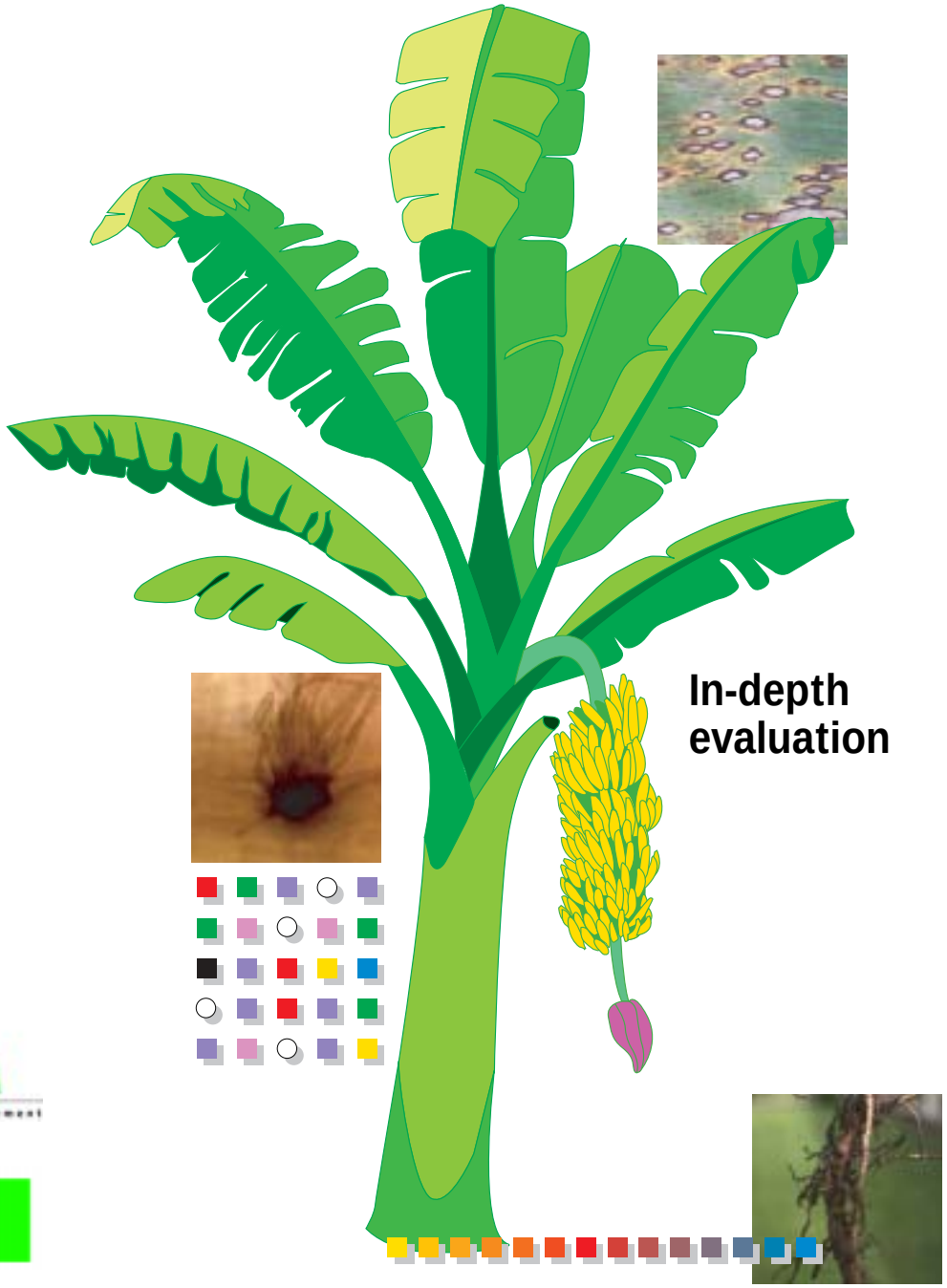
INIBAP Technical Guidelines 6

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

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.

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

The International Plant Genetic Resources Institute is an autonomous international scientific organization, supported by the Consultative Group on International Agricultural Research (CGIAR). IPGRI's mandate is to advance the conservation and use of genetic diversity for the well being of present and future generations. IPGRI's headquarters is based in Rome, Italy, with offices in another 19 countries worldwide. It operates through three programmes: (1) the Plant Genetic Resources Programme, (2) the CGIAR Genetic Resources Support Programme, and (3) the International Network for the Improvement of Banana and Plantain (INIBAP).

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Financial support for the Research Agenda of IPGRI is provided by the Governments of Australia, Austria, Belgium, Brazil, Bulgaria, Canada, China, Croatia, Cyprus, Czech Republic, Denmark, Estonia, F.R. Yugoslavia (Serbia and Montenegro), Finland, France, Germany, Greece, Hungary, Iceland, India, Ireland, Israel, Italy, Japan, Republic of Korea, Latvia, Lithuania, Luxembourg, Malta, Mexico, Monaco, the Netherlands, Norway, Pakistan, the Philippines, Poland, Portugal, Romania, Slovakia, Slovenia, South Africa, Spain, Sweden, Switzerland, Thailand, Turkey, the UK, the USA and by the Asian Development Bank, Common Fund for Commodities, Technical Centre for Agricultural and Rural Cooperation (CTA), European Union, Food and Agriculture Organization of the United Nations (FAO), International Development Research Centre (IDRC), International Fund for Agricultural Development (IFAD), International Association for the promotion of cooperation with scientists from the New Independent States of the former Soviet Union (INTAS), Interamerican Development Bank, United Nations Development Programme (UNDP), United Nations Environment Programme (UNEP) and the World Bank.

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Citation:

Carlier J., D. De Waele and J.V. Escalant. 2002. Global evaluation of *Musa* germplasm for resistance to Fusarium wilt, *Mycosphaerella* leaf spot diseases and nematodes. (A. Vézina and C. Picq, eds). INIBAP Technical Guidelines 6. The International Network for the Improvement of Banana and Plantain, Montpellier, France.

INIBAP ISBN: 2-910810-52-6

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

In-depth 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 in-depth evaluations are concerned. Technical Guidelines for performance 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.

Contents

1. Introduction

Fusarium wilt, *Mycosphaerella* leaf spot diseases and nematodes are recognised as the most important pests and diseases of bananas[1]. This document describes protocols for the in-depth evaluation of resistance to these pathogens and parasites. Contrary to performance evaluations, which use a simplified protocol to obtain information on cultivar/hybrid performance under local conditions as well as basic information on disease resistance and tolerance (INIBAP's Technical Guidelines No. 7), in-depth evaluations are more complete disease resistance evaluations carried out on a smaller number of sites. These sites are used to screen new, improved hybrids and, if requested by breeding programmes, parental breeding lines. They can also be used to conduct basic research on the pathogen and its relationship with its host, such as epidemiological studies and host-pathogen relationships for different strains. The protocols in this guide apply only to evaluations of the host plant's resistance and tolerance, adaptability and productivity.

The guidelines have been written with the collaboration of the Fusarium, Sigatoka and Nematology working groups of PRO*MUSA* 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.

1 Throughout the text, bananas is used for bananas and plantains.

2. Technical guidelines for the 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 Suzanne Sharrock at INIBAP (e-mail: s.sharrock@cgiar.org). For information on how to handle plant material, see Appendix I.

Establishment of plots

The selected site should have a severe natural infestation of Fusarium wilt. Edaphic factors such as soil type and soil pH should be determined. The soil should be analysed for mineral content and the cropping history of the site should also be documented.

If they are low, inoculum levels of *Foc* should be increased by growing a crop of appropriate susceptible cultivars on the site prior to establishing the trial. To avoid contamination with other strains, it is recommended that infected plants and soil from other sites not be introduced into the evaluation site. Diseased pseudostems/corms should be cut up after harvest and incorporated into the

soil. Before planting the trial, the site should be deeply ploughed and ripped to evenly distribute the inoculum. The soil should be tested for nematodes and treated if severe infestations are found.

Planting should be done in shallow furrows or basins. These should be filled with soil once the plants are established. For successful establishment, plants will need to be monitored to ensure that the soil in the root zone remains moist. Ideally, an irrigation system should be installed. Depending on local requirements and the results of soil analysis, fertilizer should be added to boost growth in the early stages. During establishment, measures should be taken to prevent losses from weevil borers.

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.

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).

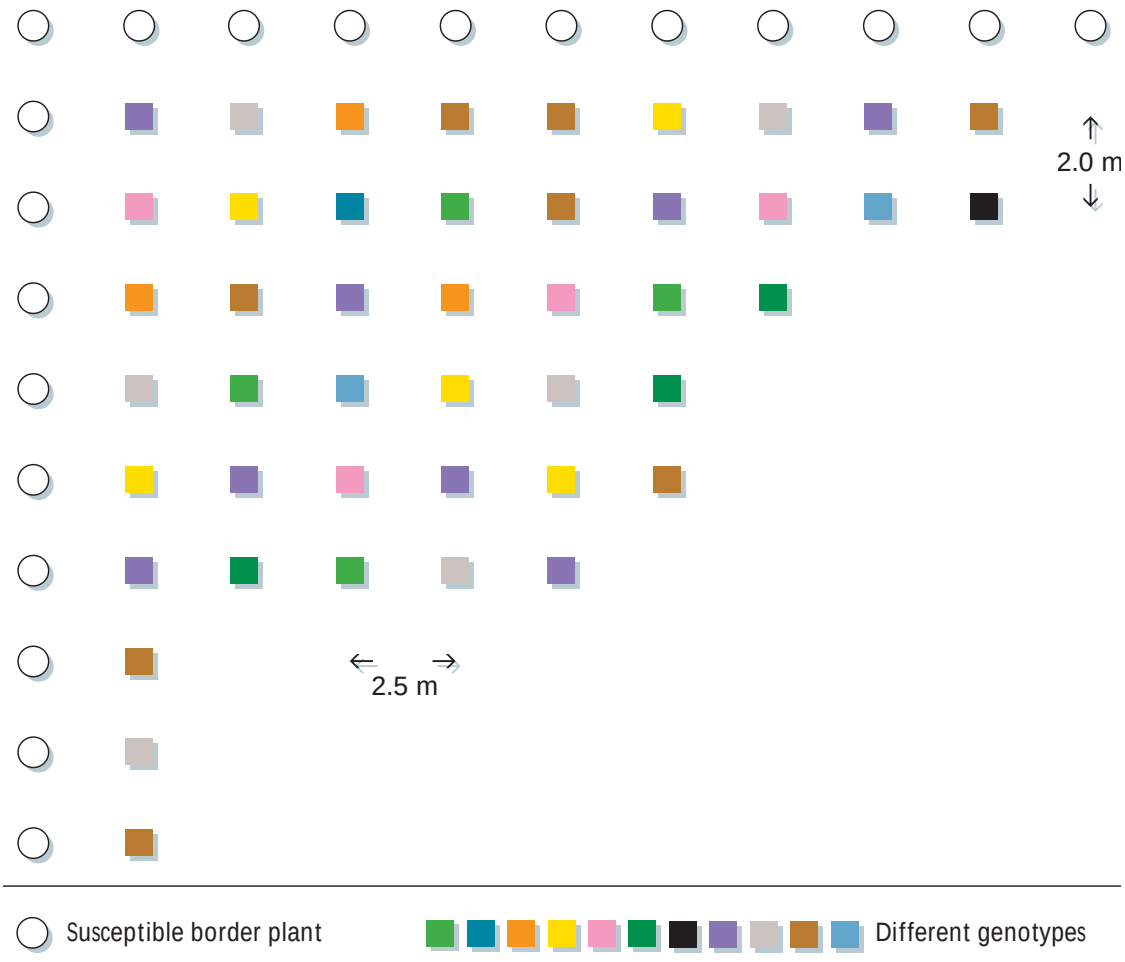


Figure 1. Layout of the completely randomized design for the evaluation of Fusarium wilt.

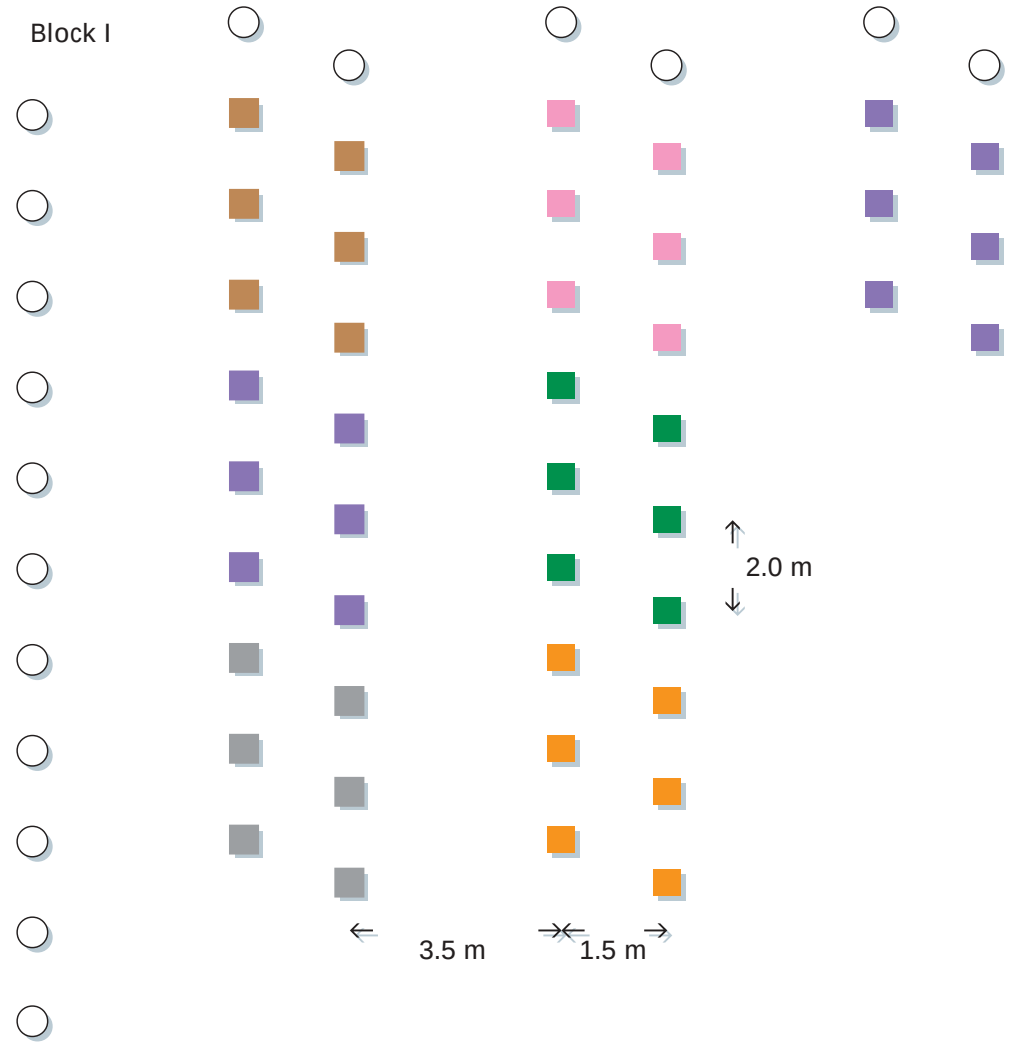


Figure 2. Layout of the randomized complete block design for the evaluation of Fusarium wilt.

Agronomic practices

The trial should be managed according to the agronomic practices of the National Agricultural Research Systems (NARS) or the institute. All management practices should be applied uniformly over the whole trial site. The data should be collected on the mother plant and first sucker. Only the first sucker should be allowed to develop. Other suckers should be removed as they appear. Fungicides should not be applied.

Data to be collected

Disease evolution data

Monthly readings from three months after planting until harvest will give the rate of disease development. Close to flowering time, the fields should be inspected weekly in order to accurately determine the date of flowering. External symptoms may develop between flowering and harvest.

External symptoms

Field form 1 in Appendix III is provided to record the data (1 = symptoms are absent; 2 = symptoms are present) on the following four external symptoms.

Yellowing of foliage (YF)

Splitting of pseudostem base (SP)

Changes in new leaves (CHN)

The changes are: irregular pale margins, narrowing of lamina, burning plus ripping of lamina and the latter becoming more erect.

Petiole collapse (PC)

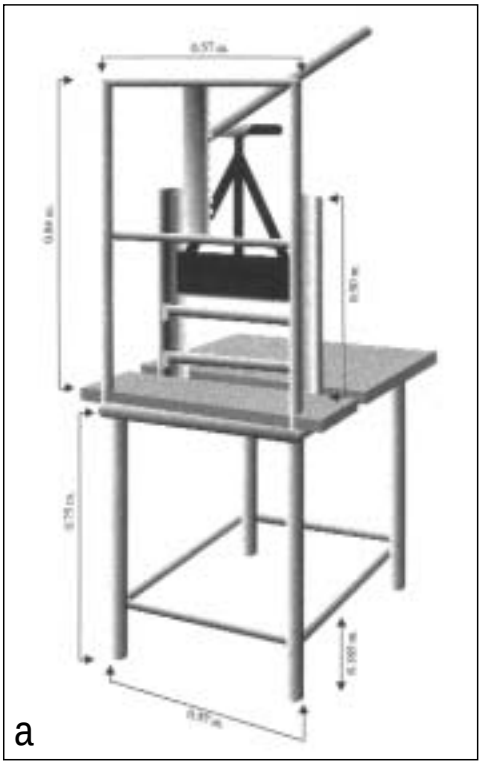
Internal symptoms

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.

For the first sucker, internal symptoms in the pseudostem and the corm should be collected. 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.

Field forms 2 and 3 in Appendix III are provided to record the data on internal symptoms.

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



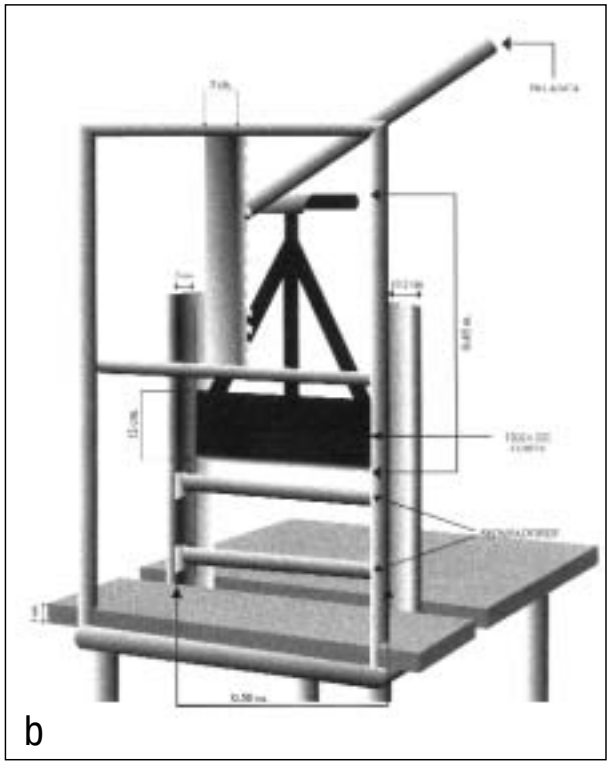


Figure 3. Guillotine for cutting corms.
3a. Global view of the guillotine.
3b. Details and measurements of the guillotine.
3c. Cutting area showing the blade and the notches which support the iron bars used to define the width of the slice.
3d. Detailed view of the lever and the rack.
3e. The guillotine in action.
(Photographs and design of the guillotine courtesy of Dr Mauricio Rivera, FHIA)

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-CNPMF).

Agronomic data

The following agronomic traits should be recorded. Table 1 details when data should be collected, and field forms 7 and 8 in Appendix XIV are 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.

Fruit characteristics

Length, diameter and weight of individual fingers should be taken for the third and seven hands. For varieties with a small number of hands (e.g. plantains), these measurements should be made on the second oldest and second youngest hands.

Table 1. Timetable for recording disease evolution and agronomic data

Type of data	Growing phase (from 3 months after planting)	Shooting phase	Harvest
Disease evolution data			
External symptoms	Once a month	Once a month	
Internal symptoms			X
Agronomic data			
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
Fruit characteristics			X
Number of fruits			X
Weight of fruit			X

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 9 in Appendix XIV is provided to record environmental data.

Management practices

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

3. Technical guidelines for the 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 Suzanne Sharrock at INIBAP (e-mail: s.sharrock@cgiar.org). For information on how to handle plant material, see Appendix I.

Establishment of plots

The experimental fields must be established in areas where there is sufficient presence of the pathogen. Moreover, the field layout must intersperse susceptible clones between the plots. 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

The experiment is a randomized complete block design with four to six clones per plot and five replications. Each plot should be surrounded by a row of susceptible border plants. The clones are independently randomized within each of the five replications. One possible example of a field layout is shown in Figure 4. The layout of blocks in the field should aim to minimize variability (e.g. soil changes, such as pH). There should be a 2.5-meter space between plants in each row and three meters between rows.

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

Agronomic practices

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

Data to be collected

Disease evolution data

Evaluating resistance to *Mycosphaerella* leaf spot diseases requires knowledge of the stages of both the process of leaf unrolling (Appendix V) and symptom development. Readings for the evaluation of resistance should begin three

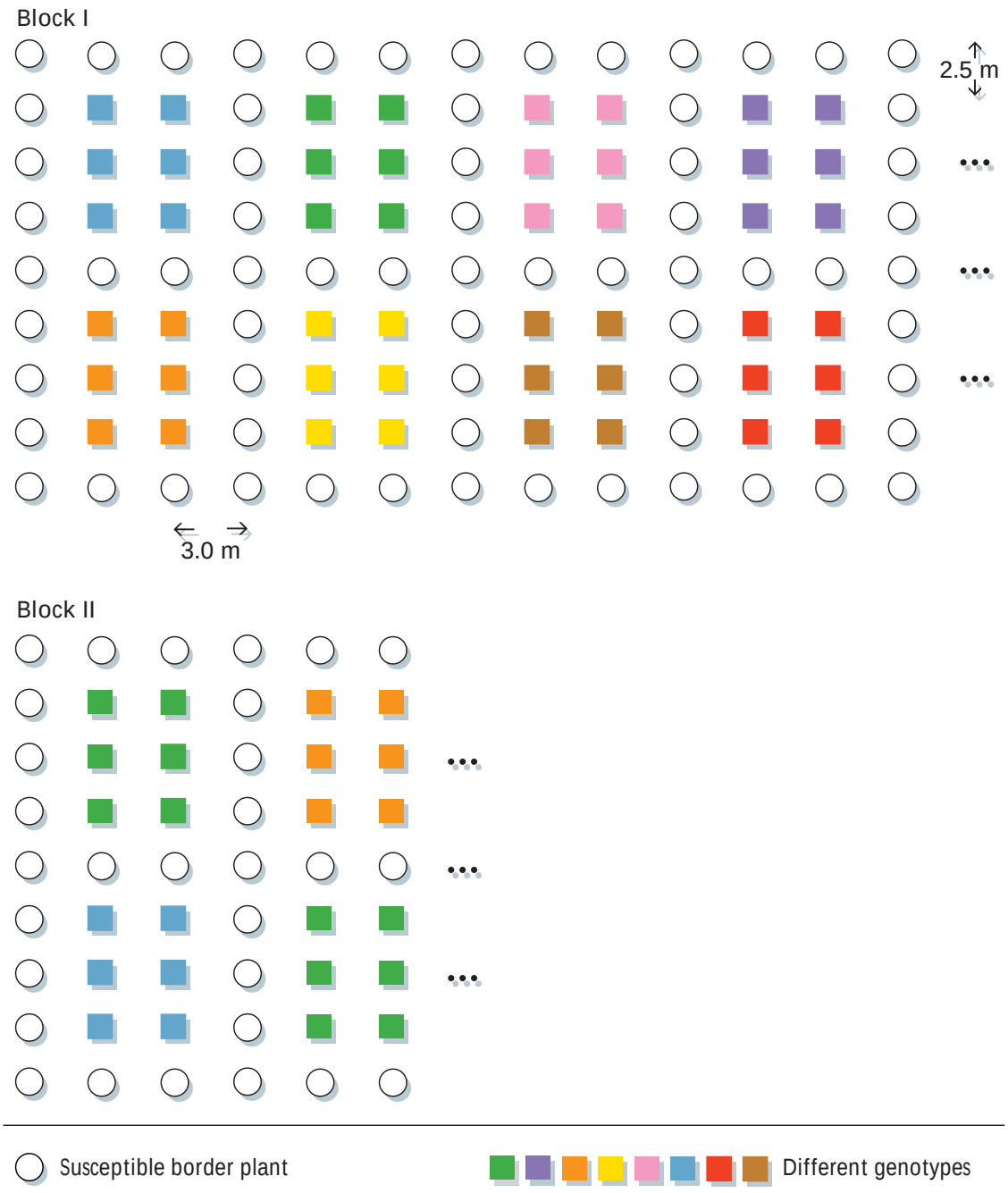


Figure 4. Layout of the randomized complete block design.

months after planting. Every test plant, except the extra plants at the ends of rows, should be used for data collection. Table 2 details when data should be collected, and field forms 4 and 5 in Appendix IX are provided to record the data.

Disease development time (DDT)

The disease development time (DDT) is the time, measured in days, between stage B of cigar leaves (a stage of the unrolling banana leaf) and the appearance of at least 10 mature necrotic lesions of stage 6 on that leave (Fouré 1982).

Field form 4 in Appendix IX is provided to make your recordings of DDT. You should use as many forms as you have plants in your experiment and use the same form for each plant throughout the cycle.

Plants should be inspected once a week. Plants with cigar leaves near Brun's stage B (Appendix V) should be selected and marked (indelible black felt-tip pen, coloured ribbon, tags) with the date at which it was estimated that the leaf was at Brun's stage B. Note the date on the form.

These leaves should be inspected once a week until the ultimate necrotic stage of the disease (stage 6) or one large necrotic area with at least 10 light-coloured dry centres is visible (Appendix VI). This date should be recorded. The time at which mature lesions appear should be estimated if this occurs between inspections. The DDT in days can then be worked out for this leaf and recorded on the form.

This process should be repeated every week, i.e. every week plants with cigar leaves at Brun's stage B are selected for observations. Observations to check for 10 mature lesions on these leaves should also be made once a week.

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 VII).

This information should be recorded for each leaf that has been used to assess DDT. After shooting, when leaves cease to be produced, the YLS value should be recorded weekly until harvest in field form 4 in Appendix IX.

Leaf emission rate (LER)

The leaf emission rate (LER) refers to the number of leaves produced per week. It should be less than one and should be calculated regularly (at least monthly) for each test and reference plant, beginning three months after planting and finishing at bunch emergence (shooting).

LER can be worked out from DDT readings. Count the number of new leaves produced between marked leaves at Brun's stage B on each plant and divide by the number of weeks between observations. Record your calculation in field form 4 in Appendix IX.

Disease severity

Disease severity is the amount of leaf area affected by the *Mycosphaerella* leaf spot pathogen and can be expressed in disease grades or in percentage. Leaves should be graded using Gauhl's modification of Stover's system (Appendix VIIIa). Disease grades should be recorded for each leaf on each test plant. Field form 5, Appendix IX is provided to help you record these data.

This information should be recorded:

- six months after planting
- at bunch emergence (shooting)
- at harvest.

Only upright leaves should be recorded. After disease severity has been recorded, the infection index for each test plant should be calculated as shown in Appendix VIIIb.

Agronomic data

Pleaser refer to page 13 for a complete list of variables. Fruit characteristics need not be recorded. Table 2 details when data should be collected, and field form 7 in Appendix XIV 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)	Shooting	Shooting to harvest phase	Harvest
Disease evolution data				
Disease development time	X			
Youngest leaf spotted	X	X	X	
Leaf emission rate	X			
Disease severity	6 months after planting	X		X
Agronomic data				
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 9 in Appendix XIV.

Management data

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

4. Technical guidelines for the 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 Quénéhervé 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* genepool (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 *Radopholus similis*	ITC0312
Yangambi km 5	Resistant to *Radopholus similis*	ITC1123
Grande naine	Susceptible to *Radopholus similis*	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 PRO*MUSA* Nematology working group are:

Grande naine*	AAA	ITC1256
Yangambi km 5*	AAA	ITC1123
Paka	AA	ITC0320
Selangor	AA	ITC1060
Kunnan	AB (Ney poovan)	ITC1034
Musa balbisiana	BB (Honduras)	ITC0247
Musa balbisiana	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 Suzanne Sharrock at INIBAP

(e-mail: s.sharrock@cgiar.org). 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 5), 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.

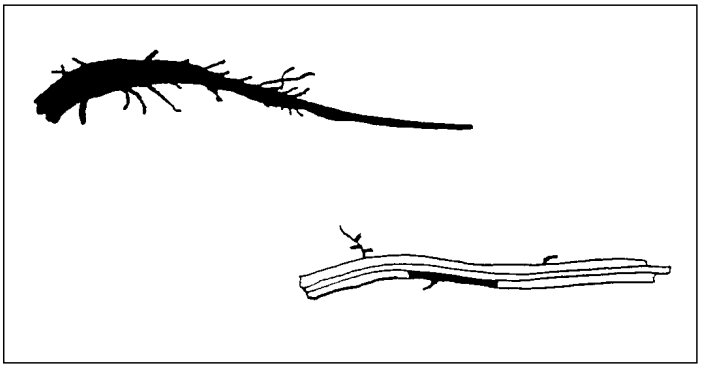


Figure 5. A dead root (top) and a functional root (bottom).

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 X.

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 XI. The advantage of these methods is that they require little equipment. In field screening experiments, the number of juveniles 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 XII contains the protocol to assess the damage caused by the nematodes. Table 3 details when data should be collected, and field form 6 in Appendix XIII is provided to record the data.

Agronomic data

Please refer to page 13 for a complete list of variables. Table 3 details when data should be collected, and field forms 7 and 8 in Appendix XIV 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
Disease evolution data						
Nematode reproduction		X	X	X	X	X
Damage assessment		X	X	X	X	X
Agronomic data						
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		

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

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 9 in Appendix XIV 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_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2HPO_4	400
Micronutrients	H_3BO_3	6.18
	$MnSO_4.H_2O$	16.90
	$ZnSO_4.7H_2O$	8.60
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.24
	$CoCl_2.6H_2O$	0.024
	$CuSO_4.5H_2O$	0.025

Table 1. (cont.)

		mg/L
Iron	$FeSO_4.7H_2O$	27.80
	$Na_2.EDTA.2H_2O$	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^6-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 ± 2°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 ± 2°C and in a 12 to 16-hour light cycle. High light intensity between 5000 and 10 000 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®. 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® 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® chamber at one vertical edge.
- Carefully remove the plantlet from the Cultu sak® 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

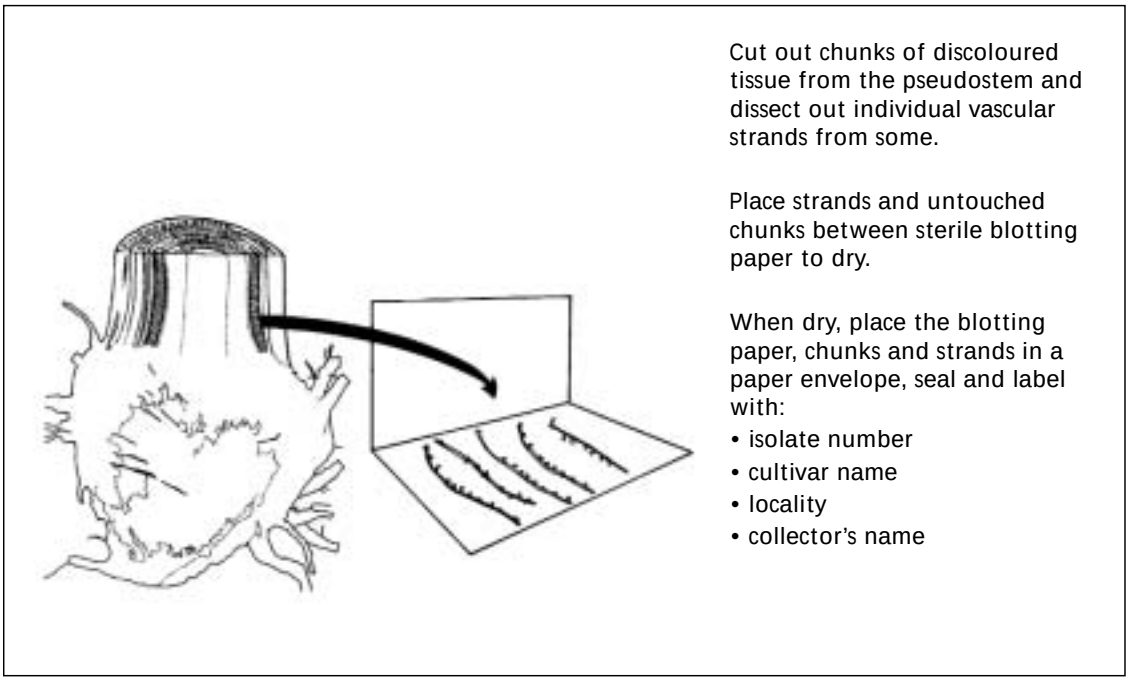


Figure 1. Preparation of samples for mailing (Courtesy of Dr. K. Pegg, QDPI).

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 III. Field forms for recording data during screenings for resistance to Fusarium wilt

Field form 1. External symptoms of Fusarium wilt disease
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

Planting date: .....

Experimental design: .....

ITC #	Replication	Plant #	MAP	YF	SP	CHN	PC

MAP: months after planting
YF: yellowing of foliage
SP: splitting of pseudostem
CHN: changes in new leaves
PC: petiole collapse

Field form 2. Internal symptoms of Fusarium wilt disease in the pseudostem
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

Planting date: .....

Experimental design: .....

ITC #	Replication	Plant #	Extent of discoloration at harvest (cm)

Field form 3. Internal symptoms of Fusarium wilt disease in the corm
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

Planting date: .....

Experimental design: .....

ITC #	Replication	Plant #	Slice # *				
			1	2	3	4	5

* 1 is the basal slice and 5 is the upper slice.

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

Table 2. (cont.)

Species (anamorph)	Conidiophores	Conidia
Pseudocercospora eumusae	(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 $CaCO_3$, 20 g agar per litre of medium, pH 6). Cultures are incubated at 20°C for 10 to 14 days under 60 μmoles m^{-2} s^{-1} 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.

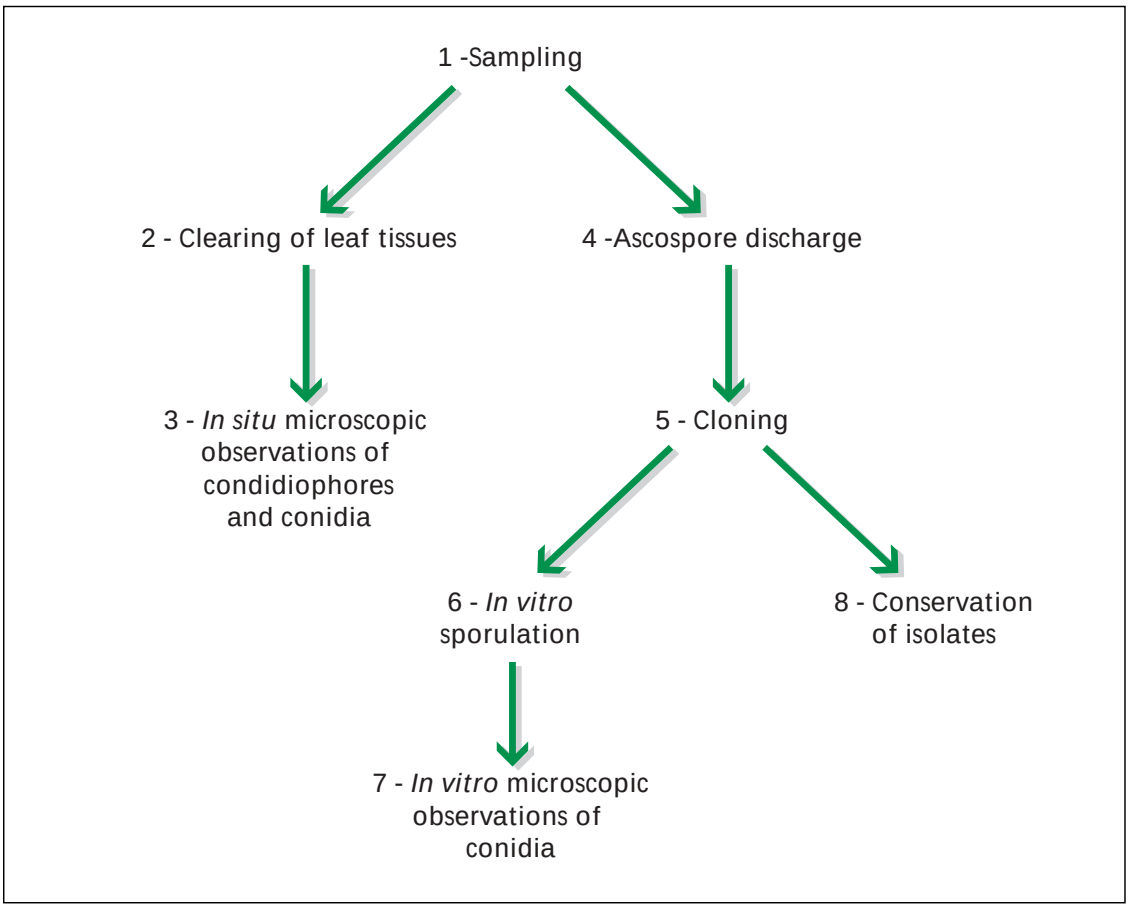
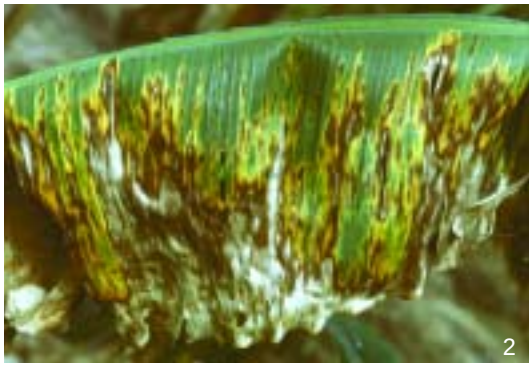


Figure 2. Flow chart for identification of *Mycosphaerella* leaf spot pathogens

(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)

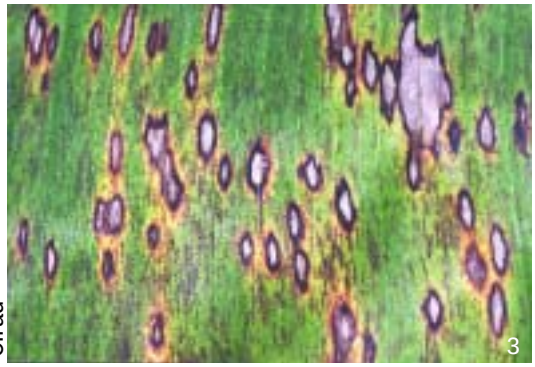
Plates 1-6: Leaf symptoms

Cirad



Cirad

Black leaf streak disease
(Mycosphaerella fijiensis)



Cirad

Cirad

Sigatoka disease
(Mycosphaerella musicola)

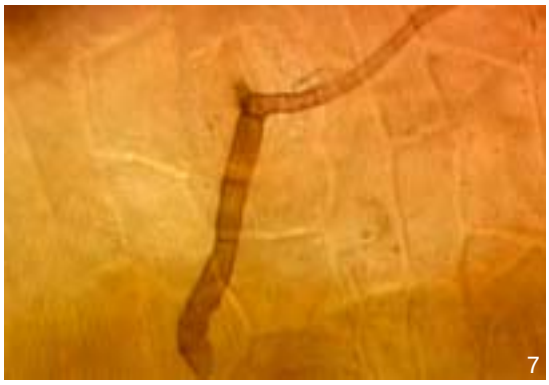
Cirad

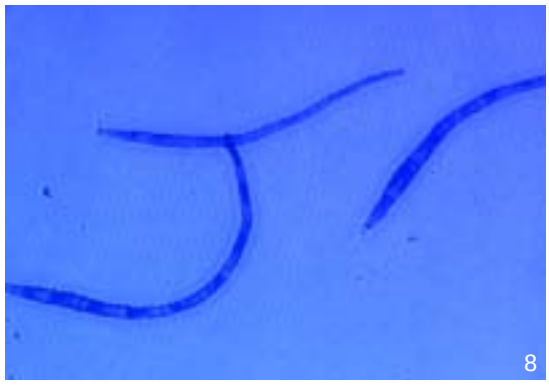
D. Jones

Eumusae leaf spot disease
(Mycosphaerella eumusae)

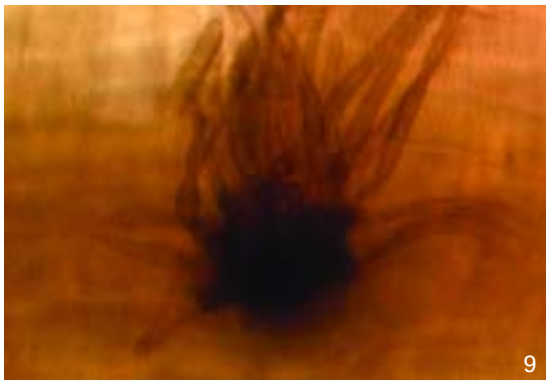
Plates 7-12: Anamorphs of banana leaf spot pathogens

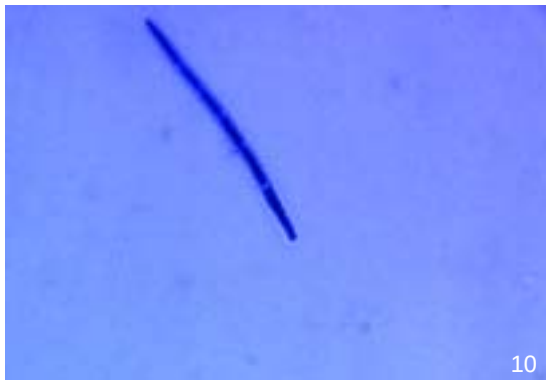
Conidiophores **Conidia**



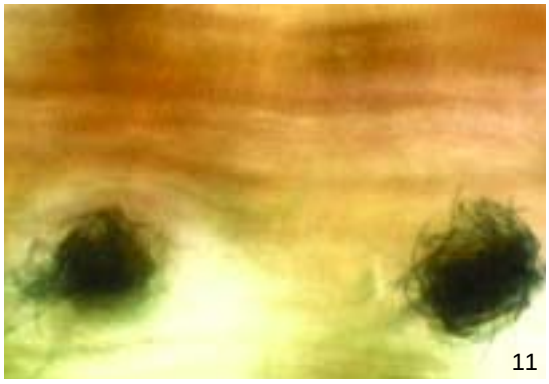


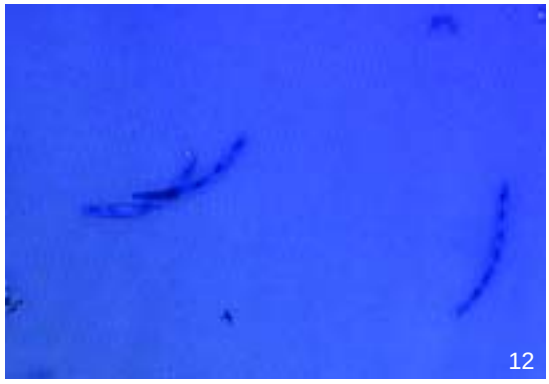
Black leaf streak disease *(Paracercospora fijiensis)*





Sigatoka disease *(Pseudocercospora musae)*





Eumusae leaf spot disease *(Pseudocercospora eumusae)*

Appendix V. Stages of an unfolding banana leaf (Brun 1963)

The young unfolded leaf is coiled into a double spiral. The right half of the leaf is situated in the hollow of the central petiole, while the left half of the leaf covers both the petiole and the right side.

The lapse of time in which a leaf unfolds varies. Under favourable climatic conditions, it takes about seven days, but it can take up to 15 to 20 days under poor conditions (drought, malnutrition, etc.).

In order to understand the unfolding process, it is important to recall that the formation of the leaf takes place within the pseudostem before shooting. The new leaf is tightly coiled, whitish, and particularly fragile.

The shooting of the leaf results in an extraordinarily rapid growth of the leaf sheath (4 m in 10 days for 'Gros Michel'). The young leaf slips into the petiolar canal of the preceding leaf and the development of a leaf corresponds to two successive phenomena, that of 'growth' and that of 'unfolding'.

To facilitate the description of the unfolding process, the latter has been divided into five successive stages (Figure 3). These stages are defined arbitrarily, since the process is in reality a continual one. The first two stages can be considered to correspond to the 'growth' phase, the third stage represents the end of the growth and the beginning of the unfolding process, and the fourth and fifth concern the unfolding itself. These different stages have been defined as follows:

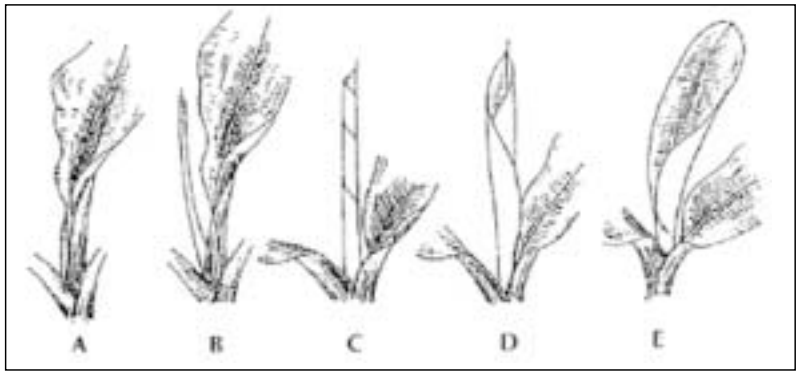


Figure 3. Stages of an unfolding leaf.

Stage A: The 'cigar', about 10 cm in length, is still joined to the preceding leaf.

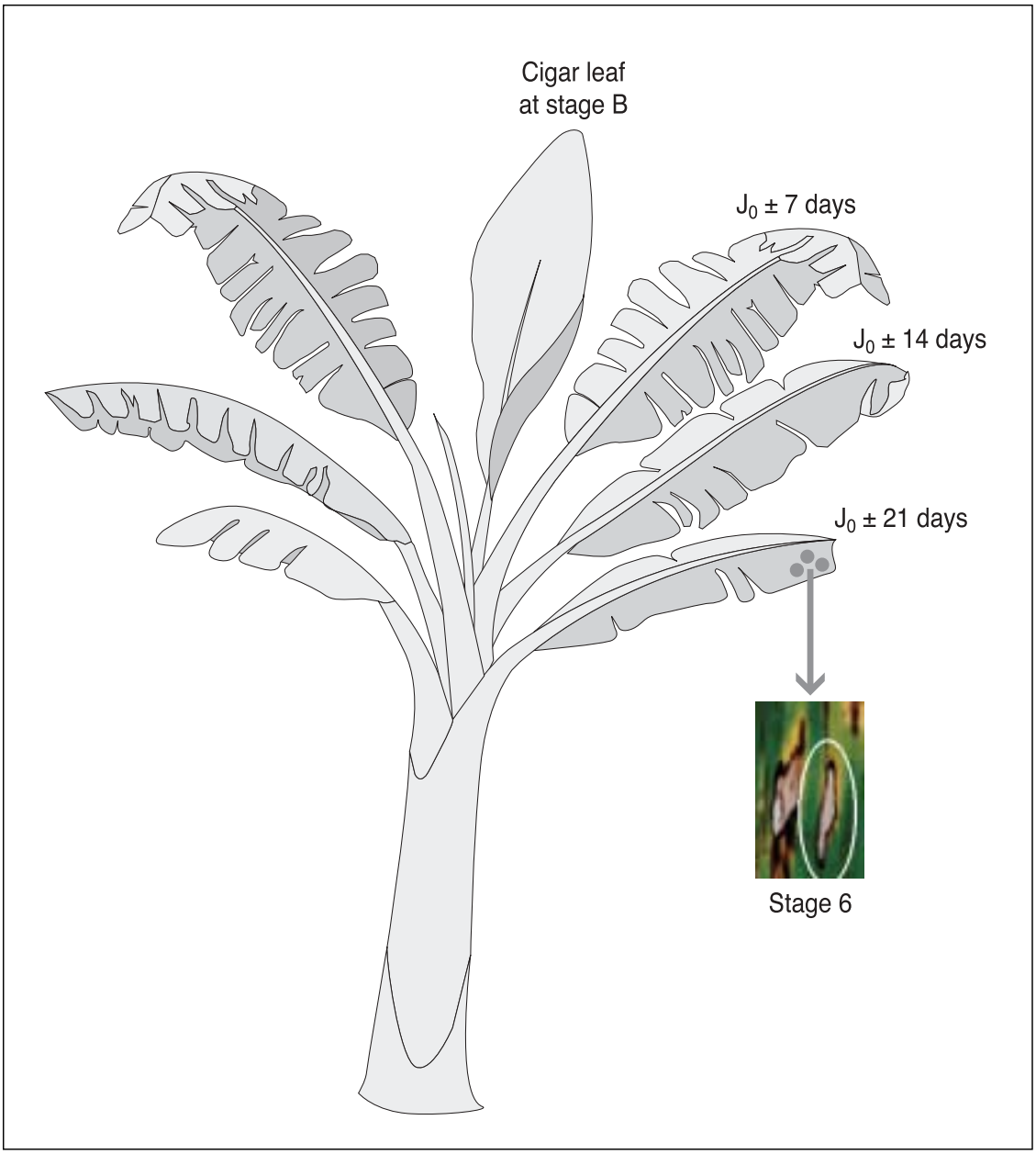
Stage B: The 'cigar' is bigger, but has not yet reached its full length.

Stage C: The 'cigar' is completely free. It reaches its full length and the diameter of its apex has considerably increased following the loosening of the spiral.

Stage D: The left-hand side has already unfolded, and spreading takes place at the extreme apex.

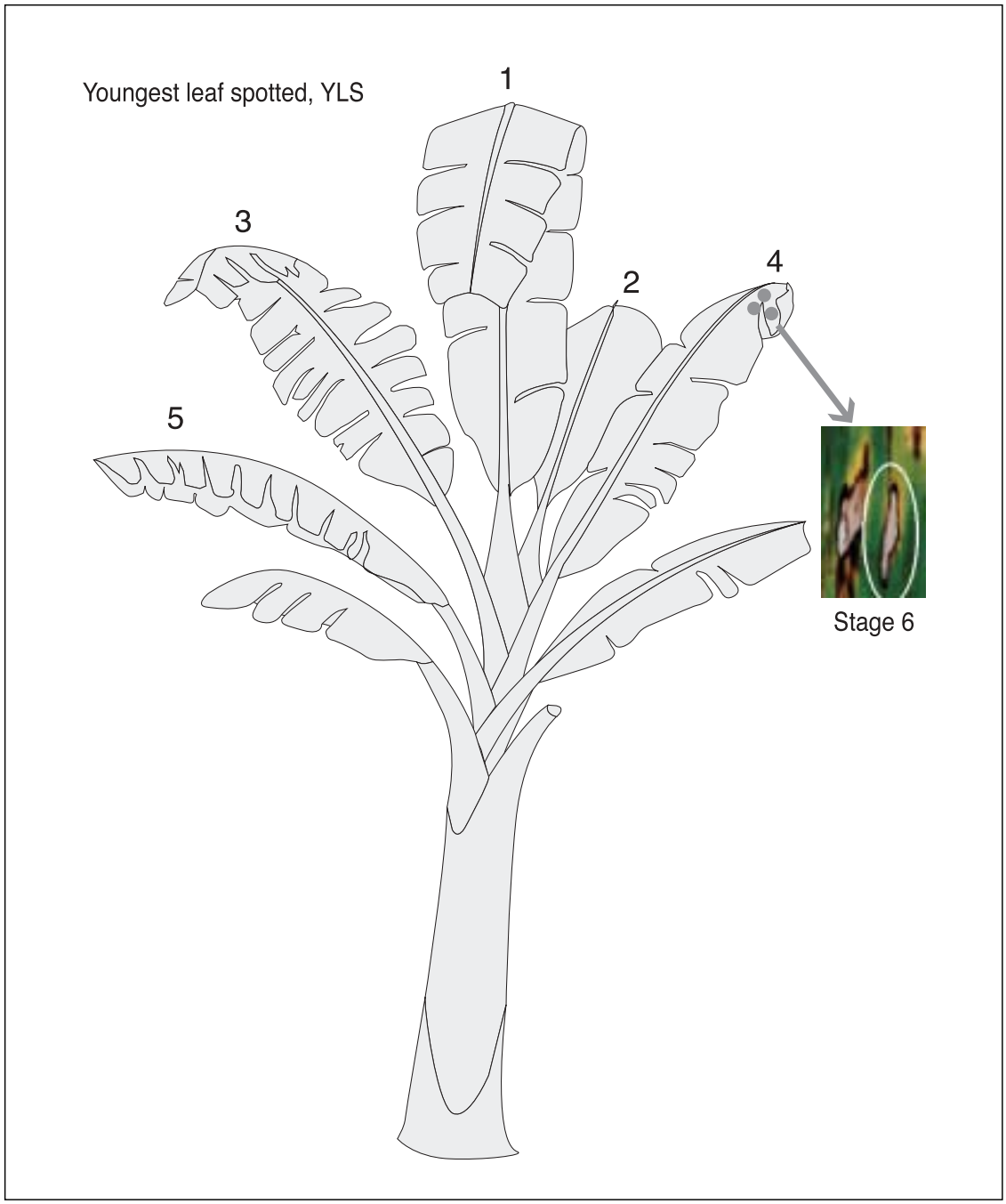
Stage E: The upper part of the leaf has unfolded and the base is in an open cornet shape.

Appendix VI. Disease development time



DDT is the elapsed time between the development of the leaf cigar at stage B and the development of at least 10 necrotic lesions at stage 6.

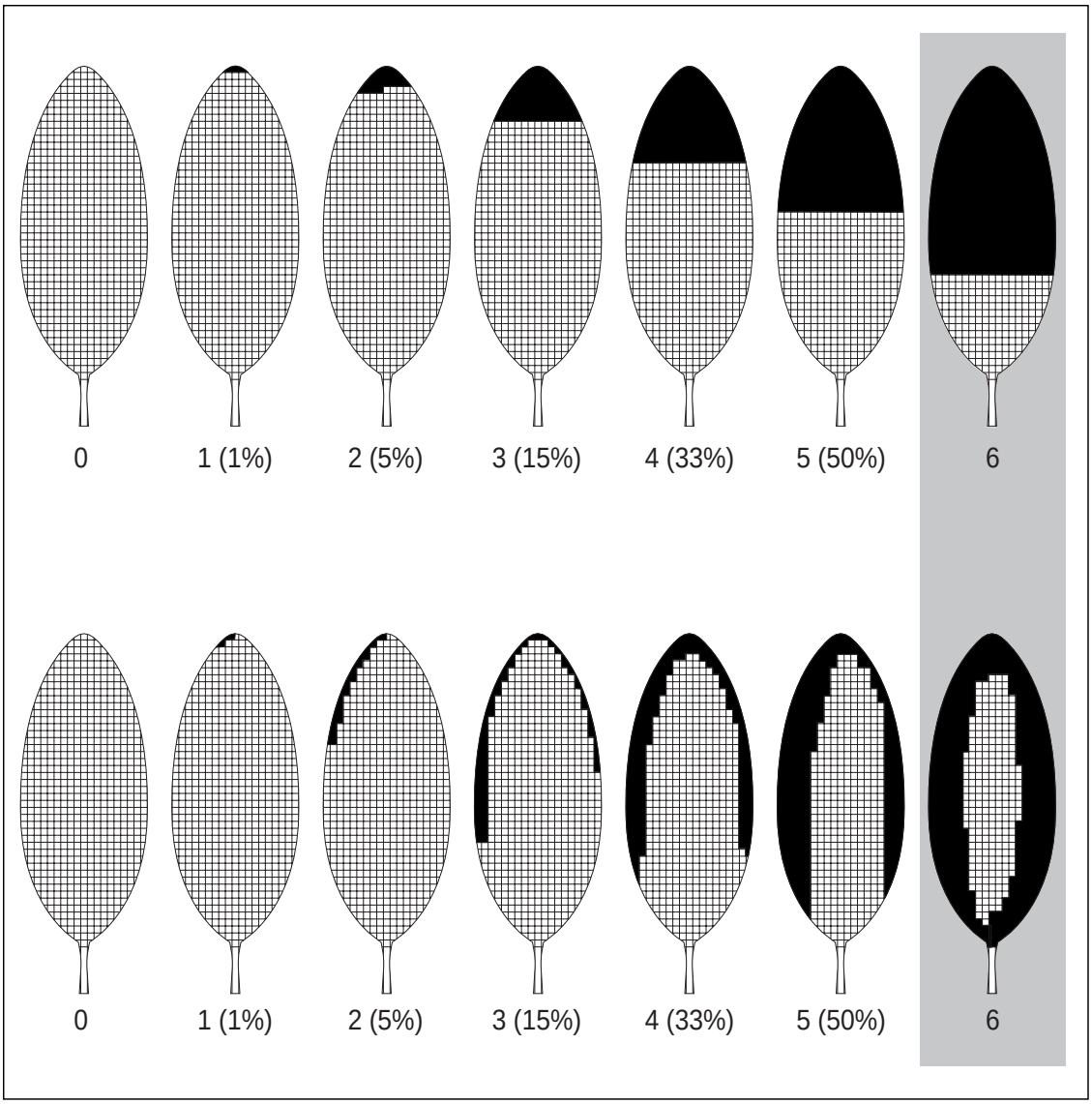
Appendix VII. Youngest leaf spotted



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

Appendix VIII. Disease severity

Appendix VIIIa. Gauhl's modification of Stover's severity scoring system



Appendix VIIIb. Infection index

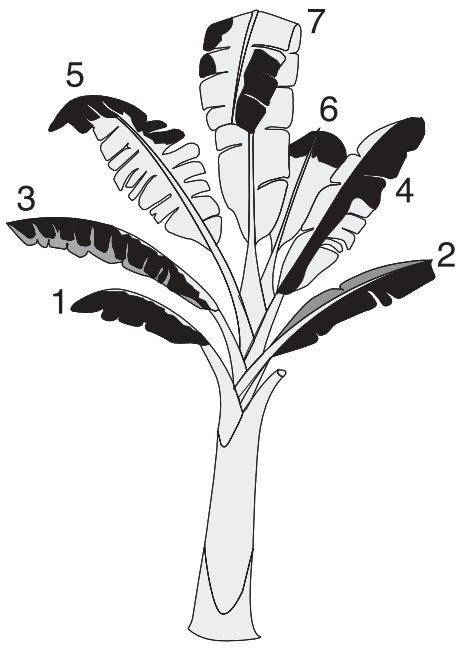
Calculation of infection index

Calculate the infection index for each plant in each replication at each growth stage:

$$\text{Infection index} = \frac{\sum nb}{(N - 1)T} x100$$

Where

n = number of leaves in each grade
b = grade
N = number of grades used in the scale (7)
T = total number of leaves scored



Example of calculation

$$\frac{3(0) + 2(2) + 1(5) + 2(6)}{(7-1)7} \text{ X } 100 = 50$$

Infection index = 50

Gauhl's modification of Stover's severity scoring system is shown in Appendix VIII (a).

Appendix IX. Field forms for recording data during screenings for resistance to *Mycosphaerella* leaf spot diseases

Field form 4. Disease development time (DDT), youngest leaf spotted (YLS) and leaf emission rate (LER). (To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

Planting date: ..... ITC #:.....

Experimental design: ..... Unique identifier: .....

Week #	**Date of stage B** (dd/mm/yy)	**Date of 10 or more lesions** (dd/mm/yy)	**DDT**	**YLS**	**New leaves**	**LER**

Field fom 5. Disease severity
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

Planting date: .....

Experimental design: .....

ITC #: .....

Unique identifier: .....

Table 3. Sigatoka severity scoring to be completed for upright leaves on each test plant at the growth stages indicated (scores have been included as example).

Leaf Number	1*	2	3	4	5	6	7	8	9	10	11	12	13	14
Growth stage														
Six months	0	0	0	1	2	2	2	3	5	6	6	6	–	–
Bunch emergence	0	0	0	1	2	2	–	4	5	6	6	6	–	–
Harvest	0	1	2	5	5	6	6	–	–	–	–	–	–	–

Key:
* = youngest completely unfurled leaf

Gauhl's modification of Stover's disease severity scoring system:
0 = no symptoms
1 = less than 1% of lamina with symptoms (only streaks and/or up to 10 spots)
2 = 1 to 5% of lamina with symptoms
3 = 6 to 15% of lamina with symptoms
4 = 16 to 33% of lamina with symptoms
5 = 34 to 50% of lamina with symptoms
6 = 51 to 100% of lamina with symptoms
– = missing leaf or dead leaf hanging down the pseudostem (when a leaf is missing or dead and hanging down the pseudostem, it should not be included in the infection index calculations).

		Leaf number													
Evaluation at	**Date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**...**	**Sum**
Six months															
Bunch emergence															
Harvest															

Appendix X. 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 4 and Figures 4 to 6.

Table 4. Morphological characters of banana migratory endoparasitic nematodes.

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 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).

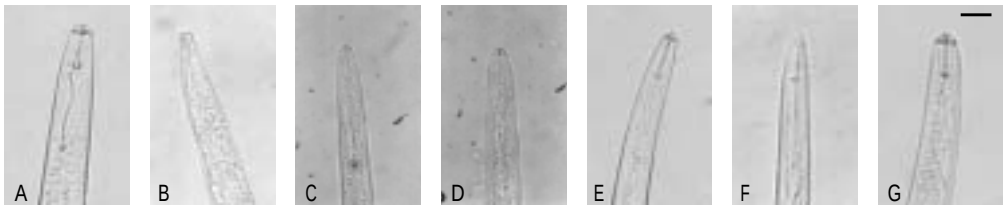


Figure 4. Head region of *Radopholus similis* (A: female; B: male), *Pratylenchus coffeae* female (C), *Pratylenchus goodeyi* female (D), *Helicotylenchus female* (E) and *Meloidogyne* (F: J4-stage-juvenile; G: male). Bar = 20 microns.

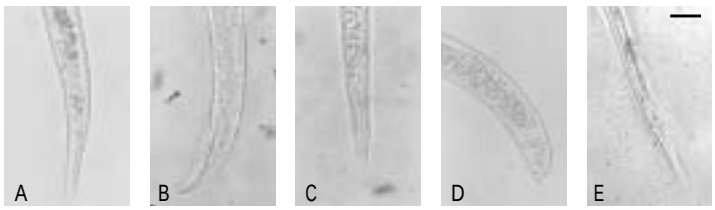


Figure 5. Female tail region of *Radopholus similis* (A), *Pratylenchus coffeae* female (B), *Pratylenchus goodeyi* (C), *Helicotylenchus multicintus* (D) and J4-stage-juvenile tail region of *Meloidogyne incognita* (E). Bar = 20 microns.

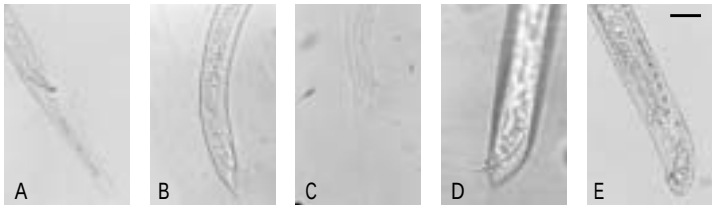


Figure 6. Male tail region of *Radopholus similis* (A), *Pratylenchus coffeae* (B), *Pratylenchus goodeyi* (C), *Helicotylenchus multicintus* (D) *Meloidogyne incognita* (E). Bar = 20 microns.

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 XI. 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 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 8).

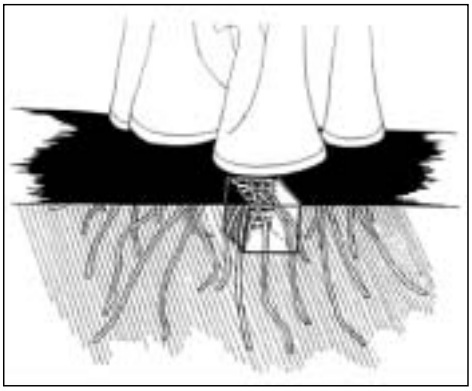


Figure 7. Collection of roots from a standard-size excavation of 20 x 20 x 20 cm extending outward from the corm of the banana plant.

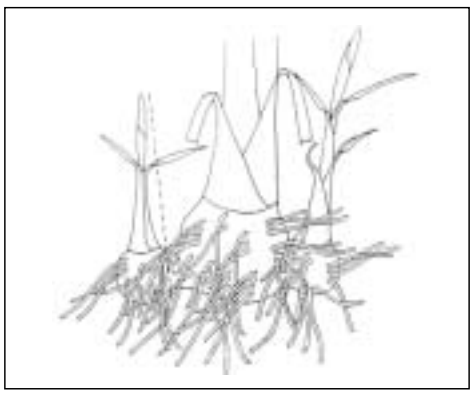


Figure 8. Removal of a sucker from a mat.

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-μm sieves and rinse the sieves with tap water
- using distilled water, collect in a beaker the nematodes from the 40-μm 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 4ºC .

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 XII. 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 8).

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 9).

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 10) or internally as pit-like structures (Figure 11) on the roots.

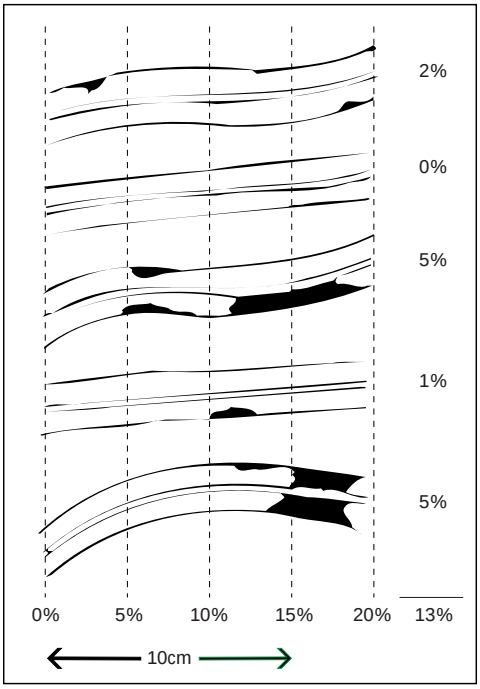


Figure 9. Example of scoring five length-wise sliced banana roots for root necrosis (% of root cortex surface showing necrosis) caused by migratory endoparasites.

Figure 10. Galling of banana roots caused by root-knot nematodes.

Figure 11. Banana roots infected with swollen root-knot nematode females.

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

Appendix XIII. Damage caused by nematodes

Field form 6. Root damage assessment
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

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

Appendix XIV. Field forms for recording agronomic and environmental data

Field form 7: Agronomic data
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

ITC #	Replication	Plant #	Date of planting (dd/mm/yy)	Time from planting to shooting (days)	Plant crop cycle (days)	Height of pseudo-stem at shooting (cm)	Girth of pseudo-stem (cm)	Height of following sucker at shooting (cm)	Weight of bunch (kg)	Number of hands in bunch at harvest	Number of fruits at harvest	Weight of fruit (g)	Number of functional leaves at flowering	Number of functional leaves at harvest

Field form 8. Agronomic data: Fruit characteristics
(To be sent in Excel format. If needed, electronic forms can be provided by INIBAP).

Site: .....

Surveyor: .....

ITC #	Replication	Plant #	Fruit characteristics of 3rd hand				Fruit characteristics of 3rd hand			
			Hand #*	Length (cm)	Diameter (cm)	Weight (g)	Hand #*	Length (cm)	Diameter (cm)	Weight (g)

* Use 'Hand #' column if data is taken from hands other than the 3rd and the 7th.

Field form 9. 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: .....

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 himidity $\geq$ 90%					

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Layout and cover illustration by Roberto Hamm - Crayon & Cie
Printed by Arceaux 49, Montpellier, France

ISBN: 2-910810-52-6

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