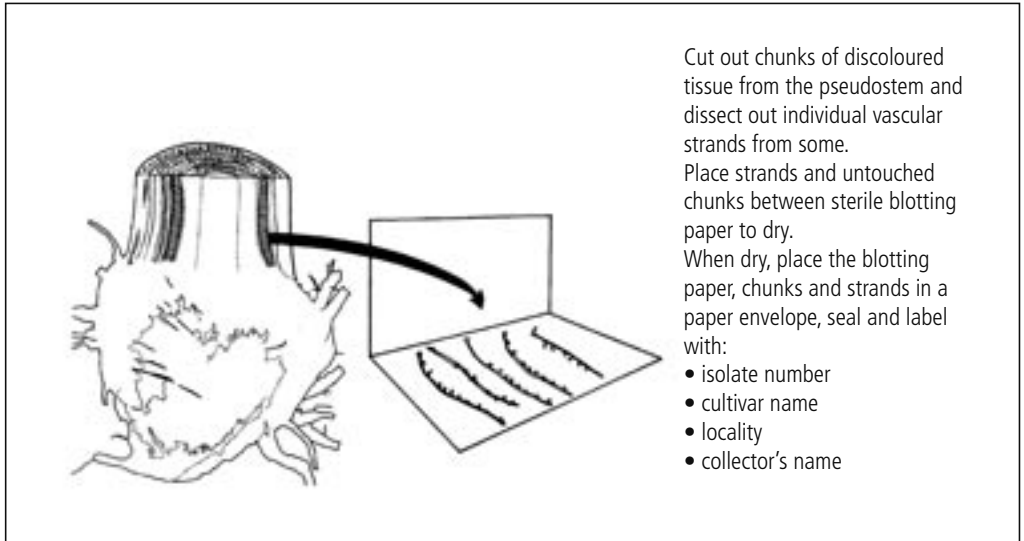
*In vitro* rooted plantlets are distributed as sterile cultures, grown on a hardening medium in watertight Cultu saks<sup>®</sup>. Plantlets that are 5 to 10 cm tall and have well developed roots are ready for planting in pots. If plantlets are smaller or if transplanting is not immediately possible, it is advisable to place the plantlets in the Cultu saks<sup>®</sup> in an upright position under sufficient light (not direct sunlight), at a temperature of between 20 and 30°C. Under these conditions the plantlets can be kept for a few weeks.

To transplant rooted plantlets to the soil requires some care. Please proceed as follows or use your own proven method.

- Cut open each Cultu sak<sup>®</sup> chamber at one vertical edge.
  - Carefully remove the plantlet from the Cultu sak<sup>®</sup> by holding the base gently with blunt-end forceps. Place the plantlet on the palm of your hand.
  - Remove the culture medium adhering to the roots and leaves by placing the plantlet in a container of water (bucket) and shaking gently. Do not damage the stem nor the root system.
  - Transplant the plantlet to plastic pots or bags (15-cm diameter) filled with a pasteurized mixture 30:70 peat:sand of which the upper 2 to 3 cm are fine (sifted). The upper roots should be covered by 2 to 3 cm of soil.
-

- After transplanting, water plantlets immediately.
  - Keep the plants in a high humidity atmosphere.
    - A simple humidity chamber can be constructed by enclosing a wooden frame in strong transparent plastic. The humidity chamber (about 40 to 60 cm high) is placed over the pots in a shaded area where the temperature is kept at 25 to 32°C.
    - The humidity inside the chamber is maintained by spraying water regularly to saturate the air. To minimize heat build up inside the chamber, leave a 2 to 3 cm opening at the base to allow the air to circulate.
    - During the first week after transplanting, mist the humidity chamber twice a day to saturate the atmosphere. This is very important as low relative humidity at this stage could easily destroy the plantlets. Water the plants once a day with a little tap water.
    - One week after transplanting, spray the humidity chamber and the plants once a day.
  - One month after transplanting, remove the plants from the humidity chamber.
  - Keep the plantlets on a raised surface (not directly on the ground) in a nursery in a shaded area until they are about 30 cm tall (2 to 3 months before establishment in the field).
  - Re-potting into a larger pot may be required.
  - Transplant the plants into the field during the wet season, but not later than six weeks before the onset of the dry season.
-

## Appendix II. Preparation of *Fusarium* samples for analysis of vegetative compatibility group



**Figure 1.** Preparation of samples for mailing (Courtesy of Dr. K. Pegg, QDPI).

Samples of infected pseudostem or corm tissue from diseased plants should be prepared (Figure 1) and sent to a recognised laboratory for analysis.

If all plants of an accession become infected, samples should be taken from 4 to 5 randomly selected plants. If only a few plants of an accession become infected, samples from all diseased plants should be sent for analysis. Eight strands per plants should be prepared.

Loss of viability of the samples can be minimized if you prepare them soon after collection and send them as soon as the excised strands are dry (approximately three days in sterile blotting paper).

You should send them in paper envelopes (plastic ones cause transpiration and promote the growth of bacterial contaminants). To preserve the viability of the fungus inside the vascular strands, the strands should not get too hot, too cold or too wet. Samples should be kept in paper at all times and dried on the bench under normal laboratory conditions **not in ovens**.

(Protocol courtesy of Dr Natalie Moore, QDPI, Australia)







## Appendix IV. Identification of *Mycosphaerella* pathogens

The three pathogens *Mycosphaerella fijiensis* (anamorph *Paracercospora fijiensis*), *M. musicola* (anamorph *Pseudocercospora musae*) and *M. eumusae* (anamorph *Pseudocercospora eumusae*) are respectively the causal agents of black leaf streak disease, Sigatoka disease and eumusae leaf spot disease (Jones 2000, Crous and Mourichon 2002). The pathogens are not only difficult to distinguish on the basis of symptom expression (plates 1 to 6, p. 40), but their sexual stages (teleomorphs) are also similar. However, the species can be identified on the basis of morphological differences (plates 7 to 12, p. 41) between their asexual stages (anamorphs) whether they are directly observed on diseased leaves or after being isolated and cultured. The morphological characteristics of the three pathogens are presented in table 2. Care should be taken not to confuse these pathogens with other fungal species that also attack the foliage of bananas (Jones 2000, Wardlaw 1972).

**Table 2.** Morphological characteristics of the anamorphs of *Mycosphaerella*

Species (anamorph)	Conidiophores	Conidia
<i>Paracercospora fijiensis</i>	(Plate 7) First appearance at early streak stages [Fouré's stages 2 to 3 (Fouré 1982)]	(Plate 8) Obclavate to cylindro-obclavate, straight or curved, hyaline to very pale olivaceous, 1 to 10 septate, distinct basal hilum (scar)
	Mainly lower leaf surface	
	Emerge singly or in small groups (2 to 6), sporodochia and stromata absent	Between 30 to 132 x 2.5 to 5 µm
	Straight or bent geniculate, pale to light brown 0-5 septate, occasionally branched, slightly thickened spore-scars (between 16.5 to 62.5 x 4 to 7 µm)	
<i>Pseudocercospora musae</i>	(Plate 9) First appearance at spot stages (Brun's stage 4)	(Plate 10) Cylindric to obclavato-cylindric, straight or curved, pale to very pale olivaceous, 0 to 8 septate, no distinct basal hilum
	Abundant on both leaf surface	
	In dense fascicles (sporodochia) on dark stromata	Between 10 to 109 x 2 to 6 µm
	Straight, hyaline, mostly without septation, and geniculation; no spore scars (between 5 to 25 x 2 to 5 µm)	

**Table 2. (cont.)**

Species (anamorph)	Conidiophores	Conidia
<i>Pseudocercospora eumusae</i>	(Plate 11) First appearance at spot stages  Mainly on the upper leaf surface  Pear-shaped, immersed, more or less erumpent, ostiolated when young but often acervular-like when mature (31 to 42 µm)	(Plate 12) Fusiform, hyaline, cylindrical and curved, 3 to 5 septate  Between 21.2 to 41.6 x 2.5 µm

Adapted from Wardlaw 1972, Carlier *et al.* 2000, and Crous and Mourichon 2002.

The procedure to isolate the pathogens is explained below and illustrated in Figure 2.

## 1. Sampling of diseased tissue

For *in situ* microscopic observations, the specimens should be from leaves at the early streak stages for *P. fijiensis* (plate 1), and spot stages for *P. musae* (plate 3) and *P. eumusae* (plate 5). For fungal isolation and *in vitro* microscopic observations the specimens should come from completely necrotic leaves regardless of the species (plates 2, 4 and 6). The leaf should be thoroughly dried between sheets of newspaper.

## 2 and 3. Tissue clearing and *in situ* microscopic observations

The lesions are cleared in a solution of KOH 10% overnight and washed five times in water for 10 minutes each time. The conidiophores associated with these lesions can then be directly observed on slides without staining. To observe conidia, cleared tissues are stained for 1 minute with a solution of 0.5% blue cotton and 1:1 lactic acid glycerol, and washed in water.

## 4 and 5. Ascospore discharge and cloning

Necrotic banana leaves are dried at room temperature for 48 hours and then soaked in distilled water for 15 minutes. Leaf sections are secured to the underside of the lids of Petri dishes containing water agar at 3%. Ascospores will discharge overnight onto the agar surface (the ascospores of the three *Mycosphaerella* species have two cells and measure between 12 to 18 µm x 2.5 to 4.5 µm). The next morning, ascospores are transferred one by one to a fresh PDA medium. If no ascospore is obtained, leaf sections can be incubated for 48 hours on wet filter paper in a Petri dish, soaked in distilled water for 5 minutes and then transferred onto the lids of Petri dishes as described above. Cultures are incubated at 25°C.



## 6 and 7. *In vitro* sporulation and microscopic observations of conidia

Conidial sporulation is induced by culturing small pieces of mycelia on modified V8 medium (100 ml V8, 0.2 g CaCO<sub>3</sub>, 20 g agar per litre of medium, pH 6). Cultures are incubated at 20°C for 10 to 14 days under 60 μmoles m<sup>-2</sup> s<sup>-1</sup> of continuous and cool-white fluorescent light. Cultures are then scraped with a scalpel and conidia are suspended in a solution of blue cotton directly on the slide for microscopic observation.

## 8. Conservation

Mycelium fragments from developing colonies are placed in 15% glycerol, kept for 2 hours at 4°C and then transferred to a freezer for long term storage at -80°C.

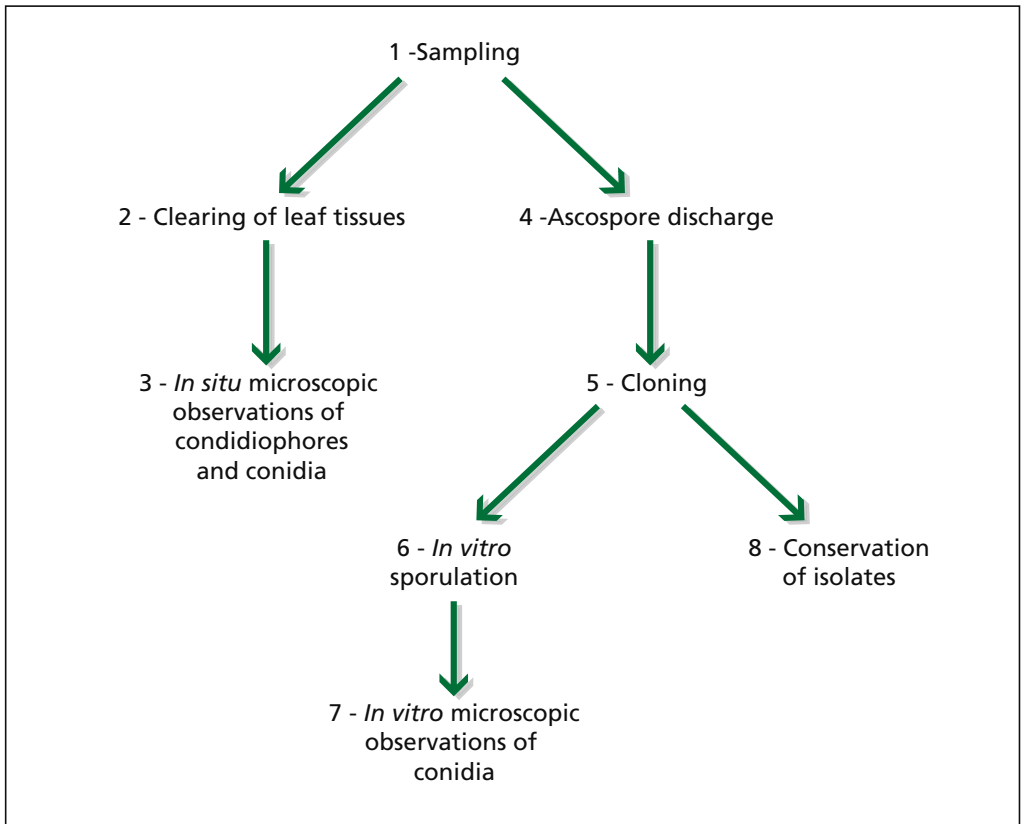


Figure 2. Flow chart for identification of *Mycosphaerella* leaf spot pathogens.

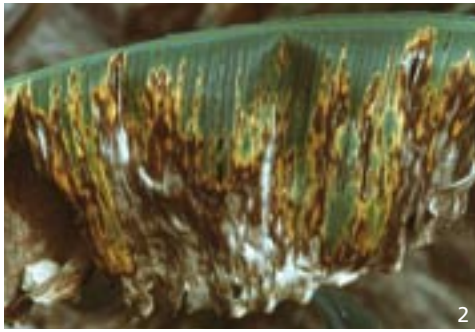
(Protocol courtesy of Marie Françoise Zapater, Jean Carlier and Xavier Mourichon. CIRAD, TA 40/02, avenue d'Agropolis, 34398, Montpellier, France, jean.carlier@cirad.fr)

Plates 1-6. Leaf symptoms

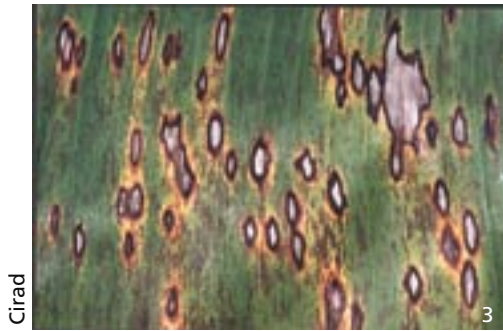


Cirad

Cirad



Black leaf streak disease  
(*Mycosphaerella fijiensis*).



Cirad

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Sigatoka disease  
(*Mycosphaerella musicola*).

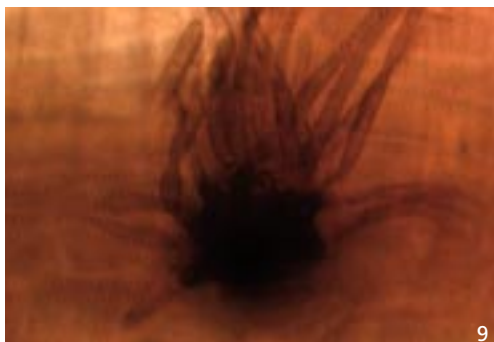


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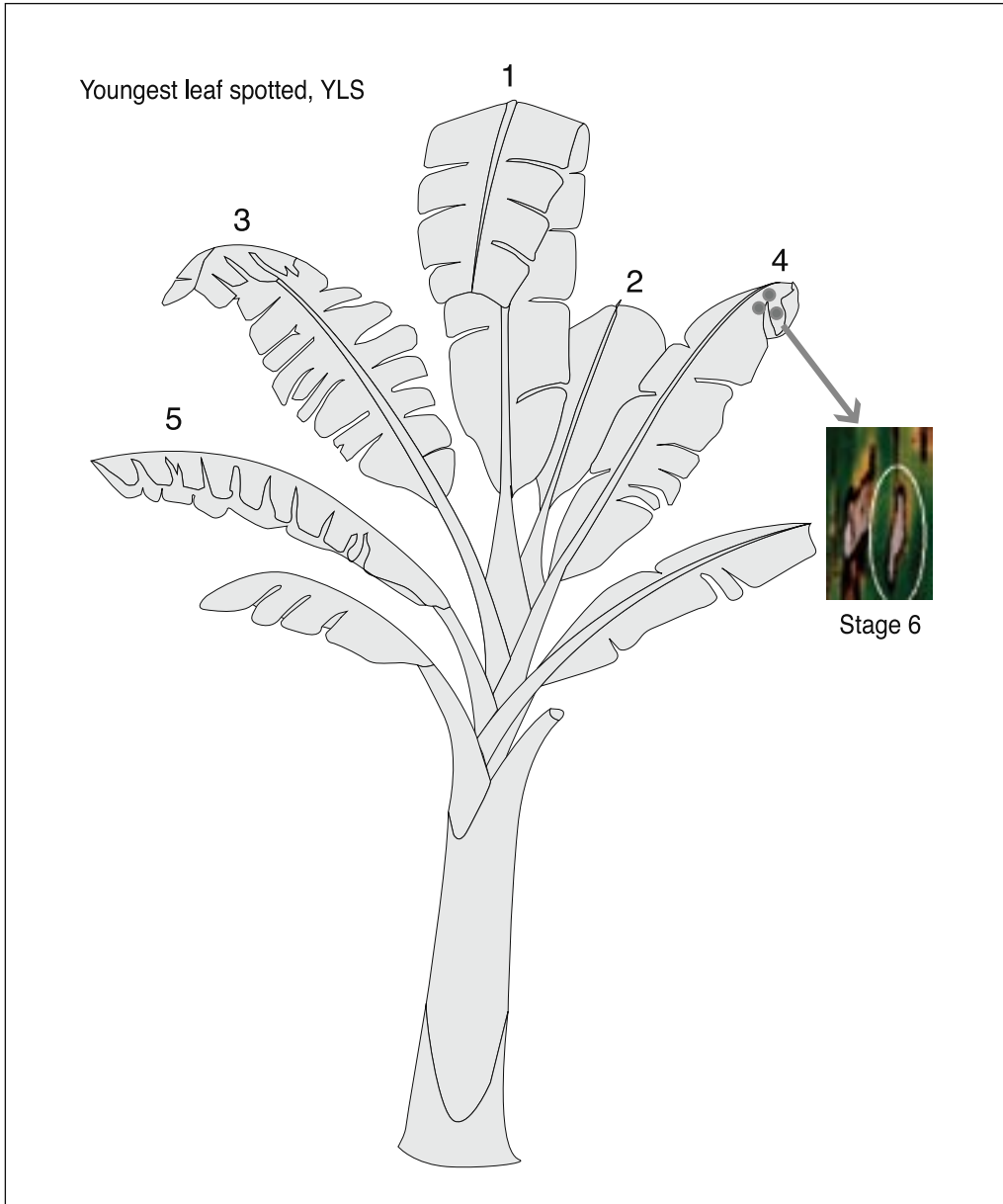
D. Jones



Eumusae leaf spot disease  
(*Mycosphaerella eumusae*).

**Plates 7-12. Anamorphs of banana leaf spot pathogens****Conidiophores**Black leaf streak disease (*Paracercospora fijiensis*).**Conidia**Sigatoka disease (*Pseudocercospora musae*).Eumusae leaf spot disease (*Pseudocercospora eumusae*).

## Appendix V. Youngest leaf spotted



YLS = leaf showing at least 10 spots with necrotic/dry center.



## Appendix VII. Identification and preparation of endoparasitic nematodes

Migratory endoparasitic nematodes can be identified on the basis of a combination of morphological characters. The most important distinguishing characteristics are described in Table 3 and Figures 3 to 5.

**Table 3.** Morphological characters of banana migratory endoparasitic nematodes.

Characteristics	<i>R. similis</i>	<i>P. coffeae</i>	<i>P. goodeyi</i>	<i>H. multicinctus</i>
<b>Occurrence of males</b>	rather rare	common	common	common
<b>Position of vulva</b>	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
<b>Number of genital branches in females</b>	2 equally developed	only the anterior branch is developed	only the anterior branch is developed	2 equally developed short, somewhat cylindrical
<b>Shape of the tail in females</b>	slightly 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
<b>Shape of the tail in males</b>	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 finger-like projection, bursa extending until tail tip

Sexual dimorphism in the 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.

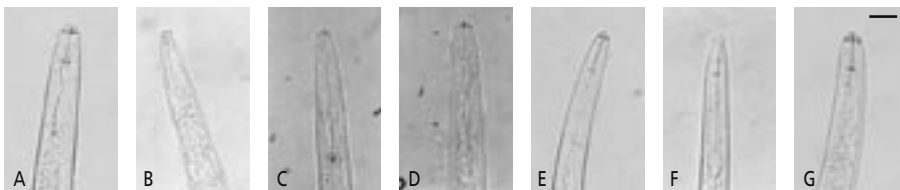
*Helicotylenchus multicinctus* can be recognised as the bodies of both females and males have distinct annules and when killed and fixed they arcuate to a C-shape.

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

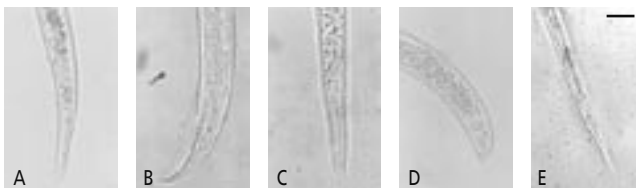
Females of *Meloidogyne* spp. are sedentary (spherical body with a slender neck) while the males and juveniles are vermiform. Males are rare. The head region is high, cone shaped, not offset and clearly annulated; strong stylet and cephalic sclerotization; the tail is short and hemispherical, and the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

absent. The shape of the head in juveniles is similar to males'; cephalic sclerotization is weak; tail tapering.

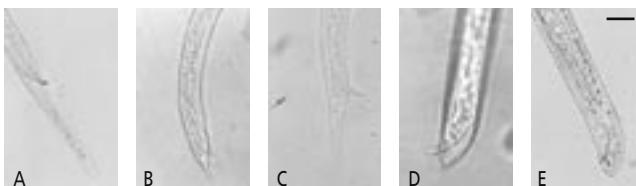
Because of the extensive morphological variation among and within *Meloidogyne* spp. and the existence of host plant races, accurate identification of these nematodes is difficult. Guidelines for the preparation of the perineal patterns of females for light microscope observation and for conducting the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



**Figure 3.** Head region of *Radopholus similis* (A: female; B: male), *Pratylenchus coffeae* female (C), *Pratylenchus goodeyi* female (D), *Helicotylenchus* female (E) and *Meloidogyne* (F: J4-stage-juvenile; G: male). Bar = 20 microns.



**Figure 4.** Female tail region of *Radopholus similis* (A), *Pratylenchus coffeae* female (B), *Pratylenchus goodeyi* (C), *Helicotylenchus multicinctus* (D) and J4-stage-juvenile tail region of *Meloidogyne incognita* (E). Bar = 20 microns.



**Figure 5.** Male tail region of *Radopholus similis* (A), *Pratylenchus coffeae* (B), *Pratylenchus goodeyi* (C), *Helicotylenchus multicinctus* (D) *Meloidogyne incognita* (E). Bar = 20 microns.

## **Protocol 1. Preparation of nematodes for identification using a 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 (Seinhorst 1966), transferred to glycerol by the ethanol-glycerol method (Seinhorst 1959) and mounted on glass slides with the wax-ring method (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 to 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**

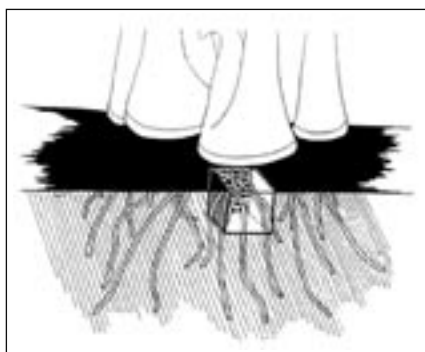
- Heat the 1.5 cm diameter tip of a copper tube in a flame
  - dip the hot tip in paraffin wax
  - when the paraffin wax has melted, press the tip on a glass slide making a thin wax ring which will soon solidify
  - put a small drop of glycerol in the middle of the wax ring
  - transfer the nematodes 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).
-

## Appendix VIII. Protocols for estimating nematode reproduction

### Protocol 2. 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 6). Take only roots from the selected plant; do not include roots from adjacent plants. In the case of a young plant, it is often easier to remove the complete sucker from the mat (Figure 7).



**Figure 6.** 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 7.** Removal of a sucker from a mat.

Roots should be removed at a specific plant stage (STG). Specific plant stages are:

- EG early growth: 10 to 12 weeks after planting
- RF recently flowered plant (less than 14 days) or plant with emerging flowers
- 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 roots from the soil
- carefully wash the soil from the roots with tap water
- cut the roots into 10 cm long pieces of and dry with paper tissue

- if the total root system has been removed, determine the total root fresh weight and cut the roots in 1-cm pieces
- 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 of distilled water in a kitchen blender
- macerate the roots 3 times for 10 sec (separated by 5 sec intervals)
- pour the macerated suspension through 250, 106 and 40- $\mu$ m sieves and rinse the sieves with tap water
- using distilled water, collect in a beaker the nematodes from the 40- $\mu$ m sieve.

## **3. Assessment of the nematode population**

- Using distilled water, dilute to 200 ml the nematode suspension in a graduated cylinder
  - 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.
-

## **Protocol 3. Estimation of the reproduction of root-knot nematodes**

### **1. Determination of root fresh weight**

- Remove the roots from the soil
- carefully wash with tap water the soil from the roots
- cut the roots in 10 cm long pieces and dry them with paper tissue
- if the total root system has been removed, determine the total root fresh weight and cut the roots in 1 cm pieces
- take a subsample of 5 g
- add 100 ml of 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 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.
-

## Appendix IX. Protocol for assessing the damage caused by nematodes

### Protocol 4. Root damage assessment

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

EG early growth: 10 to 12 weeks after planting

RF recently flowered plant (less than 14 days) or plant with emerging flowers

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 and plant number of the sampled plant. In addition to the plant stage, also note the genotype, the circumference or girth of the pseudostem at 1 m, the plant height and the number of standing leaves. Plant height is the distance from the pseudostem base to the youngest leaf axil. At harvest, record the bunch weight.

*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 7). Take only roots from the selected plant; do not include roots from adjacent plants. In the case of a young plant, it is often easier to remove the complete sucker from the mat (Figure 7).

*Step 2.* Divide the collected roots into two categories:

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

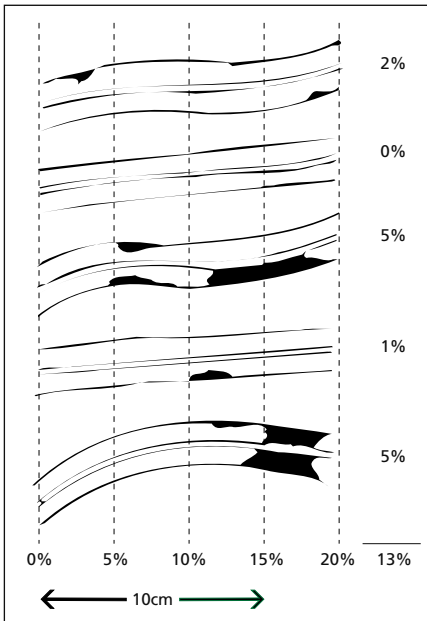
and count the number of roots in each category.

*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 condition of the secondary and tertiary roots attached. These roots are referred to as feeder roots. Then reduce the length of the five selected functional roots to 10 cm and slice the roots length-wise (Figure 8).

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 halves together. Record the necrosis of the individual roots (RN1 to RN5). The sum is the total root necrosis of the sample (Total RN).

Root-knot nematodes: The presence of root-knot nematodes (RK) can be observed either externally as galls (Figure 9) or internally as pit-like structures (Figure 10) on the roots.



**Figure 8.** Example of scoring five length-wise sliced banana roots for root necrosis (% of root cortex surface showing necrosis) caused by migratory endoparasites.



**Figure 9.** Galling of banana roots caused by root-knot nematodes.



**Figure 10.** Banana roots infected with swollen root-knot nematode females.

Photographs courtesy of Dr. D. De Waele, KULeuven.

## Appendix X. Damage caused by nematodes

Field form 5. Root damage assessment

(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP.)

Site: .....

Surveyor: .....

Planting date: .....

ITC #: .....

Experimental design: .....

Unique identifier: .....

ITC #	Replication	Plant #	Roots		RN					Total RN	RK
			DE	OK	1	2	3	4	5		

Root damage assessment:  
 Count all dead roots (DE)  
 Count all functional roots (OK)  
 Slice primary roots length-wise

Score % root necrosis (RN)  
 Score root-knot nematodes (RK):  
 0=absent  
 1=present





Field form 7. Environmental data to be collected at each site from planting to harvest

(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP.)

Site: .....

Surveyor: .....

Latitude: ..... Longitude: ..... Altitude: .....

Date to be collected	Week				
	1	2	3	4	5
Rainfall (mm)					
Highest temperature (°C)					
Lowest temperature (°C)					
Average temperature (°C)					
Highest relative humidity (%)					
Lowest relative humidity (%)					
Average relative humidity (%)					
Number of days with rain					
Number of hours during which relative humidity $\geq$ 90%					

Note: if the experimental design is RCBD, the table should be presented by block.

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