Practical Guidelines for Early Screening and Field Evaluation of Banana against Fusarium Wilt, *Pseudocercospora* Leaf Spots and Drought

Edited by Miguel Dita

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Guidelines for the evaluation of resistance to *Pseudocercospora* leaf spots of banana

Luis Pérez-Vicente[1]*, Francoise Carreel[2]*, Véronique Roussel[3], Jean Carlier[3] and Catherine Abadie[3]

[1] Instituto de Investigaciones de Sanidad Vegetal (INISAV), Cuba
[2] CIRAD, UMR AGAP, F-34398 Montpellier, France.
AGAP, Univ Montpellier, CIRAD, INRAE, Institut Agro, Montpellier, France
[3] CIRAD, UMR BGPI, F-34398 Montpellier, France.
BGPI, Univ Montpellier, CIRAD, INRAE, Institut Agro, Montpellier, France
* Both first authors

These guidelines reflect the consensus and knowledge of the authors at the time of writing, but it is expected that they will further develop as they are used. To help improve the guidelines, please send your feedback to Nicolas Roux, Alliance of Bioversity and CIAT, Parc Scientifique Agropolis II, 34397 Montpellier Cedex 5, France. Email correspondence can be sent to the MusaNet Secretariat, at musanet.secretariat@gmail.com

1. INTRODUCTION

Leaf spot diseases of bananas (*Musa* spp) include three related pathogenic ascomycete fungi: *Pseudocercospora fijiensis*, causing black leaf streak disease (BLS; also known as black Sigatoka), *P. musae*, responsible for Sigatoka disease (SD; also known as yellow Sigatoka) and *P. eumusae,* the causal agent of eumusae leaf spot disease (ELS; Crous and Mourichon, 2002).

P. fijiensis and *P. musae* can cause extensive defoliation, but *P. fijiensis* is characterized by its stronger pathogenicity on a broader range of hosts, making BLS the most destructive leaf disease of bananas and considered among the ten most destructive diseases to world agriculture (Pennisi, 2010). In general, the fungi are disseminated locally due to ascospores and conidia. The disease is believed to be spread by the movement of infected germplasm (suckers, leaves) and wind-borne ascospores.

The effects of SD and BLSD on growth, production and fruit quality are similar and have been clearly described together with the reaction of cultivars in past and present reviews (Churchill, 2011; Firman, 1972; Guzmán et al., 2013; Jones, 2000; Marín et al., 2003; Meredith, 1970; Meredith and Lawrence, 1970; Pérez et al., 2002; Stover, 1972). Leaf infection caused by both pathogens reduces photosynthesis (Hidalgo et al., 2006; Rodríguez-Gaviria and Cayón 2008), which leads to a lower fruit weight, finger length reduction and early ripening of fruits (creamy pulp) in the field or during transport to final markets (Guzmán et al., 2013), accompanied by a general deterioration of plant development. A second impact is the abandonment of plantations by small growers due to the impact of disease on production costs (Pérez Vicente et al., 2002 and 2016).

Two types of interactions and three types of phenotypes are described in *Musa* against BLS (Fouré et al., 1990; Fouré, 1994): 1) incompatible interaction characterized by a high resistance or hypersensitivity observed in wild species of *Musa*; 2) compatible interaction with two types of reactions: a) partial resistance expressed by a slow disease evolution cycle and a reduction in pathogen reproduction; this type of interaction was observed in partially resistant FHIA hybrids, expressed as a longer transition period from the first streak symptoms to spots and a drastic reduction of sexual body production (pseudothecia and spermogonia) in the mature spots (Hernández and Pérez, 2001; Pérez-Miranda et al., 2006) and b) susceptibility observed in cultivars of subgroups Cavendish (AAA), Plantains (AAB) and many other genotypes with a rapid disease evolution and intense reproduction of pathogens in the host.

Before evaluating new hybrids or selected clones, it is very important to know exactly which *Pseudocercospora* species is present at the site and, if possible, in the country. The three pathogens *P. fijiensis*, *P. musae* and *P. eumusae* are difficult to distinguish by symptom expression (particularly *P. fijiensis* and *P. eumusae*, Figures 3 and 5), but their sexual stages (teleomorphs) are also similar. However, the species can be identified by morphological differences between their asexual stages (anamorphs), whether they are directly observed on diseased leaves or after being isolated and cultured (see scheme in Figure 9), and by molecular diagnostic procedures (Arzanlou et al., 2007; Henderson et al., 2006). The morphological characteristics of the three pathogens are presented in Table 1 and Figures 8, 9 and 10. Attention should be taken to avoid confusing these pathogens with other fungal species that also attack the foliage of bananas (Guzmán et al., 2018; Jones, 2000; Wardlaw, 1972).

2. Identification of *Pseudocercospora* leaf spot pathogens

Symptoms of Sigatoka (*P. musae*), black leaf streak (*P. fijiensis*) and eumusae leaf spot (*P. eumusae*) diseases.

2.1 Sigatoka leaf spot disease (SD) caused by *Pseudocercospora musae*

Brun (1958, 1963) described the five different stages in the evolution of SD spots caused by *P. musae* on susceptible plants (Figure 1) as follows:

Figure 1. Cavendish affected by Sigatoka leaf spot disease (SD). (Photo: L. Pérez-Vicente).

Stage 1: Streaks of pale green color smaller than 1 mm length, parallels to leaf nerves, visible on the upper side of the leaf at transmitted light. Frequently, if not previously known, are unnoticed to the observers. In this stage, the infection hyphae are established in the sub-stomatic chamber and the occlusive stomatic and annexes cells are dead (Figure 2A).

Stage 2. Streaks enlarge and reach several milimeters long of a pale-yellow color. Mycelia that penetrate stomata become superficial, then emerge again and appear on both sides of the leaf as 3–4 µm brown hyphae that penetrate other stomata close to the initial infection site (Figures 2A-B).

Stage 3. Streaks enlarge and at the same time increase in length. The borders are not well defined and are confused with the normal leaf color. The color starts to change to reddish brown, and the hyphae invade the palisade parenchyma. Stroma start to develop in the sub-stomachic chamber in the center of lesions (Figures 2B-C).

Stage 4. Spots develop on a well-defined long elliptic shape of dark brown color. The center of the lesion is progressively depressed and, in the external border, a bright yellow halo can be seen. A watery halo can be observed under humid conditions. In this stage, conidiophore grouped in sporodochia are produced, and conidia production takes place if relative humidity is high (Figures 2C-D).

Stage 5. Spots are oval with a length of up to 20 mm by 2 mm wide. The spot center is gray and depressed with a dark brown to black border. Surrounding the border, a yellow halo is present. Conidia production has ceased and spermogonia and pseudothecia are present (Figure 2C-D-E).

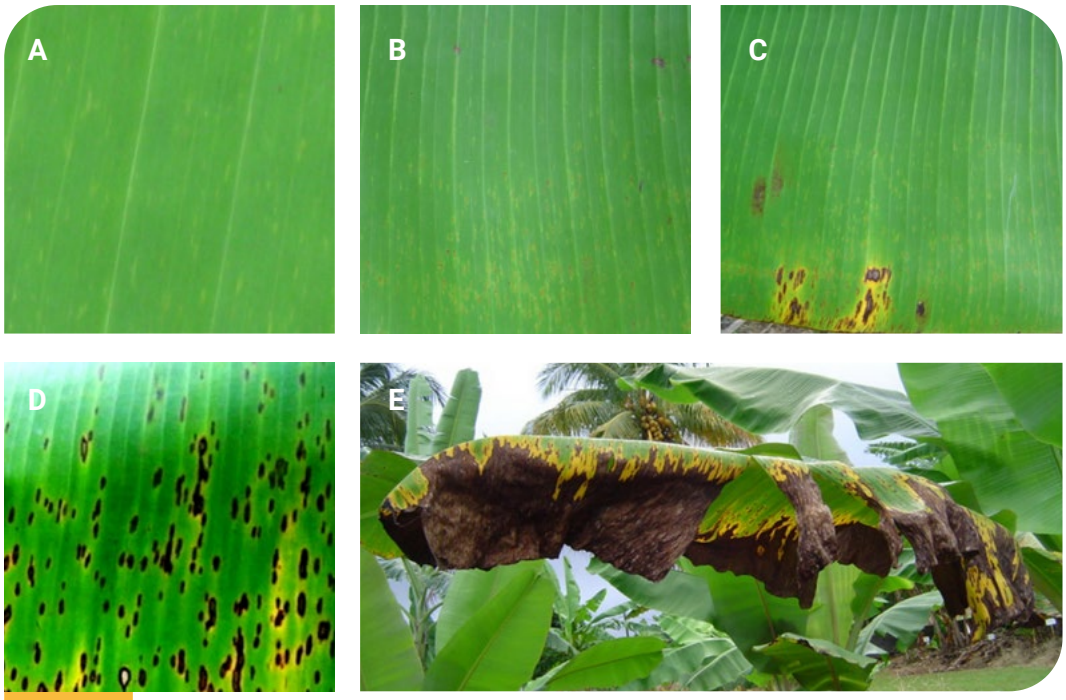


Figure 2. Five stages of evolution of symptoms according to Brun (1958), description. A) Stage 1 and 2; B) Stages 2 and 3; C) Stages 3, 4 and 5; D) Stage 4 and 5; E) Stage 5 (Photos: L. Pérez-Vicente).

2.2 Black leaf streak (BLS) or Black Sigatoka caused by *Pseudocercospora fijiensis*

Black leaf streak cause severe damages to susceptible banana cultivars in the tropics (Figure 3).

Figure 3. Cavendish plants affected by black leaf streak (BLS) (Photo: Mario Orozco).

All spots do not follow this development sequence; some do not develop further than the second to third stage. In case of high infection pressure (with a high density of spots), streaks are smaller and can coalesce after stage 3, becoming necrotic with a large amount of pseudothecia.

In BLS disease, the sword suckers' leaves can show symptoms, leading to the movement of the disease from infected fields to free regions via planting material.

Fouré (1982a) described the following stages in the evolution of BLS disease spots:

Stage 1: Appearance of small (approximate 0.2 mm in diameter) diffuse and irregular yellow pale specks or points, only perceptible on the upper side of leaves. These lesions are not always visible and usually are unnoticed in some cultivars. When environmental conditions are favorable to disease development, this stage can appear on the second youngest open leaf but are more frequently present on leaf number 3 and 4. The speck elongates and reaches 1 mm long, becomes a reddish-brown streak and is not visible on the upper side of leaves (Figure 4A-B-C).

Stage 2: Streaks elongate, reaching a variable length (from 1 to 20 mm). The main characteristic is that they are visible on the upper side of leaves and have a reddish-brown color (Figure 4C-D).

Stage 3: Streaks elongate and reach 20–25 mm length x 2 mm width, remaining the characteristic brown color. If inoculum density is high, some necrotic patches due to streak coalescence can appear, giving a darker aspect to the leaves. Streak distribution is variable but usually more frequent on the left side of the leaves. In others, they appear evenly distributed on both sides of the leaf (Figure 4D-E).

Stage 4: This is considered the first spot stage. Lesions develop to a rounded, elliptic or fusiform shape. In very young plants, the spots develop in a markedly rounded shape. The transition of streaks to spots is characterized by the development of a halo that is clearly visible early in the mornings when dew is present or after rain. In this stage, pseudothecia and spermogonia development starts (Figure 4E).

Stage 5: The reddish-brown color turns dark brown to almost black. The central area of the spot is more conspicuous due to darkening of the tissue. In this stage, the tissue around the spot turns light yellow. This stage characterizes the dark to almost black color that takes over the canopy of heavily infected plants (Figure 4F-G).

Stage 6: In this final stage, the spot center is dry and becomes clear gray and depressed. The spot is surrounded by a very well-defined dark brown or black border. Between this border and the green tissue of the leaf, there is a bright yellow halo in the transition zone. After the leaf dries, the collapsing spots remain clearly visible due to the pale center and dark border (Figure 4G-H).

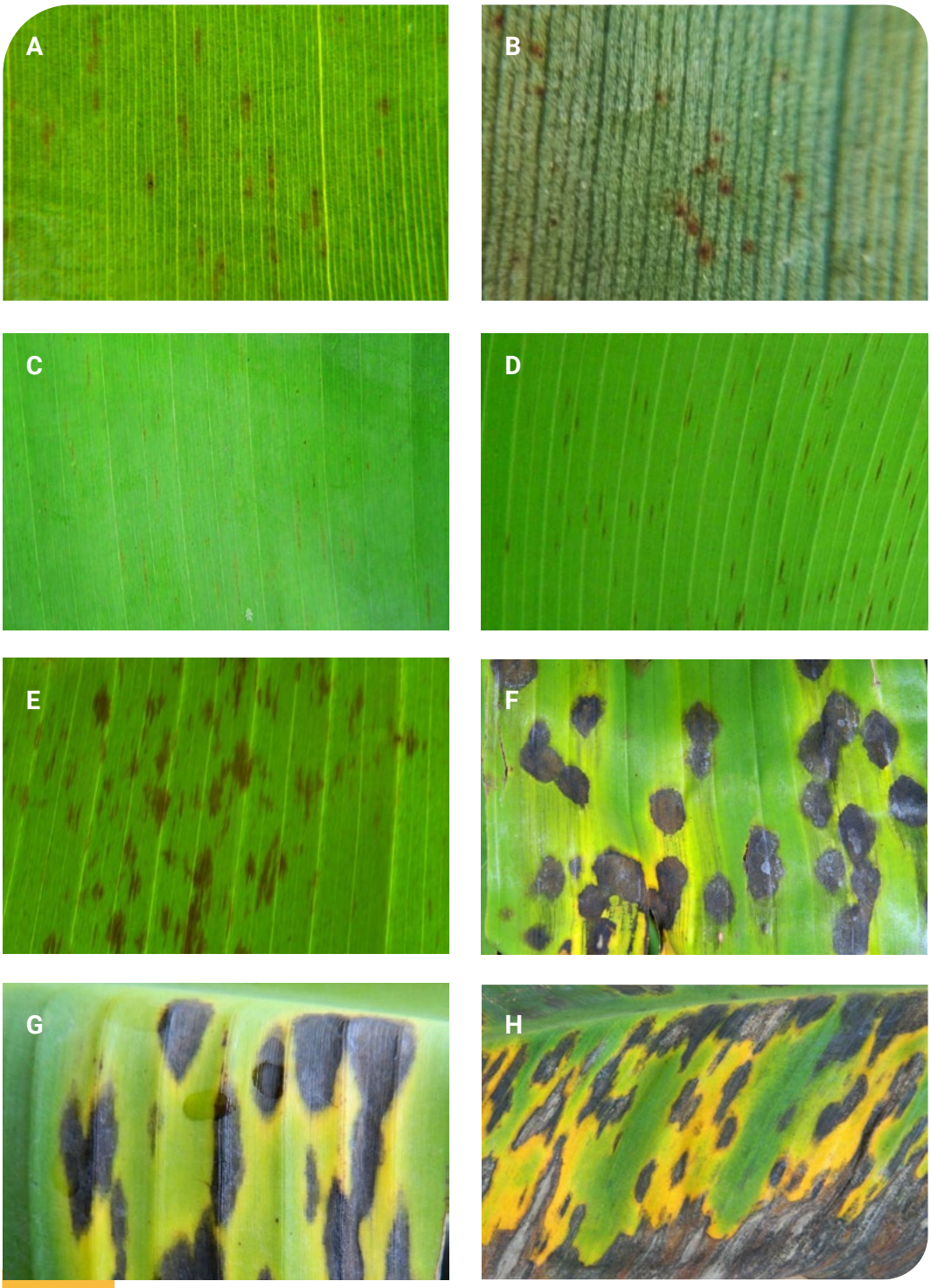


Figure 4. Six stages of symptoms evolution according to Fouré (1982b), description and details of stage 5 spots on a young (4-month-old) plant. A) Stage 1 (16x); B) Stage 1 (20x); C) Stages 1 & 2; D) Stages 2 & 3; E) Stages 3 & 4; F) Stage 5 (young plant); G) Stages 5 & 6; H) Stage 6. (Photos: L. Pérez-Vicente).

The BLS cycle has been described by Meredith and Lawrence (1969), Agrios (2005), Churchill (2011), and more recently by Guzmán et al. (2018). For details on the life cycle of *P. fijiensis,* see Guzmán et al. (2018) Figure 2.3, page 65.

2.3 Eumusa leaf spot (ELS) caused by *Pseudocercospora eumusae*

The symptoms of ELS are very similar to those of BLS (Figure 5). Accurate diagnosis has to be carried out by molecular procedures. Figure 6 presents the symptoms of ELS on Gros Michel in Thailand, Malaysia and India.

Figure 5. Gros Michel plants affected by Eumusa leaf spot in Southeast Asia. Photos L. Perez-Vicente, A. Drenth and R. Thangavelu.

2.4 Morphology of *Pseudocercospora* leaf spot pathogens

Microscopic images of the three *Pseudocercospora* pathogens appear in Figures 6, 7 and 8. Table 1 is a description of their morphological characteristics, and a scheme of the full diagnostic process appears in Figure 9.

2.5 Sampling of diseased tissue

For *in situ* microscopic observations, the specimens should be leaves at spot stages for *P. musae* (Figure 2) and *P. eumusae* (Figure 5) and early streak stages for *P. fijiensis* (Figure 4). For fungal

isolation and *in vitro* microscopic observations, the specimens should come from completely necrotic leaves regardless of the species. The leaves should be thoroughly dried between sheets of newspaper.

2.6 Tissue cleaning and *in situ* microscopic observations

The lesions are cleaned in a solution of KOH 10% overnight and washed five times in water for 10 minutes each time. They can also be cleaned in lactophenol in a boiling water bath for 5 mins. The conidiophores, spermogonia and pseudothecia associated with the lesions can then be directly observed on slides without staining (Figure 7, D and E). To observe conidia, cleaned tissues are stained for 1 min with a solution of 0.5% blue cotton and 1:1 lactic acid glycerol and washed in water.

To measure the intensity of reproduction, some spotted leaves are tagged at emergence and observed until they reach stage 5. Five spots at stage 5 of each of 10 plants (50 in total) are collected and cleaned following the procedures described above. After they are individually mounted on glass slides and the number of pseudothecia, spermogonia present in three fields of observation at 40 x10 magnification in each lesion are counted. In all cases, they are compared with data of the susceptible cultivar Grand Naine (Cavendish subgroup, AAA).

2.7 Ascospore discharge and cloning

Necrotic banana leaves are dried at room temperature for 48 hours and then soaked in distilled water for 15 mins. Leaf sections are secured to the underside of the lids of Petri dishes containing water agar at 3%. Ascospores discharge overnight onto the agar surface (the ascospores of the three *Pseudocercospora* 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 Potato Dextrose Agar (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 for 10 days under 12 h of white light.

2.8 *In vitro* sporulation and microscopic observations of conidia

Conidial sporulation is induced by culturing small pieces of mycelia on modified V8-sporulation or potato carrot leaf media. Cultures are incubated at 20°C for 10 to 14 days under 60 $\mu molm^{-2}s^{-1}$ of continuous cool-white fluorescent light. Cultures are then scraped with a scalpel, and the conidia are suspended in a solution of blue cotton directly on the slide for microscopic observation.

2.9 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.

2.10 Morphological characteristics of the *Pseudocercospora* spp. causing leaf spots in *Musa* spp.

Pseudocercospora musae

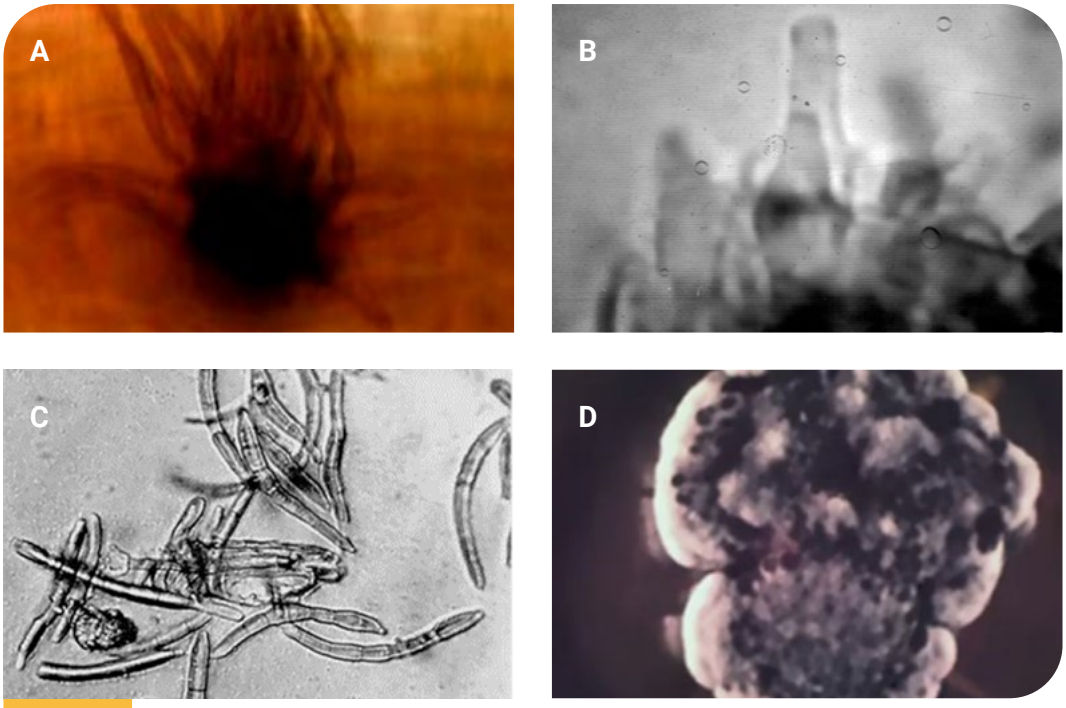


Figure 6. *Pseudocercospora musae* structures: A) Sporodochia and conidia on stroma; B) Bottle-like conidiophores grouped on sporodochia on stroma; C) Obclavate-cylindrical conidia without hilum; D) colony on PDA. (Photos: A) Carlier et al., (2002); B), C) and D) from L. Pérez-Vicente).

Pseudocercospora fijiensis

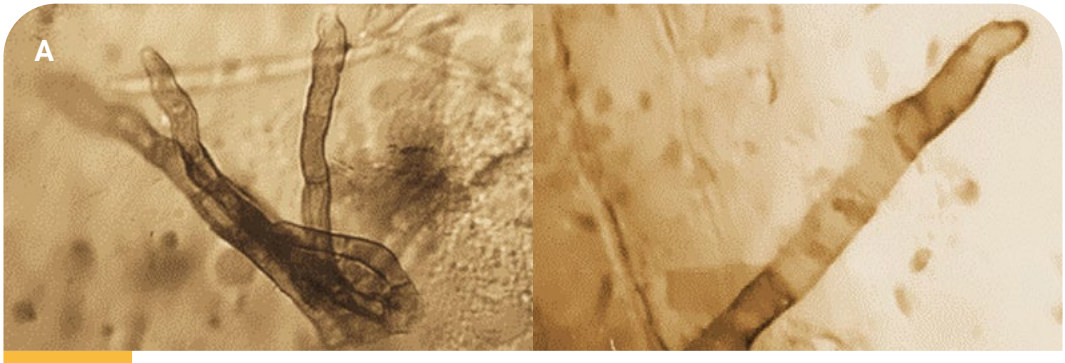


Figure 7. *Pseudocercospora fijiensis* structures: A) Group of geniculated conidiophores with scars and an engrossed basal cell; B) Obclavate conidia with a marked hilum; C) Conidiophore and conidia; D) Pseudothecia (Ps) and spermogonia (Sp) in superior side-view; E) Pseudothecia (Ps), spermogonia (Sp) and conidiophores (Cph) in inferior side-view. (Photos: L. Pérez-Vicente).

FIGURE 7 (continued)

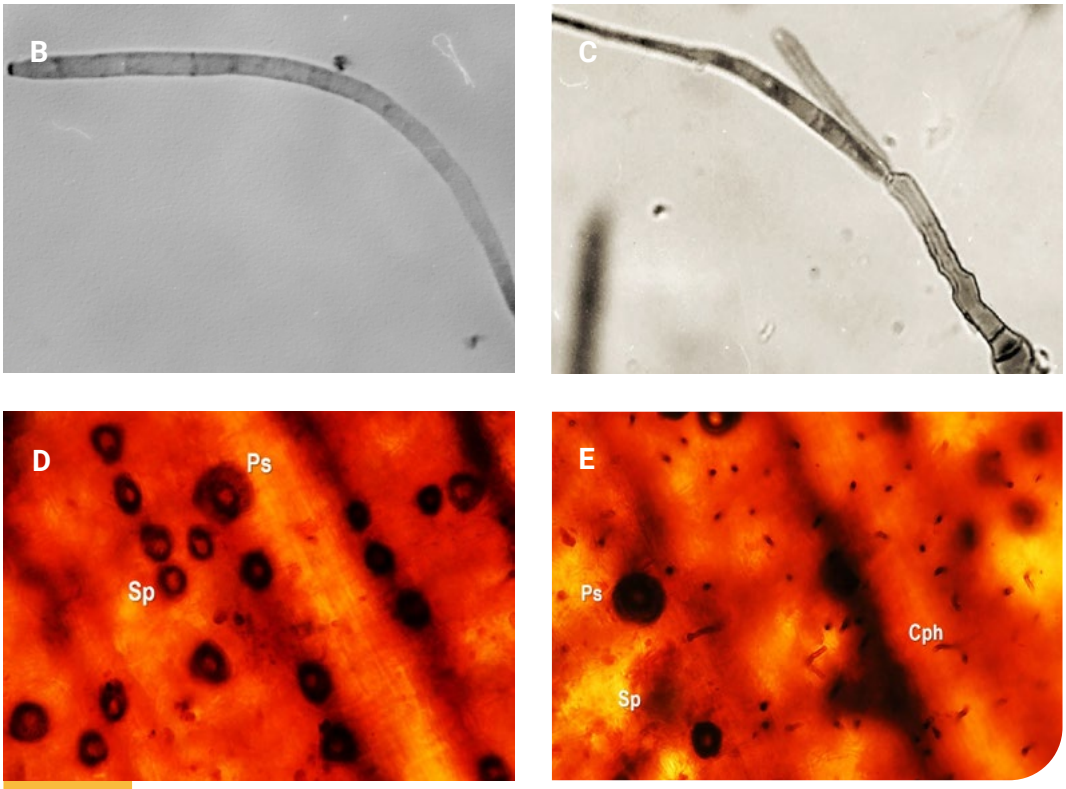


Figure 7. *Pseudocercospora fijiensis* structures: A) Group of geniculated conidiophores with scars and an engrossed basal cell; B) Obclavate conidia with a marked hilum; C) Conidiophore and conidia; D) Pseudothecia (Ps) and spermogonia (Sp) in superior side-view; E) Pseudothecia (Ps), spermogonia (Sp) and conidiophores (Cph) in inferior side-view. (Photos: L. Pérez-Vicente).

Pseudocercospora eumusae

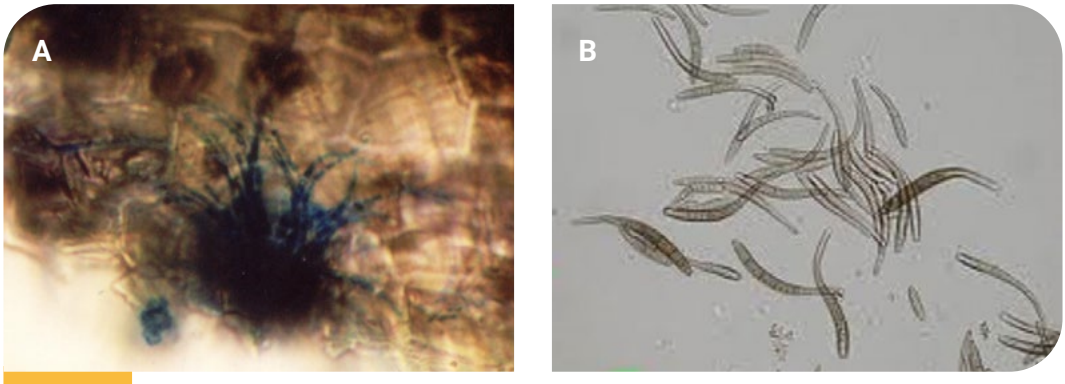


Figure 8. *Pseudocercospora eumusae* structures: A) sporodochia; B) Conidia (Photos: Tania Polanco).

Table 1. Summary of the morphological characteristics of the three *Pseudocercospora* spp. causing leaf spots in *Musa* spp.

SPECIES (ANAMORPH)	CONIDIOPHORES	CONIDIA
Pseudocercospora musae	(Figures 6A and 6B) First appearance at spot stages (in Brun stage 4) Abundant in both surfaces Conidiophore bottle shape in dense fascicles (sporodochia) on dark stromata Straight, hyaline, mostly without septation and geniculation; no spore scars Between 5-25 x 2-5µm	(Figure 6C) Cylindrical to obclavate - cylindrical, pale olivaceous, 0-8 septates, no distinct basal hilum Between 10-109 x 2-6µm
Pseudocercospora fijiensis	(Figure 7A, B and C) First appearance at early, streak stages [Fouré's stages 2 to 3 (Fouré, 1982a)] Mainly lower leaf surface hilum (scar) Emerge singly or in small groups (2 to 6), sporodochia and stromata absent Straight or bent geniculate, pale to light brown 0-5 septates, occasionally branched, slightly thickened spore-scars Between 16.5-62.5 x 4-7µm	(Figure 7D) Obclavate to cylindric-obclavate straight or curved, hyaline to very pale olivaceous, 1-10 septates, with a distinct basal hilum scar Between 30-132 x 2.5-5µm
Pseudocercospora eumusae	(Figure 8 A) First appearance at spot stages Mainly on the upper leaf surface, pear-shaped, immersed, more or less erumpent, (31-42µm)	(Figure 8 B) Fusiform, hyaline, cylindrical and curved, 3-5 septate Between 21.2 to 41.6 x 2.5µm

Adapted from Wardlaw (1972), Carlier et al. (2000), Crous and Mourichon (2002), Guzmán et al. (2018).
The scheme of the full process appears in Figure 9.

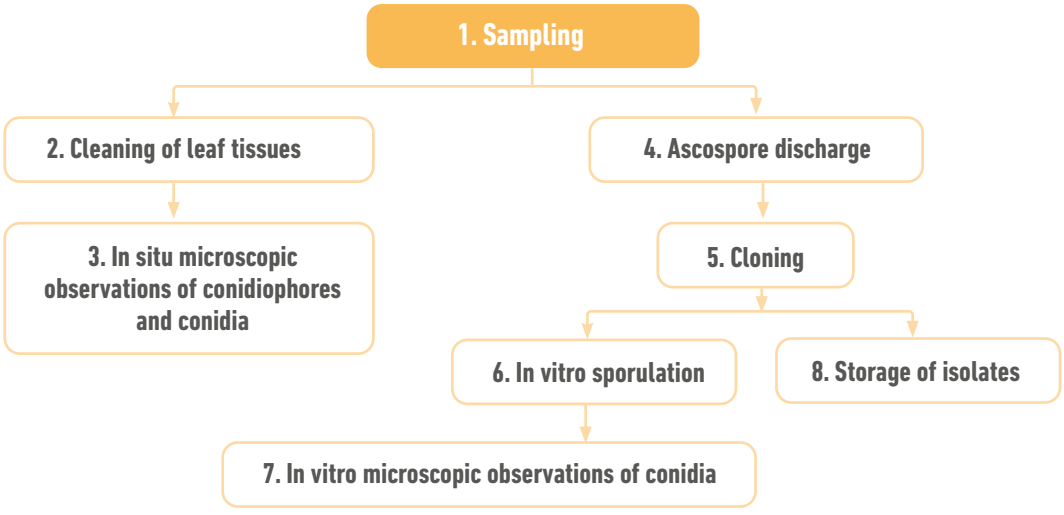


Figure 9. Flow chart for identification of *Pseudocercospora* leaf spot pathogens (reproduced from Carlier et al., 2002).

3. EARLY SCREENING

Phenotyping of the interaction *Musa – Pseudocercospora* in controlled conditions

3.1 Introduction – literature survey

Two major challenges when phenotyping the interaction of *Musa-Pseudocercopspora* are the plant size and the relatively slow growth of both the fungus and plant.

Field evaluation protocols under natural infection have been developed and reviewed by Carlier et al. (2002) and here below. Although they remain the benchmark to select new varieties, they are costly and time consuming, commonly affected by environment fluctuations and do not allow the detection of specific interactions between some strains and plant genotypes or the evaluation of pathogenicity.

For these reasons, efforts have been made to develop inoculation systems in laboratory conditions. Mourichon et al. (1987) first showed that symptoms obtained under controlled environmental conditions were very similar to those observed in fields on mature plants. Since then, several phenotyping methods have been used either to assess the pathogenicity of geographically and genetically diverse strains of *P. fijiensis* or the resistance of their *Musa* hosts for genetic or selection purposes.

Evaluation methods using artificial inoculation under controlled conditions have been developed to get early, rapid, reliable and robust tests of the *Musa* sp.-*P. fijiensis* interaction. The objective is to develop a high-throughput screening method for a large number of individuals (plants or strains).

Plant materials for phenotyping are listed as follows (according to Fullerton and Olsen, 1995; Alvarado Capó et al., 2003; Donzelli and Churchill, 2007; Twizeyimana et al., 2007; Abadie et al., 2008; Kovacs et al., 2013; Leiva-Mora et al., 2015; Torres et al., 2012; Carreel et al., 2013):

- whole *in vitro* plantlets kept under isolation in greenhouses,
- individual leaves of whole plants in greenhouses,
- detached pieces of leaf blades placed with their upper surfaces on an agar medium in Petri dishes and incubated in growth chambers (the most commonly used method), or
- as previous but with leaves from field grown plants after surface sterilization.

Twizeyimana et al. (2007) developed an evaluation test using *in vitro* plantlets growing on a culture medium in tubes and compared results with a detached leaf assay. The authors found that disease development was more rapid on *in vitro* plants than on detached leaves, but reactions were only compared for 10 *Musa* genotypes as plant age is known to influence some plant reactions.

In the bioassay based on detached leaves, to prevent chlorophyll degradation and maintain excised banana leaf squares in a non-senescent state for up to 2–3 months, the adaxial side is deposited on agar. Added to this medium are different plant hormones, such as cytokinin, benzimidazole and

gibberellic acid. Twizeyimana et al. (2007) found that gibberellic acid is the most appropriate hormone to keep leaf fragments green and it is now the standard hormone used.

As mentioned by Churchill (2011), greater awareness and understanding of the effects of the plant and leaf ages and physiologies (particularly between field, greenhouse and growth chamber) and even the environment (light, nitrogen nutrition…) are needed, particularly in the context of molecular analyses of the plant defense response and pathogen aggressiveness.

Although progress has been made, a high-throughput phenotyping protocol is still lacking for the *Musa* sp.-*P. fijiensis* interaction, which would allow monitoring the whole course of infection and all stages of the *P. fijiensis* disease cycle, including sporulation. Except in the field (Cf §disease), few *Musa* accessions have been thoroughly evaluated and studies are usually performed with few *P. fijiensis* isolates. Characterization of *Musa* accessions, in particular diploid genitors, by precise phenotyping in controlled conditions could identify different sources of BLS resistance. More knowledge is needed on specific interactions between *Musa* and *P. fijiensis* genotypes as well as more evaluation of the quantitative traits of aggressiveness.

Controlled production of ascospores in the laboratory is difficult because *P. fijiensis* is a heterothallic fungus. Mourichon and Zapater (1990) obtained some ascospores *in vitro* and used the segregating population for genetic analysis (Arango et al., 2016). Ascospores coming from necrotic banana leaves collected from the field are sometimes used to phenotype new hybrids, but isolates sources are thus not controlled.

As **inoculum source**, authors usually use:

- **mycelial fragments** (either count as fragments/ml or in mg/ml) (Alvarado Capó et al., 2003; Twizeyimana et al., 2007; Donzelli and Churchill, 2007; Leiva-Mora et al., 2015),
- and/or **conidial suspensions** with very variable concentrations which will need to be standardized (Fullerton and Olsen, 1995; Donzelli and Churchill, 2007; Twizeyimana et al., 2007; Pérez-Vicente et al., 2006; Abadie et al., 2008; Torres et al., 2012; Kovacs et al., 2013; Leiva-Mora et al., 2015, with or without filtration of larger fragments and hyphae as in Fullerton and Olsen (1995)).

Symptoms are usually higher and faster in inoculations with mycelial fragments than with spore suspensions. The level of disease was found to be more correlated with the amount of applied mycelium than the degree of fragmentation of hyphae (Donzelli and Churchill, 2007). Although stricter conditions should be followed to produce conidia, quantification of conidial suspensions is more precise, and thus should be more adapted to evaluate quantitative traits of aggressiveness or resistance (Abadie et al., 2008). However, mycelia inoculum can be used with virulent isolates deficient in conidia production *in vitro* (Donzelli and Churchill, 2007).

3.2 Preparation of inoculum suspension

Inoculum suspensions are prepared from *P. fijiensis* virulent single spore isolate cultures stored in glycerol (15%, see 2.7–2.9) at -80°C. Inoculum can consist of conidia (Mourichon et al., 1987; Fullerton and Olsen, 1995) and/or mycelia fragments (Leiva-Mora et al., 2002; Alvarado-Capó et al., 2003; Donzelli and Churchill, 2007, 2009; Twizeyimana et al., 2007). The procedure is as follows (Figure 10):

Reactivation from -80°C stock of an isolate on Potato Dextrose Agar (PDA): Inoculum can be prepared inoculating Petri plates with sterile medium of PDA (39 g/l, see Annex 1). Several small plugs of mycelium are put on PDA for 8–10 days at 25°C with a 12 h light period.

If needed, transplant the isolate on PDA every 10 days; same conditions as before.

Preparation of mycelium for production of conidia: Place four to five plugs of mycelium obtained in PDA as described above in 1.5 mL sterile water in 2 mL tubes with a ceramic bead and grind 3*20s at 4M/s in a Fast Prep.

Production of conidia: Pipette about 0.5 mL of the ground mycelium in a 55 mm diameter plate with V8-sporulation sterile medium (commercial V8 vegetable juice 100 mL/L + 0.2 g/L $CaCo_3CaCO_3$, adjusted pH to 6, then add 20 g/L of agar, see Annex 1). Put at 24 h light for 11 to 13 days at 20°C.

Conidia suspension: Glassware and low-retention tips should be used to avoid loss of conidia by adhesion to plastic. Add 10–12 mL of sterile water to sporulation plates and sonicate to get conidia suspension or lightly brush the colony surface with a spatula. The conidial solution should be filtered if too many mycelium fragments are present in the suspension.

Inoculum suspensions are usually adjusted with haemocytometer or for high-throughput screening, with a coulter to concentrations above 10^4-10^6 conidia/ml. It is usually checked by spraying conidia suspension on petri dishes with PDA, followed by colony number evaluation.

It is advisable to add Tween 80 (0.02%), Triton X-100, Silwet L-77 or gelatin, to the mycelial/spore suspensions to facilitate inoculum homogenization and/or adhesion to the leaf (Donzelli and Churchill, 2007; Abadie et al., 2008).

As BLS mostly occurs on the abaxial leaf in natural infections, the inoculation procedure is always done on the lower side of the leaf either:

- by droplets
- by camel's hair brush
- or spray

(Fullerton and Olsen, 1995; Donzelli and Churchill, 2007; Twizeyimana et al., 2007; Abadie et al., 2008; Kovacs et al., 2013; Leiva-Mora et al., 2015; Torres et al., 2012; Carreel et al., 2013).

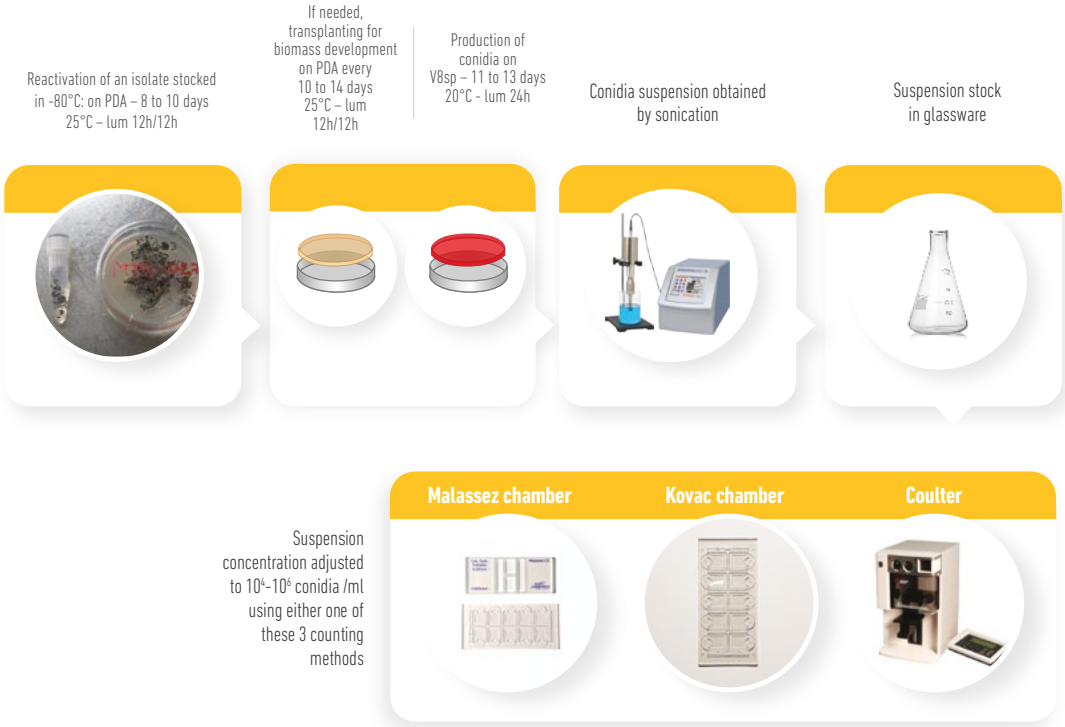


Figure 10. Scheme of conidial suspension preparation (Photos: CIRAD).

3.3 Plant material

The evaluated plants should be the same age and, if possible, should come from tissue culture.

Tissue culture plants are usually grown in isolation in greenhouses in plastic pots of approximately 500 mL capacity for 4–8 weeks under reduced luminosity (60–70%) and adequate cultural growing conditions (irrigation and nutrition) and then transplanted in 1L and 5L pots. Inoculations are carried out on non-juvenile plants between 4–9 months old. As mentioned above, more knowledge is needed on the effects on the interaction of plant age and physiology and its growth environment (light, nitrogen nutrition…); so, all these conditions should be comparable and noticed.

Reference accessions that are susceptible, partially resistant and highly resistant should be added to the assay (see 4.2).

3.4 Essays with full plants

Inoculations are carried out on the abaxial surface of the 1 to 3 youngest leaves of at least 3 plants by cultivar, with an atomizer or a fine brush. In this latter case, 1% of gelatin can be added to the suspensions to improve adhesion of infective structures to the abaxial leaf surface (Leiva-Mora et al., 2015).

After inoculation, plants are kept for 72 hours at 25°C in a saturated atmosphere. After this period, the incubation environment is then alternated from >50% relative humidity and high illumination for 9 h a day to 100% relative humidity for 15 h at night.

Assessments of disease development are carried out according to the parameters explained below (see section 4.3 *Parameters*):

- **P2.** Disease severity Index at 30, 45 and 60 days
- **P6.** Disease development time (DET)
- **P8.** Transition period from streaks to spots
- **P9.** Reproduction of spots

3.5 Essays with detached leaves

3.5.1 Plant material preparation

Select the leaf which is the youngest fully mature leaf. It is the first unfolded leaf after the cigar leaf when it is in stages between 0.6 and 0.8 and select the 2nd unfolded leaf when cigar is between stages 0.0 and 0.4 (Figure 11). This leaf gives more reproducible results but if necessary, the second youngest leaf may be used as well. Cut the leaf and bring it back to the laboratory. To conserve the leaf until needed, put the stem in water. Plant age influences some accessions' interactions with *Pseudocercospora*, so plant age must be recorded.

Leaves can come from the field, but we advise to get them from plants of same age issued from *in vitro* plantlets in greenhouse free of disease. If from the field, surface sterilize leaf pieces in 1% NaOCl solution for 90 secs and wash five to six times in sterile distilled water (Viljoen et al., 2016).

Put cut leaf pieces (6 cm × 6 cm) in a petri dish with the upper leaf surface facing down on the survival medium (0.4% bacto agar and 5 mg/L of gibberellic acid: GA3; a mother solution of GA3 at 1.25 mg/mL may be warmed up to 40°C and filtered to sterilize then added to cooled autoclave agar medium, see Annex 1).

To maintain leaf fragments in contact with the media, place plastic transparent lamina with an open square (5 cm x 5 cm) in the center on top of the leaf fragment. After inoculation, seal the plates with cellofrais.

The complete procedure scheme appears in Figure 11.

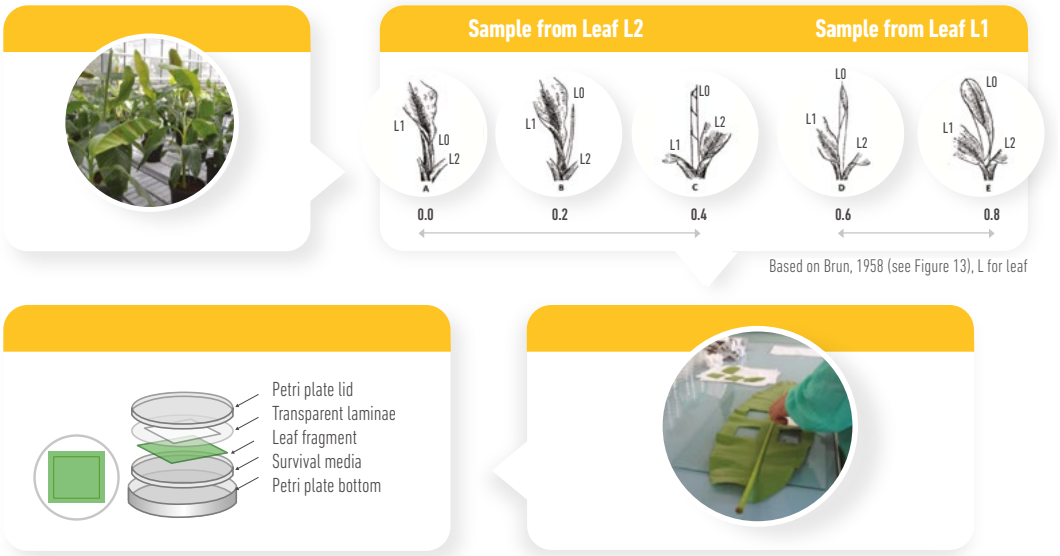


Figure 11. Procedure to prepare detached leaves fragments in survival media (Photos: CIRAD).

3.5.2 Preparation of inoculum suspension

Musa-Pseudocercospora interactions vary according to the strain, so suspension should be obtained as much as possible from a single spore culture. The strain origin must be recorded. One or two reference strains should be added to all experiments as a control: a middle aggressive CIRAD-COL064 and a highly aggressive CIRAD-GLP701. For inoculation of detached leaves, it is advisable to use conidial suspensions as explained in 3.2 and Figure 10.

3.5.3 Inoculation procedure and evaluation

Inoculation (Figure 12) is applied by 0.5 mL of inoculum suspension, with a micro sprayer (1.5 kg·cm−2) held vertically over the leaf pieces at a height of 40 cm or, alternatively, by placing 2 µL or 4 µL droplets of inoculum suspension on the leaf (Abadie et al., 2008). It is important to avoid condensation in the petri dish to keep the leaf pieces alive. It is advisable to use a climatic chamber with a circular movement of fresh air.

For the evaluation, symptoms appear earlier on *in vitro* plantlets or leaves from whole plants than on detached leaves, but standardization of the notations is easier on detached leaves.

The different methodologies are complementary and give access to different traits of pathogenicity and different events of interaction, but they will need to be compared to be able to cross drive experiments (cross reference data).

The *Musa-P. fijiensis* interaction is a long process. Observations start from few hours after inoculation to 4–6 weeks on susceptible and partially resistant plants. The evaluated quantitative traits are (and see 4.4):

- incubation time
- infected leaf area or proportion over time as the Area Under the Disease Progress Curve (AUDPC)
- symptom evolution time
- stage of symptoms
- latent period

Image analysis software has been recently used to measure symptoms number and size from which one can estimate the percentage of the infected leaf part. In image analysis software, the threshold levels to recognize the disease area can be manually or automatically adjusted (Donzelli and Churchill, 2009; Carreel et al., 2013). Donzelli and Churchill (2009) discuss different statistical transformations and analysis methods.

Figure 12. Scheme of the inoculation procedure and assessment of disease development in the detached leaf assay.

3.5.4 Data to record

Plant: name, age, culture condition, nutrition and temperature

Strain: name, origin (country and accession) and date of isolation

Inoculum: origin (mycelial suspension of conidia), concentration, product added (for example, Tween)

Incubation time (first appearance of symptoms)

And up to 6 weeks, at least once a week:

- Disease severity (surface infected)
- Number of symptoms
- Surface/symptoms
- used to calculate AUDPC (see also 4.3).

3.5.5 Troubleshooting

Survival problems of leaf pieces may be due to:

- condensation on cover plate; check the homogeneity of the temperature and air flow in the chamber and try stacking empty petri dishes.
- the quality of the petri dishes
- other diseases or bad growth of plant material; try biological control and slow release fertilizer.

Control accessions should be added to all experiments. They may be chosen among accessions with known data (see Table in 4.2).

4. FIELD SCREENING

Musa spp. and important disease traits for the assessment of cultivar reaction

4.1 *Musa* spp. leaf production and leaf emission rate (LER) assessment

Leaf development has been well studied in Cavendish cultivars (Stover and Simmonds, 1987). In order to understand the unfolding process, it is important to note that the formation of the leaf takes place within the pseudostem before shooting. The new leaf (called a cigar leaf) is tightly coiled, whitish, and particularly fragile. The shooting of the leaf results in a rapid growth of the leaf sheath (4 m in 10 days for 'Gros Michel'). The young leaf slips into the petiole canal of the preceding leaf and thus the development of a new leaf corresponds to two successive phenomena, that of 'growth' and that of 'unfolding' (Carlier et al., 2002).

The young unfolded leaf is coiled into a double spiral. The right half lamina 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 duration of leaf development varies. Leaf emission rate depends on the cultivar, environmental conditions and cultural practices. Under favorable climatic conditions, one leaf per week is emitted, but this can increase up to 20 days under unfavorable conditions (drought, nutritional deficiency, etc.).

The unfolding process has been divided into five successive stages (Figure 13) to allow a quantitative description. 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:

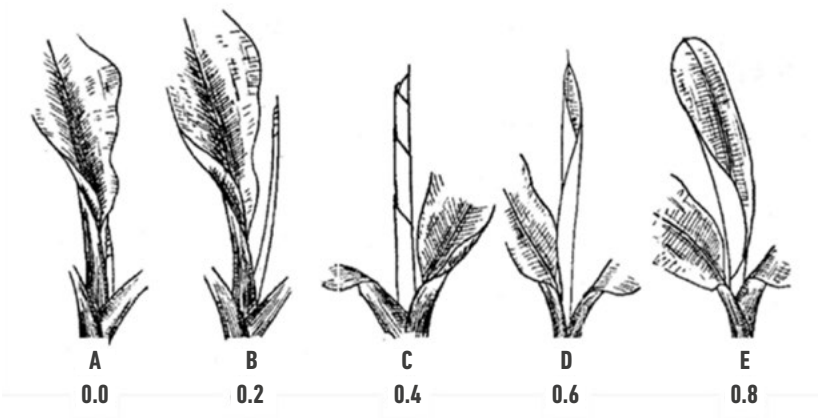


Figure 13. Stages of an unfolding leaf (according to Brun, 1958).

Stage A: The 'cigar', about 10 cm in length, is still joined to the preceding leaf value 0.0).

Stage B: The 'cigar' is bigger but has not yet reached its full length (value 0.2).

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 (value 0.4).

Stage D: The left-hand side has already unfolded, and limb deployment takes place at the extreme apex (value 0.6).

Stage E: The upper part of the leaf has unfolded, and the base is in an open cornet shape (value 0.8).

The phenological stage of a plant can then be defined by the number of open leaves completed by a decimal part defined by the stage of the unfurled leaf as defined in Figure 13. For example, a plant with 11 open leaves and a cigar leaf in stage C receives a notation of 11.4.

This is applied to each plant in a plot. The leaf emission rate (LER) of each plant is defined by the difference of leaf emissions between two consecutive assessments taking into account the loss of older or damaged leaves. It can be expressed by day or by week. The LER varies with genotypes and agronomical and environmental conditions of the crop (humidity and nutrition). It is usually less than one leaf per week (depending on environment and growth conditions) and should be calculated regularly (at least monthly) for each test and reference plant, beginning three months after planting until bunch emergence (shooting). Record data in Field Data Form 2 (Annex 2).

4.2 Reference cultivars

Diploid and triploid clones against which the new, improved hybrids are to be evaluated for their reaction to *Pseudocercospora* leaf spot diseases are listed here (with associated ITC code), depending on the ploidy of the accession/hybrid to test:

ITC CODE	CULTIVAR	LEVEL OF RESISTANCE
ITC0249	Calcutta 4 (AAw)	Highly resistant
ITC0407	Khom (AAA)	Highly resistant
ITC1587	Pisang Klutuk Wulung (BBw)	Highly partially resistant
ITC1441	Pisang Ceylan (AAB)	Highly partially resistant
ITC0258	Pisang Madu (AA)	Partially resistant
ITC0414	Pisang Sri (AAA)	Partially resistant

ITC CODE	CULTIVAR	LEVEL OF RESISTANCE
ITC0663	Pisang Kha Nai On (AA)	Susceptible
ITC1256	Grande Naine (AAA)	Susceptible
ITC1254	Paka (AA)	Variable with aggressiveness of strain
ITC1123	Yangambi Km5 (AAA)	Variable with aggressiveness of strain

And see other accessions in Guzmán et al. (2018), Figure 2.9 page 82.

It is advisable to use a well-known local cultivar at each site as an appropriate standard to compare reactions. Bioversity International, through the *Musa* Germplasm Information System (MGIS, www.crop-diversity.org), compiles a list of virus-indexed material from which reference genotypes can be selected (Carlier et al., 2002).

4.3 Parameters (P) to record to assess a cultivar's disease reactions

The evaluation of the level of resistance to *Pseudocercospora* leaf spot diseases requires knowledge of the stages of both the process of leaf unrolling (Figure 13) and symptom development. The evaluation of resistance should begin three months after planting until at least flowering, but preferably until harvest. Every test plant, except the extra plants at the ends of rows, should be used for data collection. The following parameters are used to assess the reaction of banana genotypes to *Pseudocercospora* leaf spots:

P1. Total number of leaves

Total number of erect leaves (green or necrotic) that are not pending along pseudostem (petioles erect).

P2. Disease severity index

An index to express the degree of leaf area of all standing leaves on the plant affected by *Pseudocercospora* spp. leaf spot diseases. Disease severity is the amount of leaf area affected by *Pseudocercospora* leaf spots and can be expressed in disease grades or in percentage. Leaves should be graded using Gauhl's modification of Stover's severity score system (Gauhl, 1994; Figure 14). Assessments should be carried out monthly from third month after planting until harvest. The following data should also be recorded:

- Date of bunch emergence (shooting)
- Date of harvest
- Disease grades should be recorded for each leaf on each test plant. Field forms 1 and 2 are provided to record these data (see Annex 2).

- Only upright leaves should be recorded (with petioles upright). After disease severity has been recorded, the infection index for each test plant should be calculated following the formula:
- Disease severity index (Figure 15): = [Σnb/(N-1) T]x100 (McKinney, 1923; Horsfall and Heuberger, 1942)

Where

n = number of leaves in each grade

b = grade

N = number of grades used in the scale

T = total number of leaves scored

Area under the curve of progress of the disease

Another way to determine disease progress over time is the calculation of area under the curve of progress of the disease with the formula (AUDPC; Campbell y Madden, 1990):

$S[(x_i + 1 + x_j)(t_{i+1} + t_i)/2]$ where:

x_i = proportion of disease in the ith counterparty observation

t_i = time in the ith counterparty observation

I = from 1 to N

P3. Youngest leaf with streaks (YLStr)

Counting down from the top of the plant, the youngest leaf with streaks is the youngest open leaf with lesions in stage 1 or 2 of Fouré (1982a) description. Data is recorded in field forms 1 and 2 in Annex 2.

P4. Youngest leaf spotted (YLS)

Counting down from the top of the plant, the youngest leaf spotted (YLS) is the youngest open leaf with at least 10 discrete, mature, necrotic lesions or one large necrotic area with 10 light-colored dry centers (Figure 16). After shooting, when leaves cease to be produced, the YLS value should be recorded weekly until harvest. YLS is correlated with infection severity. Data is recorded in field forms 1 and 2 in Annex 2.

P5. Proportion of healthy leaves and Index of non-spotted leaves (INSL)

The number of healthy leaves is an important indicator of the reaction of the plant to leaf spot pathogens. It can be estimated as the proportion (as decimal or percentage) of leaves rated in grade 0 of the total leaves assessed. It can be also estimated as an Index of non-spotted leaves (INSL), from YLS values obtained in the assessment by the following formula:

INSL = (YLS-1)/NL where: NL: T = total number of leaves scored

P6. 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 leaf (Fouré 1982a; see Figure 17). It is assumed that infection occurs in the first five days after leaf emergence. Plants with cigar leaves near Brun's stage B (Figure 13) should be selected and marked (permanent black felt-tip pen, colored ribbon, tags) with the date at which it was estimated that the leaf was at stage B. Note the date on the field form 3 (see Annex 2). These leaves should be inspected once or twice a week until the ultimate necrotic stage of the disease (stage 6) or one large necrotic area with at least 10 light-colored dry centers (Figures 2 and 4) is visible. 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 (or at least twice during each rainy and dry season).

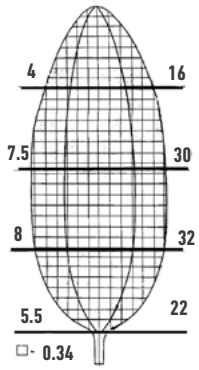
P7. Symptom evolution time

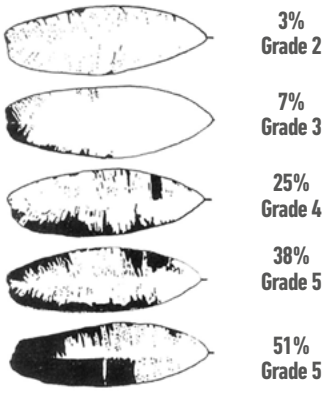
This is an approximation of incubation time. It is estimated by the formula YLStr/LER.

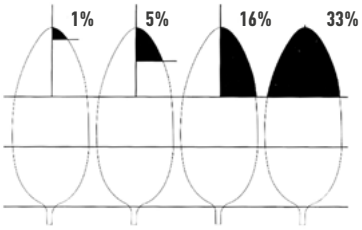
P8. Transition period from streaks to spots

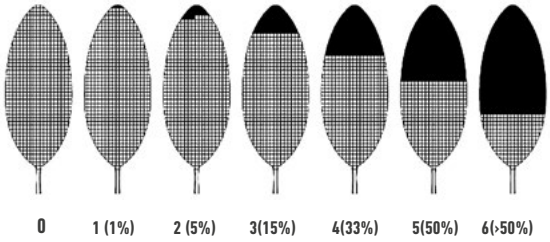
The transition period is the time in days, between the symptoms stage 1 to spots at last stage of evolution. It has been used by Simmonds (1939), Vakili, (1968), Meredith and Lawrence (1969), Firman (1972), Fouré (1982a, b), Fouré (1994), Fouré et al. (1984) and Hernández and Pérez (2001), to determine the reaction of banana cultivars to *P. musae* and *P. fijiensis* in Fiji, Hawaii, Cameroon and the Caribbean Islands. This parameter is difficult to access in very tall genotypes and is time consuming although very informative to detect partial resistance. It can be used in essays on greenhouses or in early assessments of disease development in field trials.

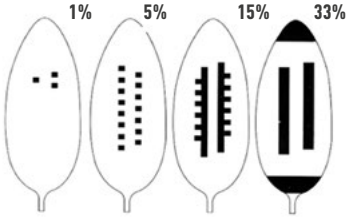
Leaves near Brun's stage A should be selected and marked with permanent black felt-tip pen, colored ribbon, or tags and date of the appearance recorded. Data are taken from 10 plants sampled in the plot. The leaves should be inspected twice a week until appearance of lesions at stage 1 of Fouré (1982b) description. The date is also recorded. The symptoms are observed until the ultimate necrotic stage of the disease (stage 6) is visible and date is recorded. The time at which mature lesions appear should be estimated if this occurs between inspections. The transition period is estimated from the difference between both dates. This process should be repeated at least twice during each rainy and dry season.

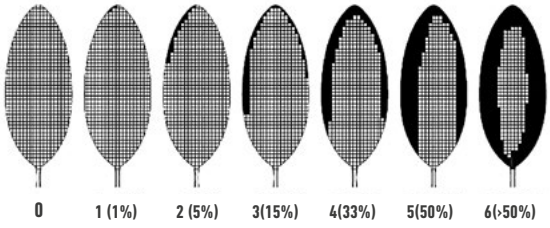








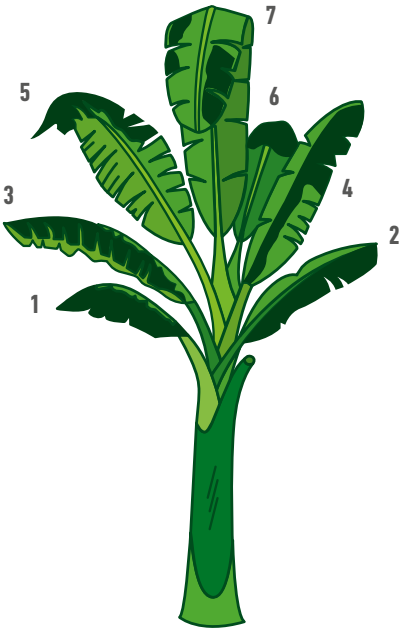




Scale of Severity Description

0 Healthy
1 < 1% leaf area affected (streaks and until 10 spots)
2 Until 5% of necrotic area
3 Between 6 and 15% of necrotic area
4 Between 16 and 33% of necrotic area
5 Between 34 and 50% of necrotic area
6 > than 51% of necrotic area

Figure 14. Gauhl's modification of Stover's severity scoring system (Gauhl, 1994).



Infection index= [∑nb/(N-1)T]x100
(McKinney, 1923; Horsfall and Heuberger, 1942)

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:

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

Figure 15. Scheme of calculation of infection index (Adapted from Carlier et al., 2002).

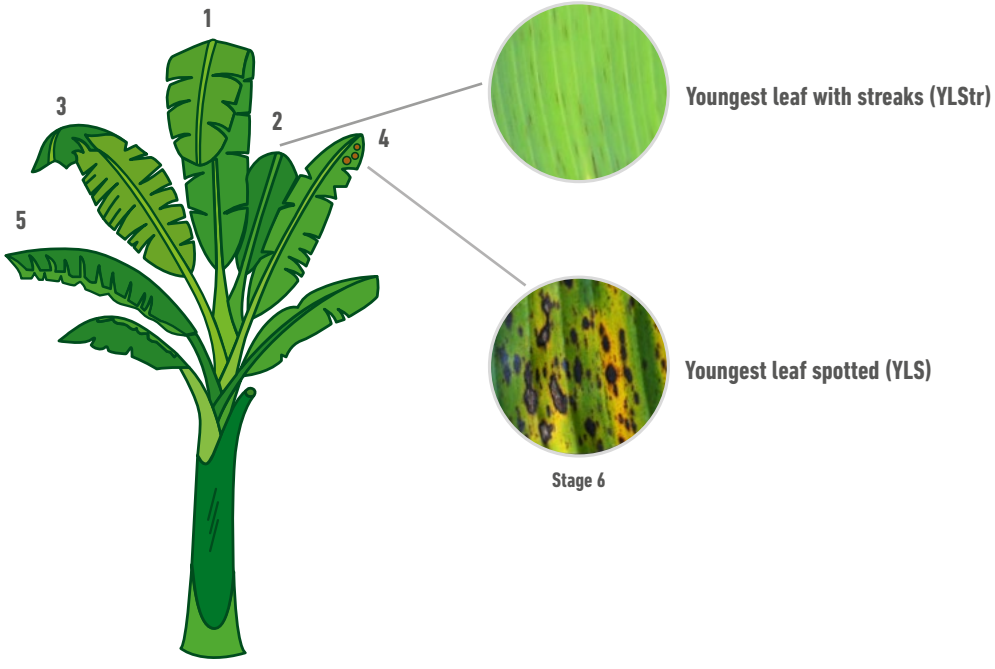


Figure 16. Scheme of the youngest leaf with streaks (YLStr) and youngest leaf spotted (YLS) determination (Adapted from Carlier et al., 2002)

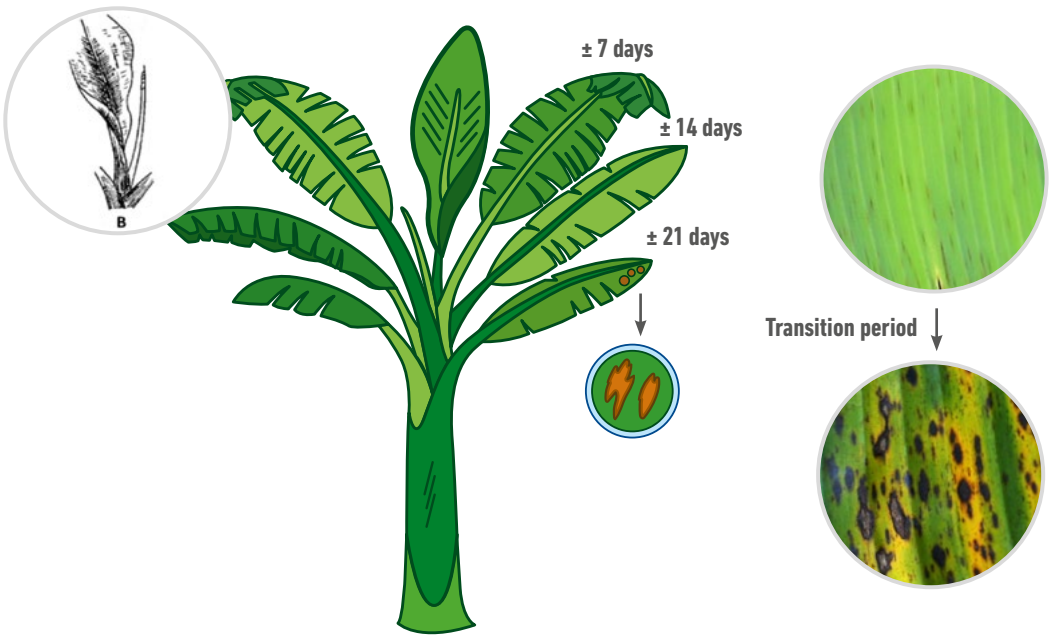


Figure 17. Scheme of disease development time determination (DDT, adapted from Carlier et al., [2002]) and transition period from streaks to spots according to descriptions of Simmonds (1939), Meredith and Lawrence (1970) and Hernández y Pérez (2001).

P9. Reproduction of spots

Partial resistance is expressed by a lengthening of the evolution duration of symptoms and a reduction of sexual reproduction of the fungus in the lesions. It is the mean of the amount of pseudothecia, and spermogonia counting in three microscope fields (40 x 10 magnification) of 50 individual spots at stages 5 and 6 of previously tagged leaves at stage A or B which have been collected from 10 plants of the banana genotype. The reproduction on a given cultivar is assessed together with the reproduction of the standards clones. The quantification of spermogonia, pseudothecia and conidiophore in lesions in each plant is carried out by tagging an unfurled leaf at stage A-B and allowing the lesions to develop to stage 5. When stage 5 is reached, five isolated spots are detached from the leaves of each of 10 plants (50 spots/banana genotype) and decolored following any of the procedures explained in section 2.3 (tissue clearing and *in situ* microscopic observations). Once the tissue is cleared (transparent, observable at microscope), the spots are mounted on slides or on the tops of glass petri plates and observed under transmitted light of a microscope. The total number of spermogonia, pseudothecia and conidiophores (shown in Figure 7) can be observed at three microscope fields (at 40 x 10 magnification) at the upper side of each spot. For each observation, data of the cultivars are submitted to ANOVA and statistically compared with data of the reference clones.

4.4 Statistical analysis

Temporal kinetics: ANOVA of quantitative parameters for each period evaluation and AUDPC.

Dunnett test: to compare the quantitative parameters for evaluated accessions and the reference cultivars control data (S, PR).

4.5 Establishment of plots

The experimental fields must be established in areas where the disease pressure is high. 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 *Pseudocercospora* leaf spot diseases. It is thus preferable to choose sites where only one leaf spot disease is present. The presence of several pathogens will not allow comparison with other evaluation sites.

All reference cultivars should be included in experiments carried out in different selection phases or to characterize resistance of genitors and elite hybrids.

4.6 Procedures for field experiments in different breeding selection phases

4.6.1 Field experiments at early selection (phase 1) with many hybrids/accessions

Layout. The layout can be the one reported by Abadie et al. (2009). Genotypes can be planted in a randomized quincunx design, where each genotype is surrounded by four plants of *Pseudocercospora* leaf spots susceptible 'Grand Nain' (AAA genome; Cavendish subgroup), at a density of 2000 plants/ha (2 x 2.5 m) in five replications. If there is a large number of genotypes to include in the early selection phase where a randomized quincunx design would take up a large surface, it is possible to put a line of genotypes to evaluate in this selection phase between the lines of susceptible Grand Nain.

No fungicides are applied. Figure 18 shows a possible layout for the early selection phase of many hybrids and accessions.

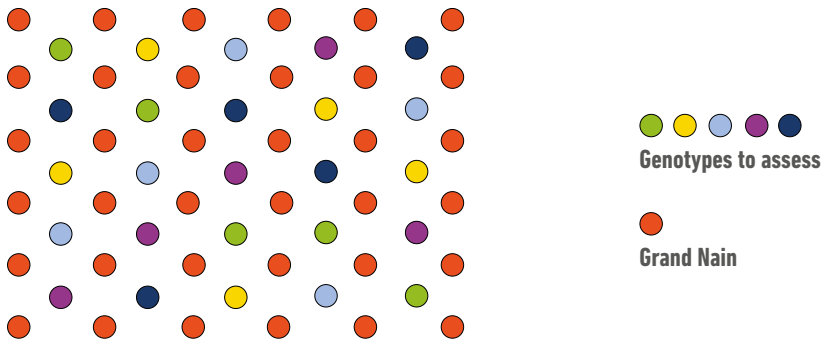


Figure 18. Layout for the early selection phase with many hybrid accessions.

Assessments. In the early screening (phase 1) with many hybrids/accessions, the parameters to assess are (see description in section Parameters (P) to record to assess cultivar's disease reactions):

- **P1.** Total number of leaves
- **P3.** Youngest leaf with streaks (YLStr)
- **P4.** Youngest leaf spotted (YLS)
- **P5.1** Proportion of healthy leaves and/or P5.2 Index of non-spotted leaves (INSL)

4.6.2 Field experiments on late selection (phase 2) and characterization of resistance of genitors and elite hybrids

Layout. The layout is a randomized complete block design with four to eight clones per block, 20–25 plants/clone/plot and 2–4 replications. Each plot should be surrounded by a row of susceptible border plants. The clones are independently randomized within each of the three to five replications. One possible example of a field layout is shown in Figure 19. 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 m space between plants in each row and 3 m between rows.

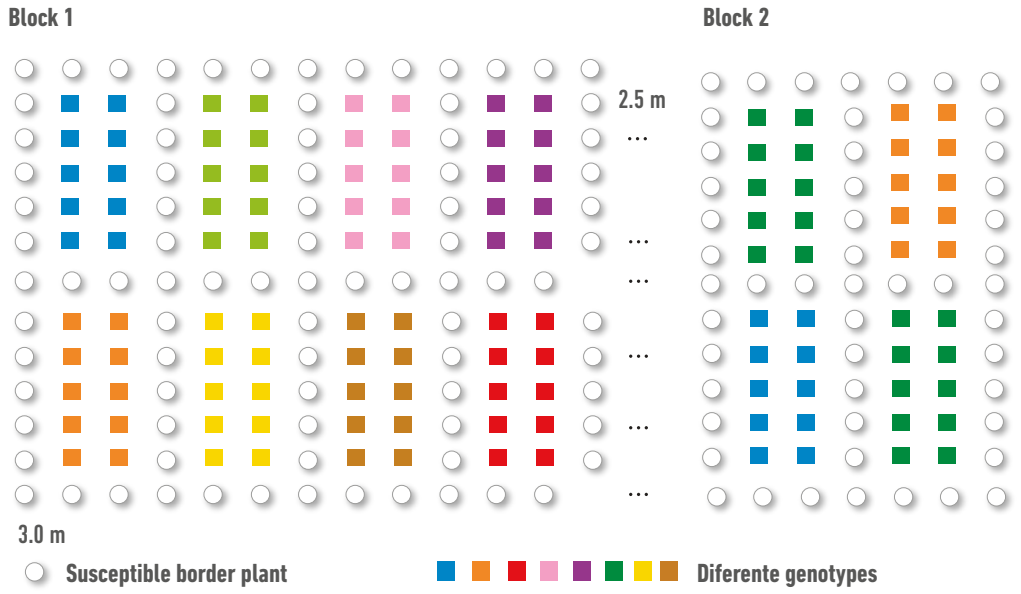


Figure 19. Layout of the randomized complete block design for phase 2.

Assessments

In field experiments on characterization of resistance of genitors and elite hybrids, as well as to determine genetic of resistance (QTL approach), the parameters to assess are (see description in section 'Parameters (P) to record to assess cultivar's disease reactions', page 51):

- **LER:** (see section 4.1: '*Musa* spp. plants leaf production and leaf emission rate assessment')
- **P1.** Total number of leaves
- **P2.** Disease severity index: before flowering and at flowering
- **P3.** Youngest leaf with streaks or symptoms evolution time (YLStr)
- **P4.** Youngest leaf spotted (YLS)
- **P5.1** Proportion of non-spotted leaves and/or
- **P5.2** Index of non-spotted leaves (INSL)= (YLS-1)/NL
- **P6.** Disease development time (DDT). Field form 3 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.
- **P7.** Symptoms evolution time (SET)
- **P8.** Transition period from streaks to spots
- **P9.** Reproduction of spots.

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. However, to obtain a reliable data on reaction of cultivar against *Pseudocercospora* spp. populations present on the sites, proper management practices regarding nutrition, weed control and irrigation should be carried out in the experimental field.

The data should be collected on the mother plant and first sucker (2 successive cycles).

Table 2 shows a complete list of variables. Fruit characteristics need not be recorded.

The following agronomic traits should be recorded (Carlier et al., 2002):

- *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% 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 seventh 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 2. Timetable for recording disease evolution and agronomic data.

TYPE OF DATA	GROWING PHASE (FROM 3 MONTHS AFTER PLANTING)	SHOOTING PHASE	SHOOTING TO HARVEST PHASE	HARVEST
DISEASE EVOLUTION DATA				
Disease development time	X			
Youngest leaf spotted	X	X	X	X
Leaf emission rate	X			
Disease severity (from 3 months after planting)	X	X	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	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 closest meteorological station to the trial plot. Where trials are conducted in the grounds of collaborating institutes this should not be a problem.

Daily fluctuations in temperature and in humidity should be monitored. Data should be taken at the same hour every day and as early as possible. Weekly rainfall can be calculated if daily readings cannot be taken. Readings should begin at planting and continue until harvest. The soil of the test site should be analyzed. 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.

A format for recording environmental data is provided on field form 4 (see Annex 3).

Management data

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

Classification of reaction

Table 3 proposes a classification of cultivars reaction according to the different parameters measured during two cycles. It includes vegetative and flowering stages until harvest. Due to the impact of nutrition and physiological stress caused by lack of proper irrigation, the classification of reaction (phenotyping) should be carried out under a balanced nutrition and proper water supply during the most favorable season for disease development.

Table 3. Reaction of cultivars to black leaf streak during most favorable conditions for crop and disease development (under balanced nutrition and no irrigation stress).

PHENOTYPE REACTION		P1.	P2. SEVERITY INDEX BEFORE FLOWERING	P2. AT FLOWERING	P3.	P4. YLS AT FLOWERING/ FUNCTIONAL LEAVES	P4. YLS AT HARVEST	P5. PROPORTION OF NON-SPOTTED LEAVES	P6.	P7.	P8.	P9.
Incompatible reaction	HR Calcutta 4 or Khom		0	0		X	All standing leaves produced by plant	100	X	No	Blockage of symptom development before stage 2	No
Compatible reaction	HPR Pisang Klutuk Wulung or Pisang Ceylan		<15	<15	>7	>10	9 or more	>80	>80	30-40	80 days or stop evolution at stage 3	Low
	PR Pisang Madu or Pisang Sri		15-25	15-25	>4	7-10	6-8	60-80	40 -80	15-30	50-80	Interme-diate
	S Grand Nain or Khai Nai On		>25	>25	<3	2-7	<6	<60	20-40	<15	<50	Intense

P1. Total number of leaves
P2. Disease severity Index
P3. Youngest leaf with streaks or symptoms evolution time (YLStr)
P4. Youngest leaf spotted (YLS)
P5. Proportion of non-spotted leaves
P6. Disease development time (DET)
P7. Symptoms evolution time (SET). It is an approximation of incubation time estimated by the formula YLStr *LER
P8. Transition period from streaks to spots
P9. Reproduction on spots

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Annexes

ANNEX 1. CULTURE MEDIA MENTIONED IN THE TEXT

Media for *P. fijiensis* culture and growth

PDA (from Tuite, 1969): Potato Dextrose Agar

PDA powder agar Difco or Oxoid 39 g

Distilled water 1 L

Add the powder to water and agitate, until dissolution in a hot water bath. Refill consumed water to 1 L.

Place 150 ml aliquots in 250 ml Erlenmeyer flasks and sterilize in autoclave for 20 min. If required, add chloramphenicol or streptomycin sulfate 100 µg and Penicillin G at 100 UI after autoclaving.

Or PDA: Potato Dextrose Agar

Peeled sliced potatoes 200g

Dextrose 20 g

Agar 12–20 g according to manufacturer

Refill consumed water to 1 L

Wash and peel potatoes and cut into pieces. Boil in 500 ml of water for an hour. Sieve the solution through a sterile cheese cloth. Dissolve agar in 500 ml of distilled water. Mix the resulting solutions and sterilize. If required add chloramphenicol or streptomycin sulfate 100 mg/L after autoclaving.

Place 150 ml aliquots in 250 ml Erlenmeyer flasks and sterilize in autoclave for 20 min. If required, add 50 ml of chloramphenicol 100 mg/L. It can be acidified with 25% lactic acid, 3–5 drops/100 ml of melted agar when used for fungi isolation. Do not re-melt after acidifying. If required, add chloramphenicol or streptomycin sulfate 100 mg/L after autoclaving.

Potato carrot juice agar

Peeled sliced potatoes 200g

Carrot pieces 20 g

Dextrose 20 g

Agar 12–20 g according to manufacturer

Refill consumed water to 1 L.

Wash potatoes and carrots, peel and cut into pieces. Boil both in 500 ml of water for an hour. Sieve the solution through a sterile cheese cloth. Dissolve agar in 500 ml of distilled water. Mix the resulting solutions and sterilize.

V8-sporulation media – for 1 L

V8 commercial juice 100 ml

$CaCO_3$ 0.2 g

Agar 20 g

Distilled water to make 1000 ml

Mix V8 juice with $CaCO_3$ and water up to 1 L of media. Adjust pH to 6. Then add Agar. Add streptomycin sulfate 100 µg and Penicillin G at 100 UI after autoclaving.

Media for detached leaf assays

Agar 4 g

Water 1 L

GA3 5mg/L for that prepare a GA3 solution at 1.25 mg/ml by warming it (<50°C) and then filtrating it with a 0.20 µm filter under sterile condition. Add 4 ml of the GA3 solution to 1 L of the autoclave and cool Agar media.

ANNEX 2. FIELD DATA FORMS

FIELD FORM 1. DISEASE EVOLUTION. LEAF SPOT SEVERITY DATA.

Site_____ *Pseudocercospora* sp. _____

Planting date_____ Assessment Date_____

Surveyor_____ ITC # or cultivar_____

PLANT NO.	OPEN LEAVES #														SUMMARY NUMBER OF LEAVES IN EACH GRADE								YLST	YLS
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	0	1	2	3	4	5	6	T		
1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
9																								
10																								
Total																								
	S (# of leaves x degree of severity)																							

TOTAL LEAVES =

SEVERITY INDEX % =

[∑nb/(N-1)T]x100

% HEALTHY LEAVES =

[S (leaves grade 0/ Total leaves) x 100]

INSL =

(YLS-1)/NL

YLSt =

YLS=

STOVER MODIFIED SCALE BY GAUHL	
0	Healthy
1	Until < 1% leaf area affected (streaks and until 10 spots)
2	Until 5% of leaf area spotted
3	From 6 - 15% leaf area spotted
4	From 16 al 33% leaf area spotted
5	From 33 al 50% leaf area spotted
6	> 50% leaf area affected

FIELD FORM 2. SUMMARY RECORD OF WEEKLY DATA ON LEAF SPOT SEVERITY

Site_____ *Pseudocercospora* sp. _____

Surveyor_____ ITC # or cultivar_____ Plot number _____

DATE	WEEK #	LER	SET	YLStr	YLS	TOTAL LEAVES		SEVERITY %	UNDPC	RAINFALL (MM) IN THE WEEK
						HEALTHY	IN PLANT			

WEEK: number of the week of the year (1-52)
LER: leaf emission rate
SET: symptoms evolution time = YLSt*LER
YLS: youngest leaf spotted
AUDPC: area under the disease progress curve

FIELD FORM 3. RECORD DEVELOPMENT TIME (DDT) AND TRANSITION PERIOD

Site_____ Planting date_____

Surveyor_____ ITC # or cultivar_____

Experimental design_____ Identifier_____

WEEK #	DATE OF STAGE B (DD/MM/YY)	DATE OF 10 OR MORE LESIONS IN STAGE 6 (DD/MM/YY)	DDT IN DAYS	TRANSITION FROM STREAKS TO SPOTS IN DAYS

ANNEX 3. ENVIRONMENTAL DATA FORM

FIELD FORM 4. ENVIRONMENTAL DATA TO BE COLLECTED IN EACH SITE FROM PLANTING TO HARVEST.

Site _____ Surveyor: _____

WEEK	DATA TO BE COLLECTED								
	RAIN-FALL (MM)	HIGHEST TEMP. (°C)	LOWEST TEMP. (°C)	AV. TEMP. (°C)	HIGHEST R.H. (%)	LOWEST R.H. (%)	AV. R.H. (%)	NUMBER OF DAYS WITH RAIN	NUMBER OF HOURS WITH R.H. ≥ 90%
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
...									
52									