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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INTRODUCTION

This book provides revised practical guidelines for the early screening and field evaluation of banana (*Musa* spp.) for resistance to three major traits: Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*), leaf spot diseases (*Pseudocercospora* spp.) and drought.

The guidelines have been produced by experts within the Evaluation Thematic Group of MusaNet, led by Chair Miguel Dita and Co-chair Kodjo Tomekpe. The co-authors are listed at the beginning of each protocol. MusaNet is an international network for *Musa* genetic resources coordinated by the Alliance of Bioversity and CIAT (www.musanet.org).

The guidelines supersede the following documents published by INIBAP:

- Carlier et al. 2002. Global evaluation of *Musa* germplasm for resistance to Fusarium wilt, Mycosphaerella leaf spot diseases and nematodes. INIBAP Technical Guidelines 6 (In-depth Evaluation).
- Carlier et al. 2003. Global evaluation of *Musa* germplasm for resistance to Fusarium wilt, Mycosphaerella leaf spot diseases and nematodes. INIBAP Technical Guidelines 7 (Performance Evaluation).
- Orjeda, G. 1998. Evaluation of *Musa* germplasm for resistance to Sigatoka diseases and Fusarium wilt. INIBAP Technical Guidelines 3.

The recommendations made in this book are intended for research programmes in *Musa* genetic resources and crop improvement by conventional and modern technologies. When collecting and transporting germplasm, International Standards for Phytosanitary Measures (ISPMs) established by the Food and Agriculture Organization's (FAO) International Plant Protection Convention (IPPC) should be considered. The Technical Guidelines for the Safe Movement of *Musa* Germplasm (Thomas, 2015)* is the standard reference for safe transfer of banana germplasm.

Guidelines structure

Each of the three protocols in this book is divided into two sections: Early Screening and Field Screening. Early screening protocols are carried out in the laboratory or greenhouse, while field screening concerns the final phase of evaluation in the field, where environmental factors play a critical role in determining levels of resistance.

These protocols reflect the consensus and knowledge of the authors, but it is anticipated that the information will need to be regularly updated as new information becomes available. We ask our readers to kindly bring to our attention any developments that may require a review of the guidelines. Correspondence regarding this publication should be addressed to the 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.

Ordering *Musa* germplasm

Germplasm used for evaluation should be obtained from the safest source possible. Clean, healthy banana germplasm is available free of charge from the *in vitro* germplasm collection at the International *Musa* Germplasm Transit Centre (ITC) in Belgium, and can be ordered online (http://www.crop-diversity.org/mgis). All germplasm moving from one continent to another should transit through the ITC or, if possible, be obtained from the ITC. Other sources of safe germplasm may be available where indexing laboratories have the capacity and expertise to test for the complete range of viruses.

* Thomas, JE. (ed) 2015. MusaNet Technical Guidelines for the Safe Movement of Musa Germplasm. 3rd Edition. Bioversity International, Rome.

Phenotyping *Musa* spp. for host reaction to *Fusarium oxysporum* f. sp. *cubense*, under greenhouse and field conditions

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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

1.1 Background

Once Fusarium wilt of banana (FWB) (http://www.promusa.org/Fusarium+wilt), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) is present in a given area, the most effective management practice is to plant disease-resistant varieties. Reliable screening protocols to identify varieties resistant to Foc are, therefore, essential. Standardized protocols can speed up and scale out the identification of sources of resistance and rank genotypes according to their resistance levels.

Screening for Foc resistance can be carried out under greenhouse and field conditions. Both practices have advantages and disadvantages, which are largely influenced by the particular objectives, the number of genotypes to be screened, the time and costs involved, and the available facilities. Field phenotyping provides information on host reaction under real-life conditions and allows the evaluation of other agronomic traits at the same time (Smith et al., 2008; Zuo et al., 2018). It does, however, require large experimental plots, is time consuming (typically 2–3 years in duration), and is highly demanding in terms of manpower. Inoculum density and soil characteristics may also vary considerably across the experimental site, and the development of symptoms can be affected by climatic conditions to varying extents.

Early screening for resistant genotypes under greenhouse conditions can potentially identify promising genotypes much more rapidly than field phenotyping and generate knowledge about the genetic and molecular basis of resistance. However, greenhouse screening is performed under artificial conditions, which often include high inoculum levels, young plants, pasteurized potting media and controlled environmental conditions, and may not reflect field conditions.

Ideally, early screening in the greenhouse should be used to identify resistant genotypes that will then be evaluated in the field for further confirmation. However, one of the issues frequently raised by breeders is the sometimes poor correlations between greenhouse and field results. The artificial and controlled environment in greenhouses may also result in disease levels that would either not occur or would be less severe in the field. Moreover, there is a wide range of protocols currently used for phenotyping bananas for Foc resistance under greenhouse conditions (Dita et al., 2011; García-Bastidas et al., 2019; Mohamed et al., 2001; Ribeiro et al., 2011; Smith et al., 2008; Sun and Su, 1984) rather than a widely accepted method that would allow comparisons between experiments and locations. Hence, there are limitations when comparing the results of screening experiments done by different researchers. Standardized protocols, easily applicable by different users, might improve how the community deals with FWB research globally.

The objective of this document is to describe the key elements required to develop a reliable, repeatable screening protocol for evaluation of banana genotypes for FWB resistance under greenhouse and field conditions. The document comprises detailed information on the necessary steps, including planting material preparation, inoculum production, inoculation, experimental design, evaluation and data analysis. It is expected that the proposed protocols can considerably improve phenotyping for FWB resistance in different locations.

1.2 Banana genotypes to be used as susceptible and resistant references

The selection of reference genotypes with well-known reaction to the pathogen strains under study is a crucial step in any phenotyping trial. A differential set of banana genotypes with resistant, susceptible and also an intermediate response to the target Foc strains need to be carefully selected for both greenhouse and field conditions. Some may consider that including three reference genotypes is excessive but the importance of doing so cannot be overstated. When interpreting the results of the trial, it is not so much the absolute amount of disease suffered by the varieties under test but the comparison of their disease response relative to the reference genotypes. Disease levels may vary from one trial to another due to effective inoculum density and climatic variables in particular, but by the inclusion of those genotypes with well-known disease reactions, these factors are taken into account. A list of banana genotypes frequently used in phenotyping for FWB resistance and the reaction to different Foc races are listed in Table 1.

Table 1. Host reaction of some banana genotypes to races of *Fusarium oxysporum* f. sp. *cubense* (Foc).

ITC CODE[a]	CULTIVARS[b]	FOC RACES[c]			
		1	2	SR4[d]	TR4
ITC1122	Gros Michel (AAA)	S	S	S	S
ITC0348	Silk (AAB)	S	S	S	S
ITC0213	Pisang Awak (ABB)	S	S	S	S
ITC0643	Bluggoe (ABB)	R	S	S	S
ITC0570	Cavendish (AAA)[e]	R	R	S	S
ITC0712	Rose (AA)	R	R	R	R

S: Susceptible - R: Resistant.

[a] ITC refers to the International Transit Centre of *Musa* Germplasm. More information at: https://www.crop-diversity.org/mgis/

[b] Names of genotypes may differ depending upon country and production region. More information at: http://www.promusa.org/Banana+cultivar+checklist

[c] Races in Foc here refers to groups of pathogenic strains (pathotypes) with a differential reaction on a set of banana cultivars. The race structure for Foc is imperfect; for example, cases of Foc R1 strains affecting Bluggoe have been reported. Foc populations are very diverse and new species have even been proposed recently. Racial differentiation of Foc is presented here only for the purpose of illustrating to users some frequently used terminology.

[d] Subtropical Race 4 are Foc populations able to infect Cavendish only under subtropical conditions.

[e] Several of the cultivars in the Cavendish subgroup of cultivars, such as Williams, Grande Naine and Poyo, would be suitable, but not any that has been selected for resistance to SR4 or TR4.

Note: One must be aware that there can be a spectrum within Resistant (R) and Susceptible (S) reactions driven by banana accessions, Foc strains, inoculation procedures and conditions. Therefore, evaluations may result in highly susceptible, moderately susceptible or even moderately resistant. For instance, Cavendish could be ranked as highly susceptible to Foc TR4, whereas a given genotype, for instance Plantains (AAB), could be ranked as moderately susceptible to Foc TR4.

1.3 Planting material

a. In order to avoid cross contamination and other confounding factors in evaluations, the use of disease-free tissue culture plants is mandatory. Depending on the objective of the study, wild banana plants with seeds may need to be multiplied by tissue culture to guarantee uniformity and enough replications (Li et al., 2015).

b. All plantlets should be raised under the same conditions. Plantlets should be acclimatized for at least 60 days before inoculation or planting in the field. All genotypes to be evaluated need to be at the same physiological stage. Plantlets should ideally range between 20–25 cm in height (from soil surface to lowest leaf axil) and have six or more leaves and a healthy root system.

c. Substrates used during the acclimatization process should be certified as free of any pathogen. Different substrates or potting mix can be used with good results. Pre-assays should be conducted to verify suitability if the quality of the substrate is unknown.

d. The presence of pests and diseases in the area should be monitored and recorded.

e. Temperature in the acclimatization room should ideally range between 26 and 30°C with regular photoperiod (12 ± 2 h light). Adequate and quality-controlled water (free of pathogens) should be given, and all plantlets need to be well nourished. Stabilized fertilizers with an accurate description of nutrient composition and reliable microbiological analyses are recommended.

f. Plantlets should not show symptoms of nutrient deficiency or biotic stress (leaf yellowing, necrosis, Pseudocercospora leaf spots) at the time of inoculation. Any trait that may imitate FWB symptoms (stunting, low number of leaves, pseudostem discoloration, etc.) should be noted, and the plants removed.

g. Symptoms related to somatic variation due to the use of *in vitro* propagation might also appear. Mix-ups can also occur and should be watched for; for example, the wrong variety or a mixed batch of varieties may accidentally be supplied. These variants/mix-ups would typically be less noticeable in greenhouse than in field studies where plants are grown to maturity.

h. Researchers should consider taking photos of varieties that are being field evaluated so that irregularities in disease reaction could possibly be retrospectively investigated.

Notes. The substrate (planting medium) should be autoclaved, sterilized or pasteurized to avoid or reduce influence of microbes. For the use of formalin (2%), the planting medium should be drenched with the chemical and covered with plastic sheeting for at least 5 days, and then air-dried for at least 5 days to allow the elimination of gases formed during the sterilization process. Phytotoxic effects have been observed when using autoclaved soil on banana experiments without appropriate post-sterilization aeration. Alternatively, washed river sand which has been autoclaved might be used to reduce microbial and chemical interference. In all cases, special attention should be paid to plant nutrition. Hoagland solution or other fertilizers could be used according to their availability. Make sure that the fertilizers do not interfere with the final results by comparing results with genotypes used as susceptible and resistant references.

2. EARLY SCREENING

2.1 Inoculum production of *Fusarium oxysporum* f. sp. *cubense*

There are many options to produce infective structures of Foc. Firstly, it is important that enough Foc macroconidia, microconidia and chlamydospores are produced. In this protocol, two culture media options are recommended: Potato Dextrose Broth at half strength (½ PDB) as a liquid medium, and a Corn Meal-Sand (CMS) as solid medium. Other culture media (e.g. sterilized grain as a solid medium) that have been successfully used in refereed literature can also be utilised (Dita et al., 2011; García-Bastidas et al., 2019; Ribeiro et al., 2011; Smith et al., 2008).

The reference (single spored) Foc isolate, hereafter named as Foc00X1, should be previously selected based on pathogenicity and virulence. Petri plates containing Potato Dextrose Agar (PDA) should be inoculated with Foc00X1 and incubated for one week at 25–27°C before initiating the inoculum production. These plates will provide the initial fungal growth to inoculate the culture medium (liquid or solid) used to produce the inoculum to be used in the screening.

2.2 Liquid medium

- Inoculate three (03) 5-mm-diameter mycelial disks from actively growing colonies of Foc00X1 into Erlenmeyer flasks (500 mL) containing 250 mL of ½ PDB culture medium. Incubate for one week at 27°C.
- Shake Erlenmeyer flasks once a day every other day to reduce mycelial growth and promote conidia production.
- After 7 days, sufficient amounts of conidia (macro and microconidia) should have been produced. To collect conidia, cultures need to be filtered through double layer sterile cheesecloth to remove the fungal mycelia. If substantial mycelia still remain, repeat the filtering process to make sure that most of the mycelium was removed.
- Adjust inoculum concentration to 10^6 conidia.ml^{-1} using a haemocytometer (or another tool) to quantify conidia.

Note: Alternatively, other liquid culture medium (i.e. mung bean broth medium) and growing conditions (i.e. rotary shakers) could be used. Inoculum production can be improved by modifying local conditions according to available infrastructure. For instance, by inoculating 50 ml PDB in an Erlenmeyer flask (100 ml), then placed on a rotary shaker at 200 rpm, after 48 h, an efficient spore suspension could be also produced (Li et al., 2013). Verify which conditions better fit your facilities and your Foc isolate. Foc is a highly variable pathogen, and inoculum production may also differ among isolates.

2.3 Solid medium

- Inoculate 5-mm agar plugs of 7-day-old Foc00x1 colonies grown on PDA into Erlenmeyer flasks containing sterilized CMS medium (200 g of cornmeal, 1000 g of washed river sand and 100 mL of distilled water). The amount of the ingredients may be changed depending on the final volume of your flask, but the proportions should be maintained and well mixed. Slight adjustments to the amount of water may be required to ensure that the medium is moist but not excessively wet.
- Verify the Foc growth every other day and shake the Erlenmeyer flasks manually to disaggregate fungal mycelia growing on substrate and distribute it evenly.
- Incubation period may depend on the size of the flask used, but after 7 days, enough infective structures should be present.
- Allow the substrate to dry out at room temperature for 24 h. Alternatively, a drying oven (28°C) may be used.
- Adjust the inoculum suspension to 10^6 cfu.g^{-1}. Inoculum concentration produced on CMS medium is normally higher than 10^6 cfu.g^{-1}. Adding more sterile dried sand is recommended to adjust the inoculum concentration when needed.
- Solid inoculum produced on CMS as above described could be stored at room temperature or cold rooms for long periods. The viability of the inoculum should be verified, and inoculum density adjusted before each inoculation.

2.4 Inoculation procedures

(1) Inoculation of potted plants (post-planting inoculation)

Plants already established and meeting the requirements described above can be inoculated by drenching (liquid) or by pouring (solid) inoculum into the pot. Follow the steps described below:

1. Three days before the inoculation, make four equidistant hollows (3–5 cm depth) around the plant base on each pot. Making the hollows in advance speeds up inoculation process and minimize the effect of any root wounding that may occur.
2. Drenching - Use the inoculum produced in liquid medium adjusted to a suspension of 10^6 conidia. ml^{-1}. Pour 5 ml of suspension into each hollow and cover it with the substrate (potting soil) present in the pot.
3. Placing solid inoculum - Use the inoculum produced in solid medium adjusted to 10^6 cfu.g^{-1}. Inoculate 10 g of solid inoculum in each hollow and cover it with the substrate (potting soil) present in the pot.
4. Maintain the plants in a greenhouse (25-28°C) with a 16-h light/8-h dark photoperiod) until disease symptoms appear in susceptible controls. Plantlets need to be watered regularly to maintain the substrate at field capacity.

(2) Inoculation by transferring plantlets to infested substrates (pre-planting inoculation)

1. Prepare a substrate for banana plants. Substrates containing three (3) parts vermiculite, one (1) part peat and half (0.5) part of coconut coir have shown efficient results.

2. Drench the substrate with 2% formalin and cover with plastic film for 7 days to remove or reduce microbes. Remove the plastic cover and allow gas volatilization for 5 days.
3. Add spore suspension to the substrate to obtain a concentration of 10^5 cfu.g^{-1} of substrate.
4. Transfer banana plantlets into the pots containing the inoculated substrate. Maintain the plants in a greenhouse (25–28°C) with a 16-h light/8-h dark photoperiod) for at least 30 days. Plantlets need to be watered regularly to maintain the substrate at field capacity.

2.5 Experimental design

Researchers should seek expert statistical advice when designing phenotyping trials. Deciding the number of plants per genotype should consider (a) prior knowledge of the variability of the key parameters to be assessed, (b) number of genotypes to be tested and (c) the level of confidence desired. Consider evaluating at least 20 plantlets per genotype. Three blocks (5 plants each) could be formed with inoculated plants using a randomized block design. One separate block could be formed with the remaining uninoculated 5 plants per genotype. Figure 1 shows a diagram of a putative experimental trial. Phenotyping trials involving a large number of genotypes may consider reducing the number of plants and replications according to experimental conditions and research needs, but without jeopardizing good statistical practices. Pre-experiment data estimating variability (if available) should be used to determine appropriate sample sizes.

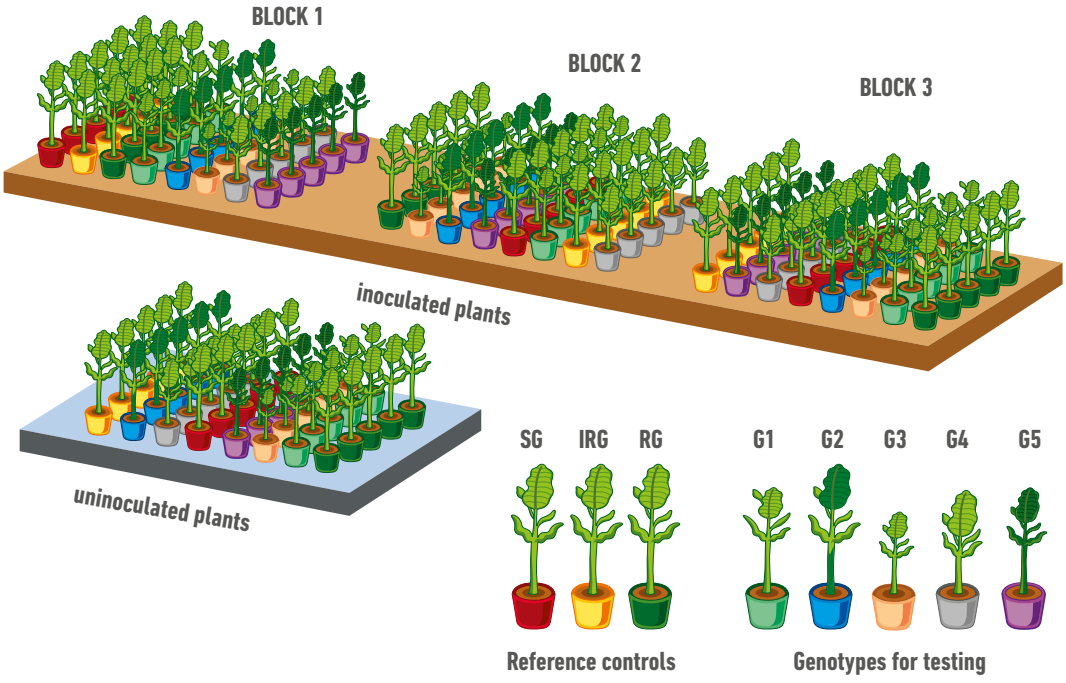

Figure 1. Diagram of a putative experimental trial with five banana genotypes (G1-G5) for testing for Fusarium wilt resistance and three genotypes with known resistance as reference controls (SG: Susceptible genotype, IRG: Intermediate resistance genotype and RG: Resistant genotype).

2.6 Variables to be measured

Evaluation consists of visual observations of typical external and internal symptoms of FWB. The following variables should be measured:

Incidence (%). The percentage of plants showing symptoms. It should be evaluated based on external symptoms first and internal symptoms (rhizome discoloration) later. Evaluations should be performed every other day or weekly depending on the disease progress rate and degree of precision needed. Use the data to calculate the incubation period (**IP50)** as described below.

Incubation period - IP50 (days). The duration in days from inoculation until 50% of the plantlets of the same genotype show typical FWB symptoms. It may be variable depending on inoculation procedure, banana genotypes used, aggressiveness of the Foc isolate or environmental condition. Evaluations should be performed every other day or weekly depending on the disease progress rate and degree of precision needed.

Severity. The degree of damage caused by the pathogen. Different scales exist for both external and internal symptoms that are used to assess disease severity. A scale to evaluate disease severity of both external and internal symptoms is proposed as shown in Figure 2. Alternatively, image analyses software could be used (Orr et al., 2019).

Disease index. Response of banana genotypes against Foc can be determined by scoring external or internal symptoms and calculating disease index (McKinney, 1923) based on this scoring.

Disease Index (DI) is calculated using the following equation:

$DI = [\sum(N_{1\text{-}5} \times S_{1\text{-}5})/(N \times S)] \times 100\%$

Where $N_{1\text{-}5}$: number of banana plants with wilt symptoms, $S_{1\text{-}5}$: value of the score of symptoms, N: total number of tested banana plants, and S: the highest value of score of symptoms.

With adjustments necessary depending especially on the disease reaction/response of the known reference cultivars in the trial, the following DI values could be used to rank varieties:

- 0 – Immune
- > 0 & ≤ 5% - Resistant
- >5 ≤ 20% - Intermediate resistant
- > 20 ≤ 50% - Susceptible
- > 50 % - Highly susceptible

2.7 Biosafety precautions

- Avoid visiting disease-free areas such as acclimatization rooms, or other banana experiments after working in a Foc inoculation area.
- Keep plants watered at field capacity and the irrigation water confined to the pot to avoid contamination on the greenhouse surfaces. Plastic plates may be used to collect drainage water.

Once the experiment is finished, thoroughly sterilize plants, substrate, pots and other tools used in the experiment. Contaminated soil/substrate should be autoclaved twice with an interval of 24 h.

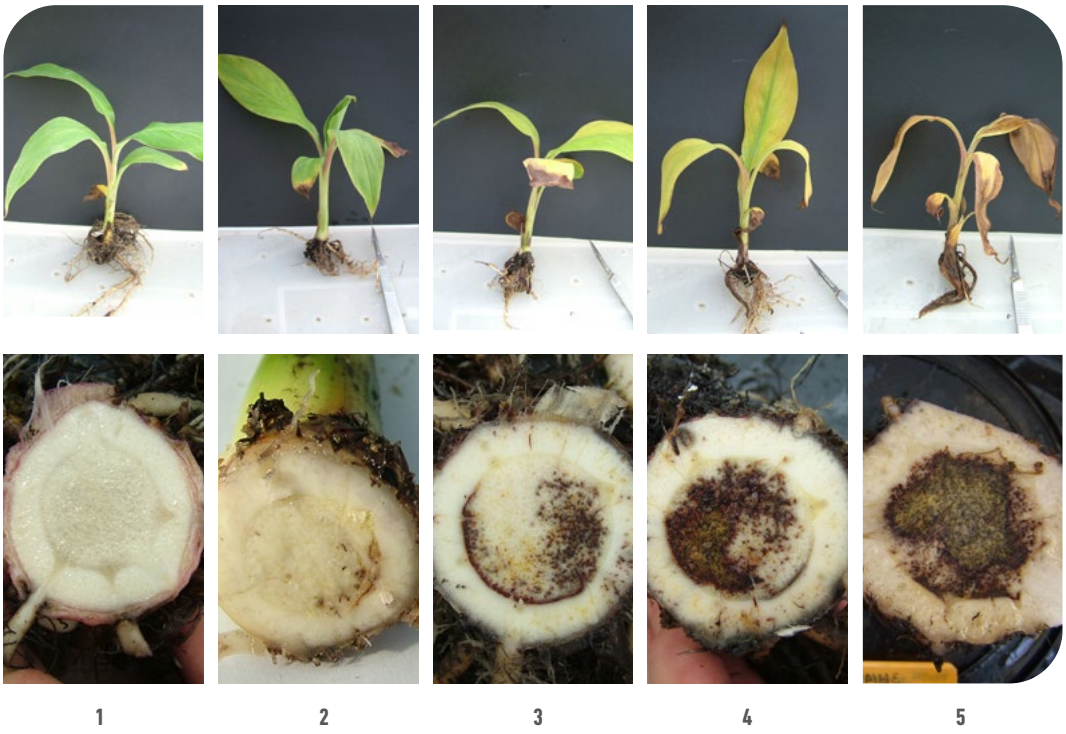

Figure 2. Scale for evaluation of Fusarium wilt of banana in greenhouse conditions based on external (upper panel) and internal symptoms (lower panel). **Classes for external symptoms are:** 1: No symptoms; 2: Initial yellowing mainly in the lower leaves; 3: Yellowing of all the lower leaves with some discoloration of younger leaves; 4: All leaves with intense yellowing; 5: Dead plant. **Class for internal symptoms are:** 1: No symptoms; 2: Initial rhizome discoloration; 3: Slight rhizome discoloration along the whole vascular system; 4: Rhizome with most of the internal tissues showing necrosis; 5: Rhizome totally necrotic. (Photos: Miguel Dita).

3. Field screening

3.1 Biotic and abiotic factors that may influence FWB intensity

In a field setting, the incidence of pests, such as weevils (*Cosmopolites sordidus*), giant borer (*Castnia* spp.), or diseases, such as Moko (*Ralstonia solanacearum*), Xanthomonas wilt (BXW), bacterial soft rot (*Dickeya* spp., *Pectobacterium carotovorum*), and viruses (i.e. Banana Bunchy top Virus-BBTV) can impact FWB development and jeopardize evaluations. These biotic factors, among others, should be systematically monitored and controlled. Areas with incidence of Moko, BXW or any other pest or disease that might mask FWB symptoms must be avoided. In addition, water deficit and soil nutrient deficiency must be monitored and corrected. Further attention should be given to soil acidity. Soil with pH values below 5 should be corrected by liming before planting as low pH values have been associated with higher FWB intensity.

3.2 Planting material

Phytosanitary and development requirements for planting material are as described in the introduction above. Once in the field, plants may initially suffer from exposure to UV radiation. Gradual exposure to full sun during final acclimatization steps may increase production of epicuticular waxes and so reduce UV damage once in the field. If there is a long distance between acclimatization facilities and field phenotyping plots, verify transport conditions to minimize injury to plants. Biosecurity measures to avoid plant or soil contamination with pathogens during transport need to be strictly followed. Plant nutrition status must be monitored and corrected from planting to harvest, following standard recommendations.

3.3 Reference genotypes

Susceptible (SG) and resistant (RG) genotypes should be included, not only as reference for comparison, but also to verify the effect of differences in the distribution of pathogen inoculum density and soil attributes. The selected SG genotype must be susceptible to the Foc strain/race that is under study (see Table 1 on p. 6). Including genotypes with intermediate levels of resistance (IRG) or susceptibility (ISG), when available, also helps further comparisons and increases the reliability of the assays.

3.4 Distribution of inoculum in the soil of the experimental area

Knowledge of the strain(s) present, inoculum load and distribution of the Foc inoculum in the soil is essential for successful field trials. The strains present at a proposed field site can be determined by analysis of diagnostic samples from diseased plants in an existing crop or specially planted "sentinel" plants. However, determining whether there is an adequate amount and homogeneous distribution of Foc inoculum is not always easy for naturally infested sites. Even if molecular tools are available for the Foc strain being studied, obtaining a meaningful picture of Foc distribution and density could be cumbersome, largely due to logistics associated with representative sampling for a large field site. Some approaches for making inoculum load more homogeneous in naturally infested sites are discussed below.

The sources of Foc inoculum for field phenotyping trials are: (1) Inoculum present in naturally infested soils (existing hotspots) and (2) Inoculum produced in the laboratory. A combination of both sources is also possible by increasing Foc inoculum already present in the soil with inoculum produced in the laboratory. Choosing one option or another will depend on many factors, such as time available for trial, proximity of commercial banana areas, Foc strains to be used, biosecurity rules in place where the experimental trial will be established, etc.

3.5 Using inoculum present in naturally Foc-infested soils (existing hotspots)

1. Cultivate a highly susceptible banana genotype for one cropping cycle in an area previously contaminated with Foc (e.g. Grande Naine for Foc TR4). Apply management practices aimed to maximize biomass production and increase FWB intensity, such as higher doses of ammonium-based nitrogen fertilizers (2X the recommendation for the crop). At the end of the cropping cycle, evaluate the incidence and spatial distribution pattern of FWB.
2. Cut and chop all the plants and distribute the infected plant material evenly in the experimental area, making sure that healthy spots receive debris from infected plants. To chop the biomass and incorporate it into the soil, a rotary tiller followed by harrowing could be used.

Note. Chopping infected plants and distributing its debris across the experimental area may reduce the effect of differential spatial distribution of Foc inoculum in the soil. However, in some cases, artificial soil inoculation might be needed. Solid inoculum produced according to recommendations described for greenhouse phenotyping may help to reduce inoculum heterogeneity in the soil.

3.6 Using Foc inoculum artificially produced

Using artificial inoculum (see recommendations described to produce solid inoculum for greenhouse phenotyping) is an important tool, not only to increase inoculum density and reduce heterogeneity of the spatial inoculum distribution in the soil, but to guarantee known minimum inoculum densities of the target Foc strains. Inoculum density may vary depending on pre-testing, but once the experimental plot is ready for planting, solid inoculum can be placed into each planting hole. One hundred grams of solid inoculum (10^6 cfu.L^{-1}) per plant is a suggested rate for addition of artificial inoculum. Field trials in Australia have used 200 ml of Foc-colonized millet for this purpose (e.g. Smith et al., 2018).

3.7 Chemical and physical soil attributes of the experimental area

Soils present spatial variations in their attributes. Soil acidity, base saturation, organic matter content, density and depth of the arable layer are some attributes that affect the intensity of the FWB. Thus, prior to the implementation of the experimental area, these attributes must be quantified, and their values mapped in the area. It is also important to consider the slope and soil drainage/aeration conditions. They can strongly affect the distribution of the inoculum and disease intensity.

All soil fertility limitations should be corrected before planting. Site-specific effects due to soil differences need to be considered from the beginning.

3.8 Experimental design

Similar to what was described for greenhouse experiments, researchers should seek expert statistical advice when designing phenotyping field trials. Knowledge of the variability of the key parameters to be assessed, number of genotypes to be tested and the level of confidence desired should be carefully considered to decide the number of plants per genotype.

Randomization of genotypes being screened (Gn) and reference controls (genotypes with known characteristics, SG, RG, IRG) is a fundamental principle and must be followed. The main objective of reference controls is to increase the reliability of collected data from the trial overall. The randomization of treatments within blocks and the location of these blocks in relation to edaphic characteristics (i.e. pH, drainage) increases the accuracy of phenotyping.

Each replicate block containing all genotypes under study should be located in portions where there is as little variation as possible of soil attributes related to FWB. For each situation, the shape of the blocks should be adjusted according to the mapped soil attributes. For example, if there is a soil acidity gradient as a function of the position on the slope, the blocks should be arranged perpendicular to the slope as shown in Figure 3 and Figure 4.

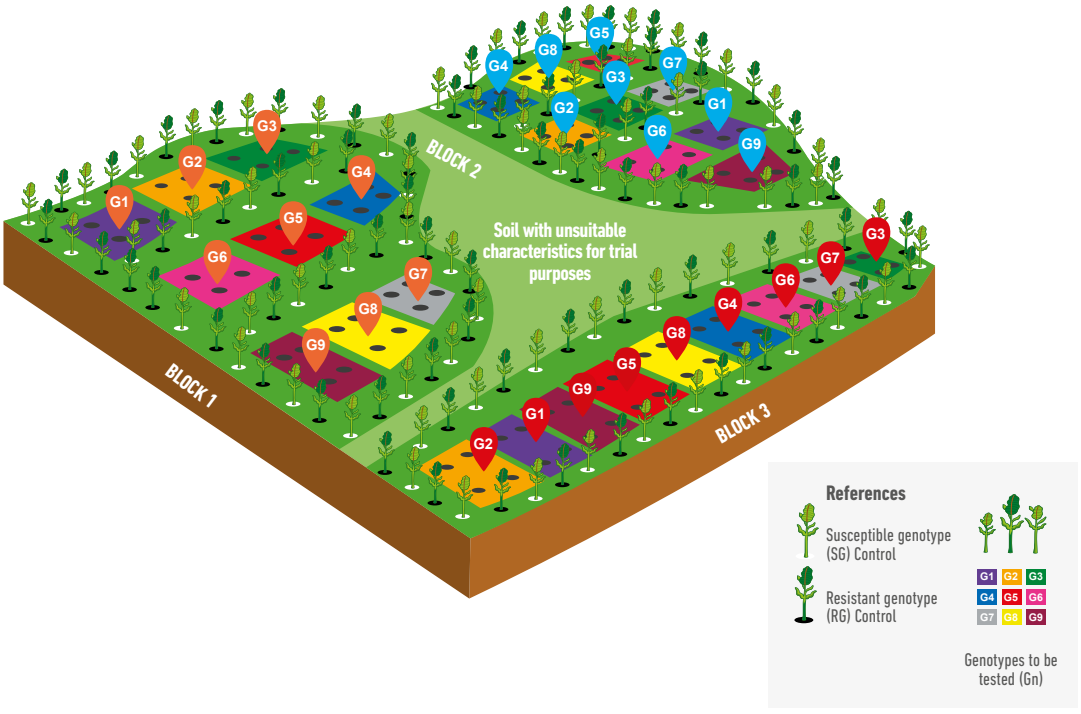

Figure 3. Example of how block distributions could be adjusted to local soil conditions.

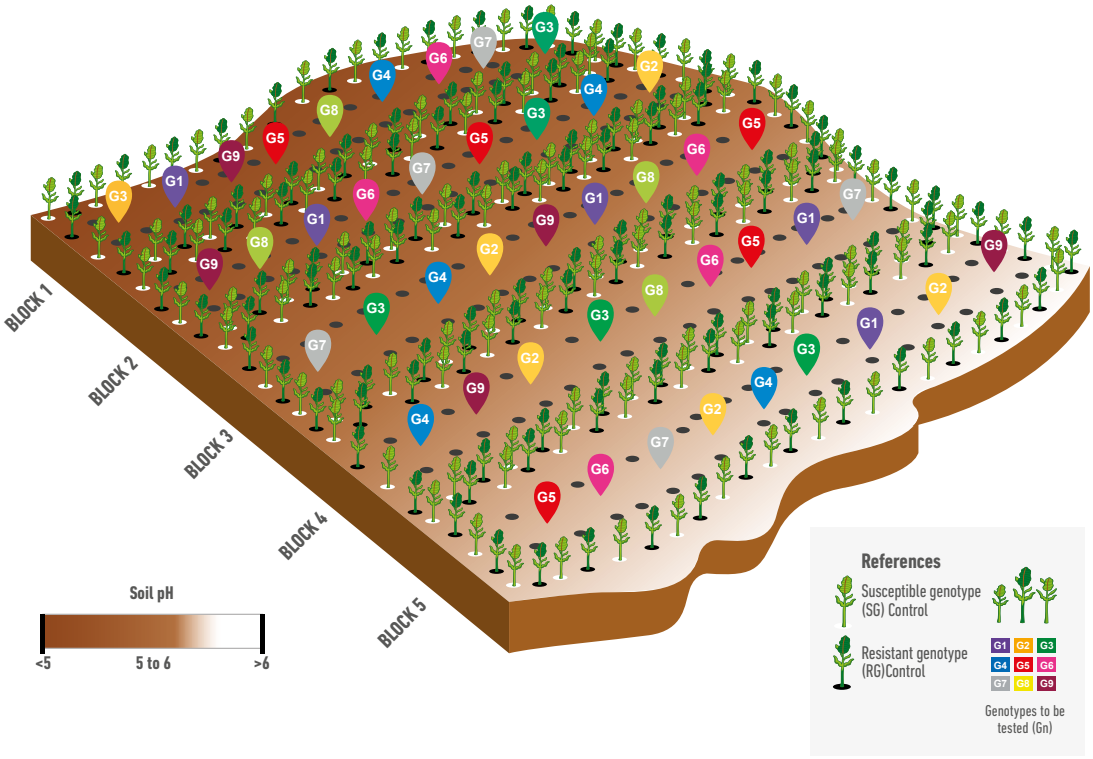

Figure 4. Example of distribution of experimental blocks with nine genotypes to minimize the effect of variation in soil acidity.

3.9 Collecting data and describing disease severity

The experimental areas should be georeferenced (latitude, longitude and altitude), and the main soil characteristics described. Weather conditions (daily temperatures and rainfall, at least) observed while conducting the experiment should also be described.

In a field trial, the severity of disease symptoms for a particular variety can vary widely due to a range of factors, including environment, level of disease inoculum pressure and plant development stage when first exposed to the pathogen. Because of this variability, the inclusion of the three reference varieties (susceptible, intermediate and resistant) is crucial to interpreting the response of the varieties under test, and this cannot be overstated.

The evaluation of disease intensity (incidence and severity) of external symptoms in the field should commence as soon as first symptoms are observed on any genotype and should be performed every other week until the plant dies or is harvested. Both external and internal symptoms should be evaluated.

Essentially, the description of the disease symptoms, recorded individually for each plant in the trial, can range from no disease symptoms at one extreme to death of the plant (no harvestable yield) at the other extreme. Between these extremes, symptoms are present, and a harvestable crop may be produced. This latter category can be further divided, if desired, but doing so can potentially

overcomplicate the disease assessment. For field trials, we therefore suggest a minimum of three ratings (1, 2 and 3 for the three mentioned above) but no more than five (1 to 5, such as illustrated in Figure 5) overall. Any external symptoms must be confirmed as being caused by Foc by the presence of the characteristic dark brown to black discoloration of the water conducting tissues within the stem. Internal symptoms should be evaluated and rated once the plant dies or is harvested. It is essential to collect samples from each variety being screened to confirm the Foc strain present, especially for field sites where there is a possibility of more than one strain occurring naturally. It is recommended to perform field evaluations during at least two cropping cycles.

Once such ratings are completed individually for each sample plant in the trial, a disease index (see Section 2.6) can be applied as for greenhouse screening. This index represents the mean for all the sample plants of each variety in each experimental unit (replicate).

3.10 Varietal ranking and categorisation

Following statistical analysis of the disease index for the varieties, they can be ranked in order and then classified into categories so that recommendations can be made to end-users. The values for the three reference varieties help to validate the scale, and categories are constructed around the reference varieties in a meaningful manner to suit the context. Choice of terms is important. We suggest at least three categories and no more than five; e.g. very susceptible, susceptible, intermediate, resistant, and highly resistant. Productivity parameters (e.g. bunch weight and its components) are also essential to support the discrimination process, mainly among those with intermediary resistance. The category assigned to each variety depends on where exactly the category boundaries are placed. This is up to the individual researcher. However, when reporting results, it is important to explain how you have integrated disease assessments to rank varieties and how category boundaries were determined with respect to the reference varieties. This will help your audience to understand the results of your study and allow a degree of comparability between field trials.

3.11 General remarks on phenotyping and ranking banana genotypes for resistance to Fusarium wilt

Both greenhouse and field phenotyping protocols have advantages and limitations. While phenotyping in a greenhouse may use inoculum densities higher than those found in the field, it gives clear responses and most of the biotic and abiotic parameters (Foc population, type of soil/substrate, pH, microbiome, temperature, water availability) are well controlled. In addition, it saves space and time and allows high throughput testing with a larger number of genotypes than in the field. On the other hand, field screening, especially when conducted in the environments where the potential new resistant varieties would be released, has the advantage to evaluate G X E (genotype –x environment) interactions and provide more essential data on agronomy, yield and market acceptability. However, field trials can be affected by extreme weather conditions and heterogenicity of soil parameters, and are also influenced by biotic factors such as nematodes and other diseases. There are promising results recently published showing the complementarity and usefulness of both greenhouse and field phenotyping protocols for FWB (Rebouças et al., 2018), screening large sets of banana genotypes (Zuo et al., 2018) and assessing variations on host resistance to Foc (Chen et al., 2019).

Finally, clear and consistent shared definitions and criteria to define resistance categories are not in place for FWB. As mentioned, these categories can vary according to the requirements of individual researchers and trial conditions, but so long as the process is transparent and reference varieties are included, then there can be some comparability between trials and ultimately some consistency in assigning genotype resistance. We understand standard protocols are difficult to establish in all scientific communities and hope the one proposed here can help to reduce asymmetries in evaluating, interpreting and reporting research findings on phenotyping bananas for FWB resistance. Standards will always evolve as long as technology evolves.

Figure 5. Disease scale for evaluating the severity of Fusarium wilt of banana under field conditions. **A.** External symptoms. **B.** Internal symptoms. 1: Healthy plant; 2 to 5 different degrees of disease severity. Classes of external and internal symptoms are not necessarily correspondent. For instance, a plant ranked with class 3 according to external symptoms could be eventually ranked with a different class when evaluated for internal symptoms. Pictures in classes either for external or internal symptoms do not represent chronological stages of the same plant (Photos: Miguel Dita).

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

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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).

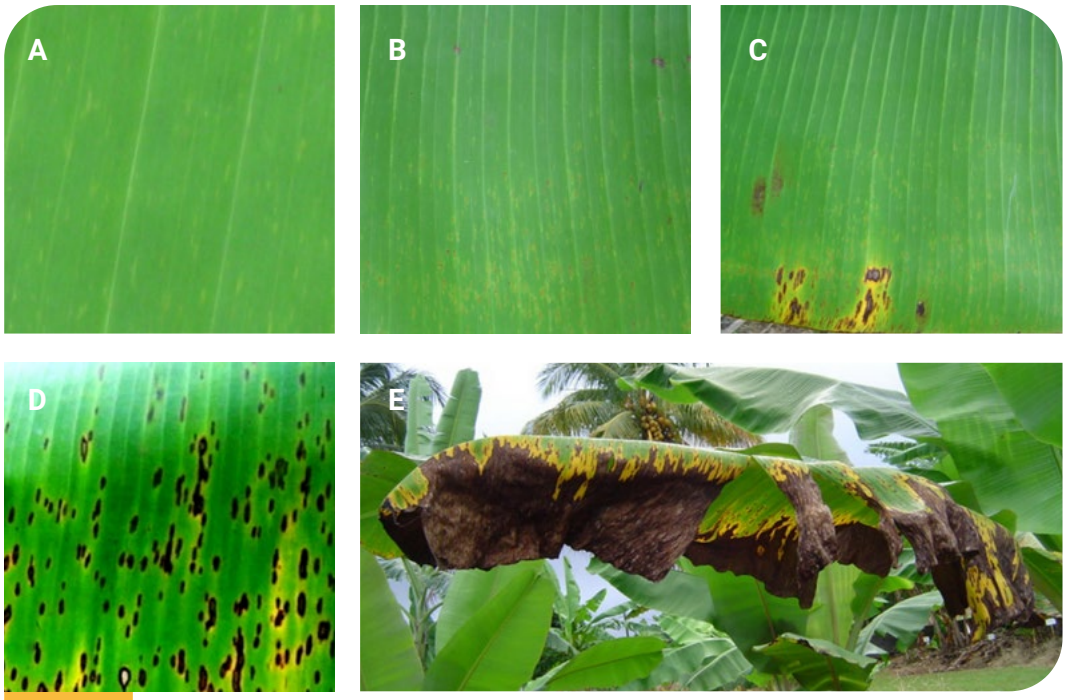


Figure 2. Five stages of evolution of symptoms according to Brun (1958), description. A) Stage 1 and 2; B) Stages 2 and 3; C) Stages 3, 4 and 5; D) Stage 4 and 5; E) Stage 5 (Photos: L. Pérez-Vicente).

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).

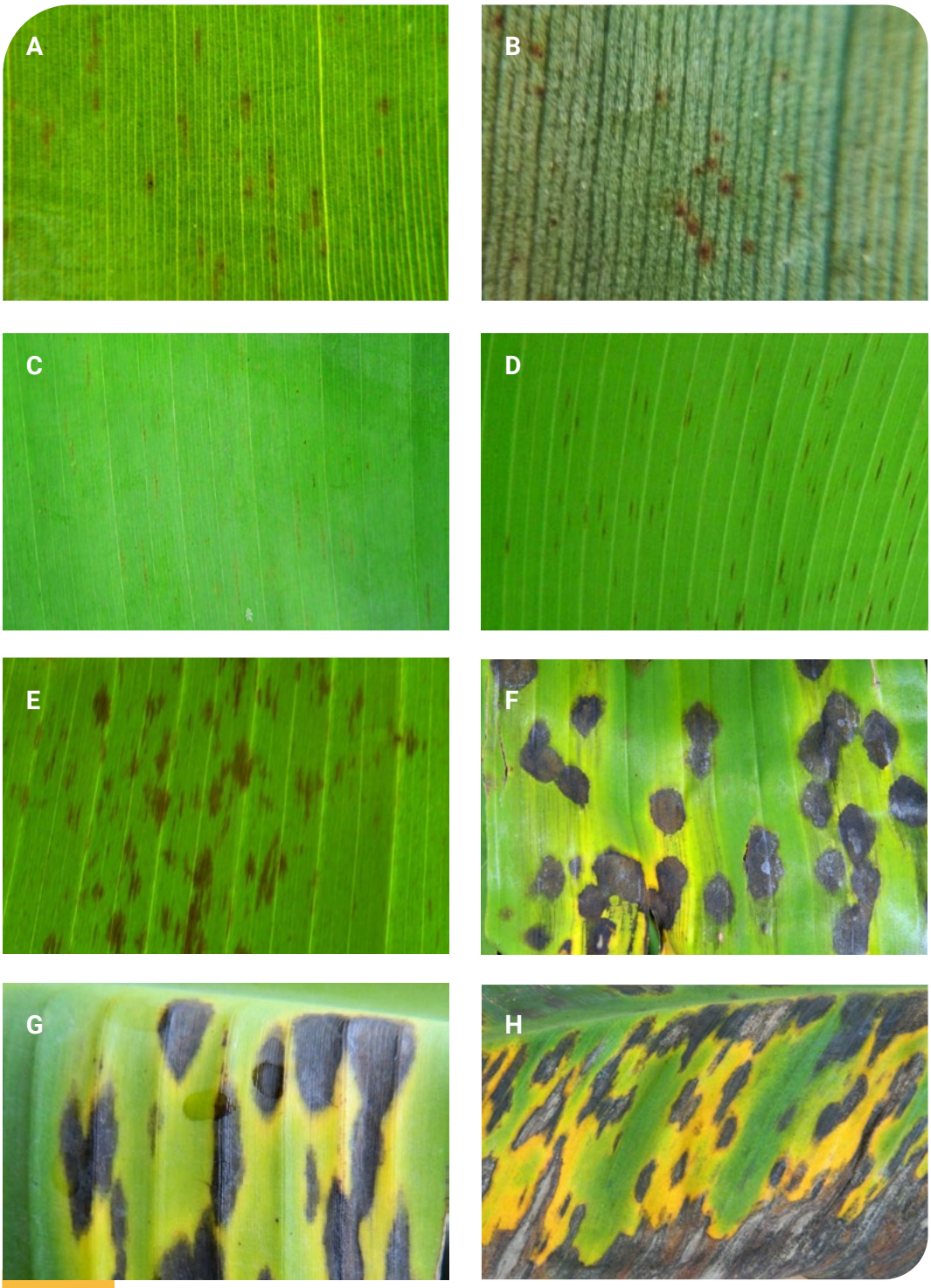


Figure 4. Six stages of symptoms evolution according to Fouré (1982b), description and details of stage 5 spots on a young (4-month-old) plant. A) Stage 1 (16x); B) Stage 1 (20x); C) Stages 1 & 2; D) Stages 2 & 3; E) Stages 3 & 4; F) Stage 5 (young plant); G) Stages 5 & 6; H) Stage 6. (Photos: L. Pérez-Vicente).

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

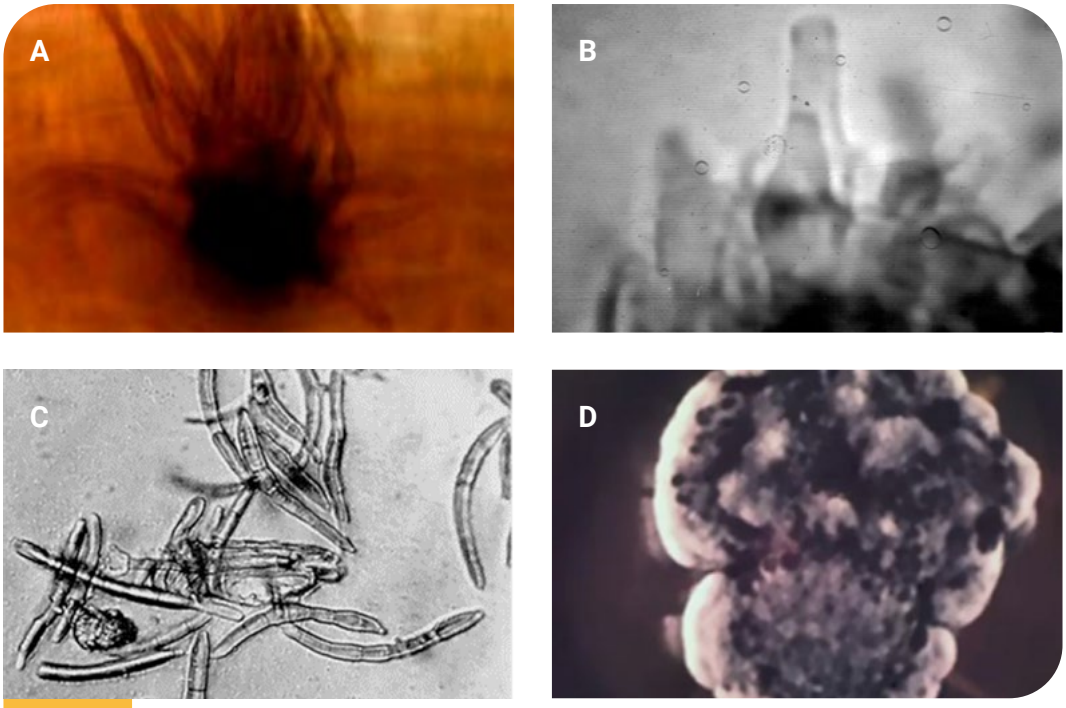


Figure 6. *Pseudocercospora musae* structures: A) Sporodochia and conidia on stroma; B) Bottle-like conidiophores grouped on sporodochia on stroma; C) Obclavate-cylindrical conidia without hilum; D) colony on PDA. (Photos: A) Carlier et al., (2002); B), C) and D) from L. Pérez-Vicente).

Pseudocercospora fijiensis

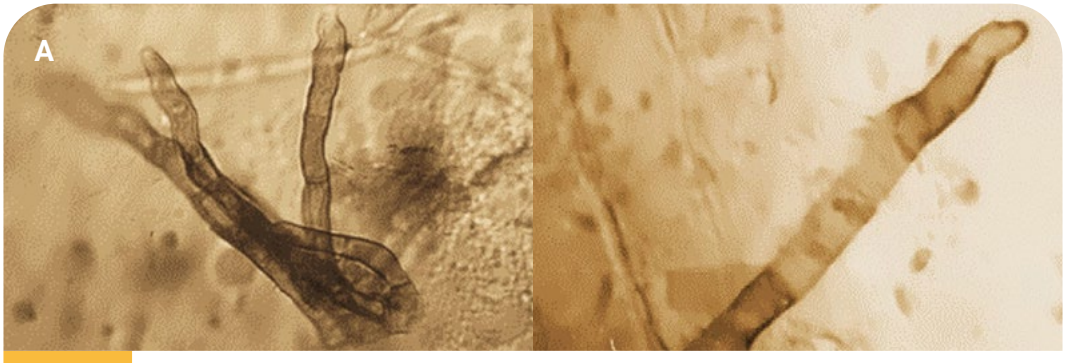


Figure 7. *Pseudocercospora fijiensis* structures: A) Group of geniculated conidiophores with scars and an engrossed basal cell; B) Obclavate conidia with a marked hilum; C) Conidiophore and conidia; D) Pseudothecia (Ps) and spermogonia (Sp) in superior side-view; E) Pseudothecia (Ps), spermogonia (Sp)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

FIGURE 7 (continued)

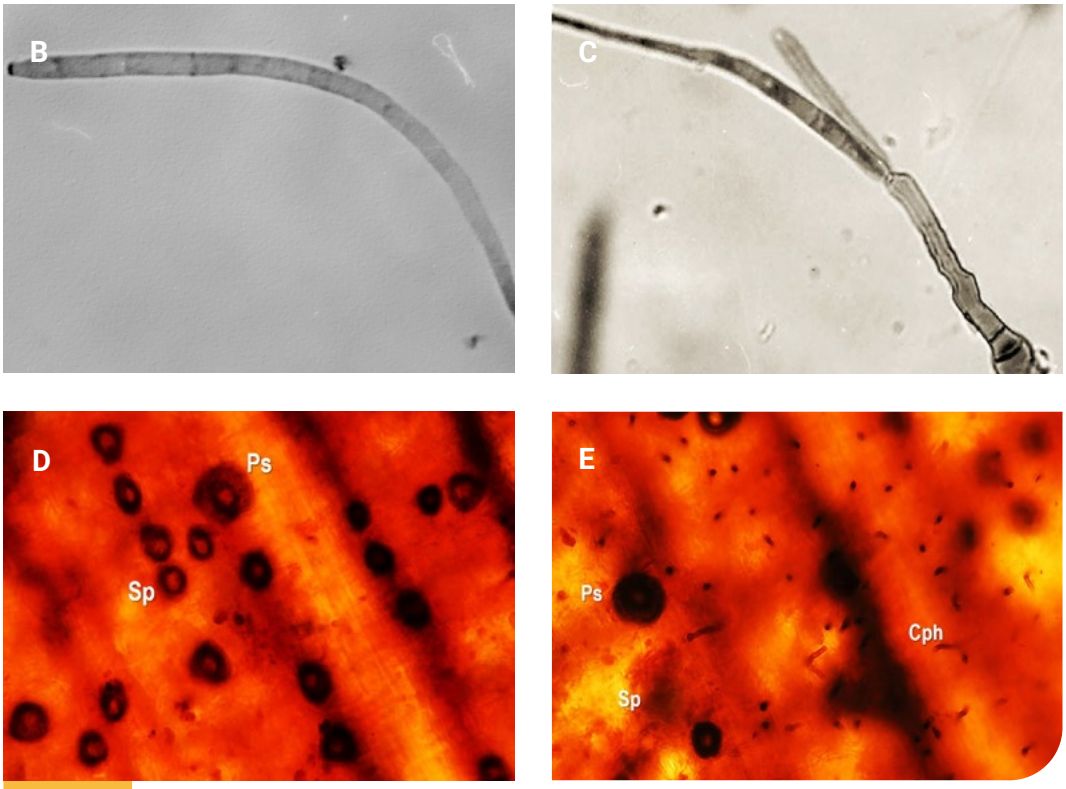


Figure 7. *Pseudocercospora fijiensis* structures: A) Group of geniculated conidiophores with scars and an engrossed basal cell; B) Obclavate conidia with a marked hilum; C) Conidiophore and conidia; D) Pseudothecia (Ps) and spermogonia (Sp) in superior side-view; E) Pseudothecia (Ps), spermogonia (Sp)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

Pseudocercospora eumusae

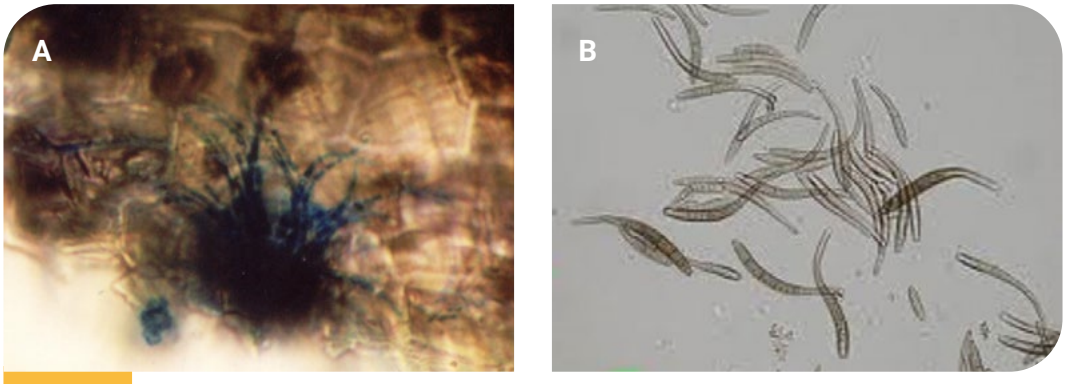


Figure 8. *Pseudocercospora eumusae* structures: A) sporodochia; B) Conidia (Photos: Tania Polanco).

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.

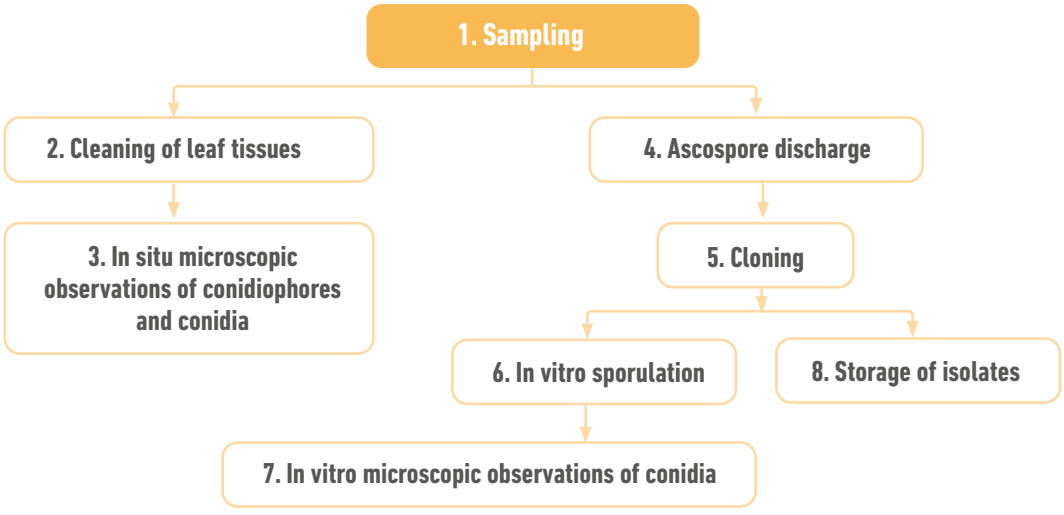


Figure 9. Flow chart for identification of *Pseudocercospora* leaf spot pathogens (reproduced from Carlier et al., 2002).

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).

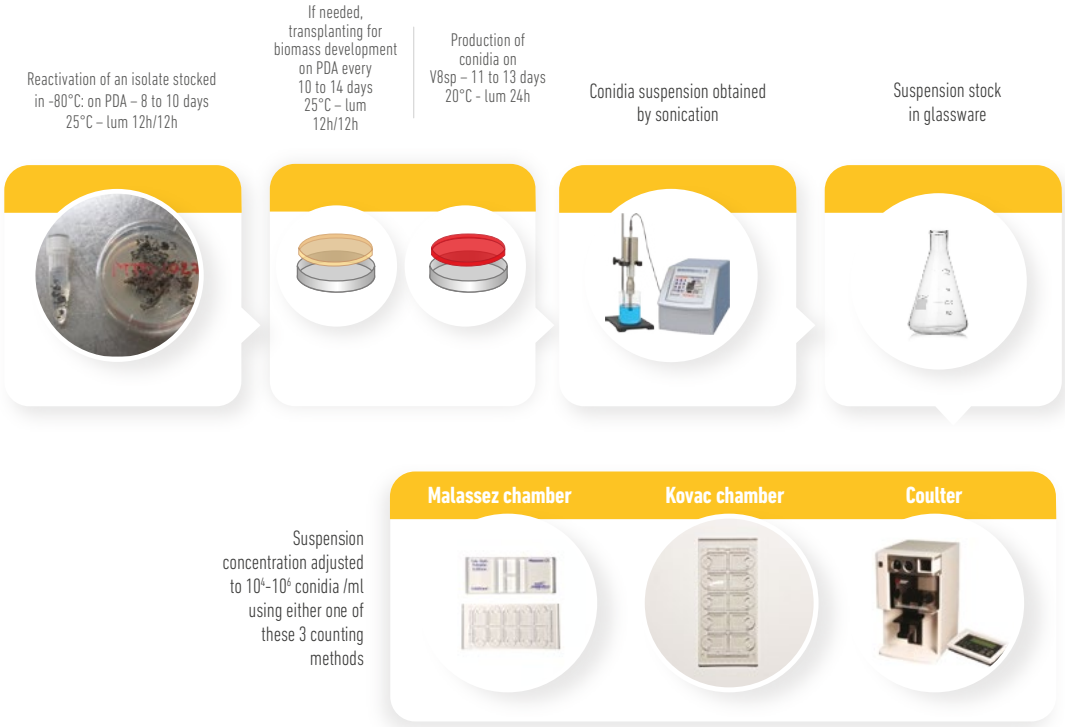


Figure 10. Scheme of conidial suspension preparation (Photos: CIRAD).

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.

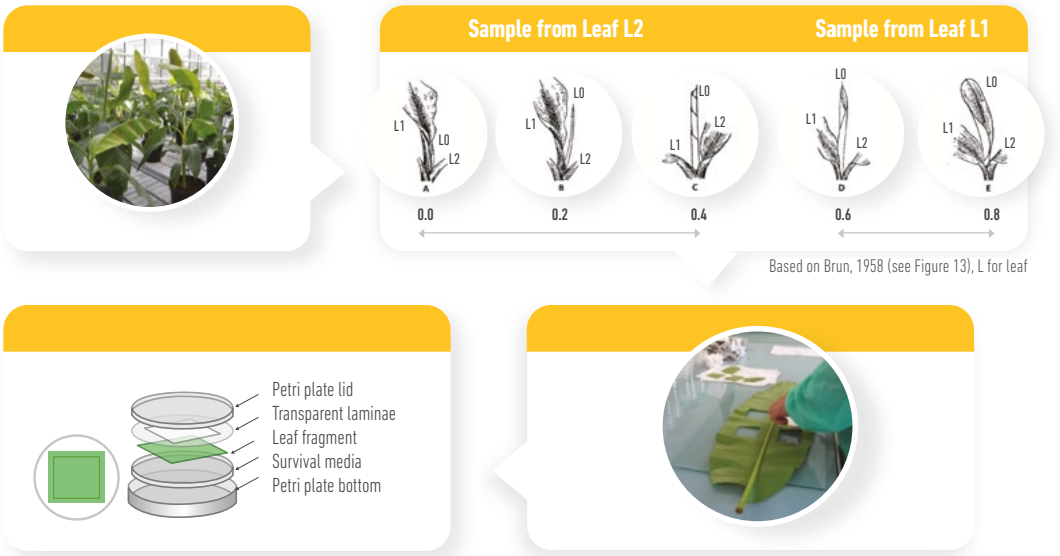


Figure 11. Procedure to prepare detached leaves fragments in survival media (Photos: CIRAD).

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:

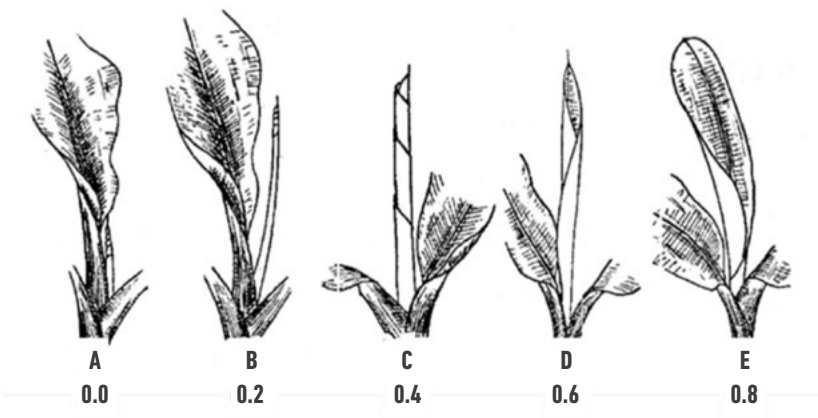


Figure 13. Stages of an unfolding leaf (according to Brun, 1958).

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).

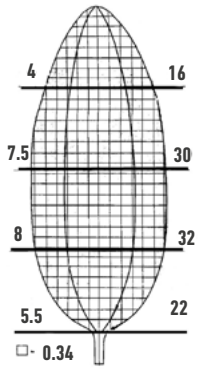
P7. Symptom evolution time

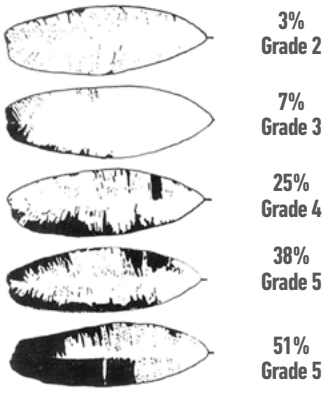
This is an approximation of incubation time. It is estimated by the formula YLStr/LER.

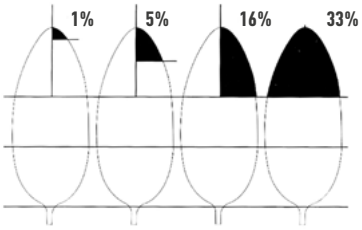
P8. Transition period from streaks to spots

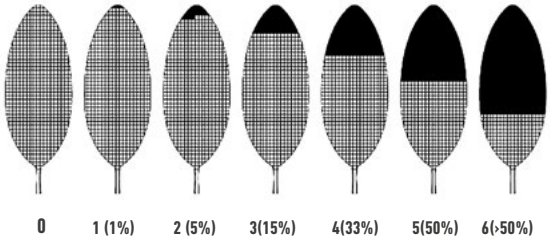
The transition period is the time in days, between the symptoms stage 1 to spots at last stage of evolution. It has been used by Simmonds (1939), Vakili, (1968), Meredith and Lawrence (1969), Firman (1972), Fouré (1982a, b), Fouré (1994), Fouré et al. (1984) and Hernández and Pérez (2001), to determine 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.

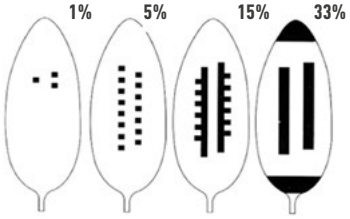
Leaves near Brun's stage A should be selected and marked with permanent black felt-tip pen, colored ribbon, or tags and date of the appearance recorded. Data are taken from 10 plants sampled in the plot. The leaves should be inspected twice a week until appearance of lesions at stage 1 of Fouré (1982b) 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.

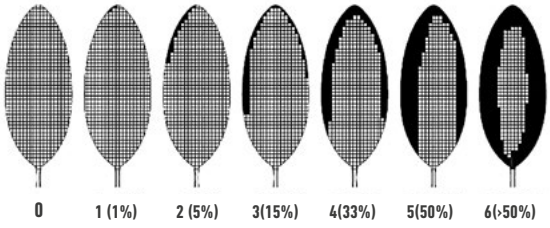








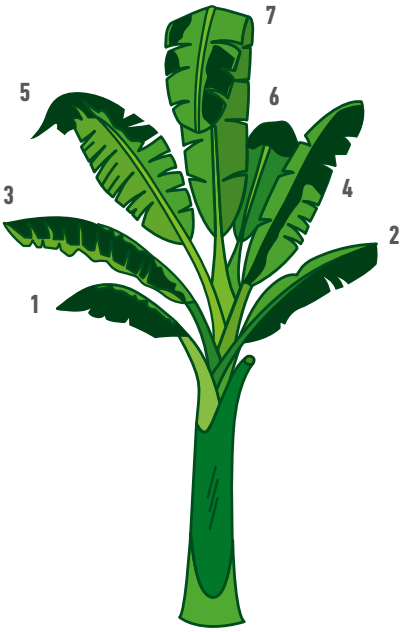




Scale of Severity Description

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).

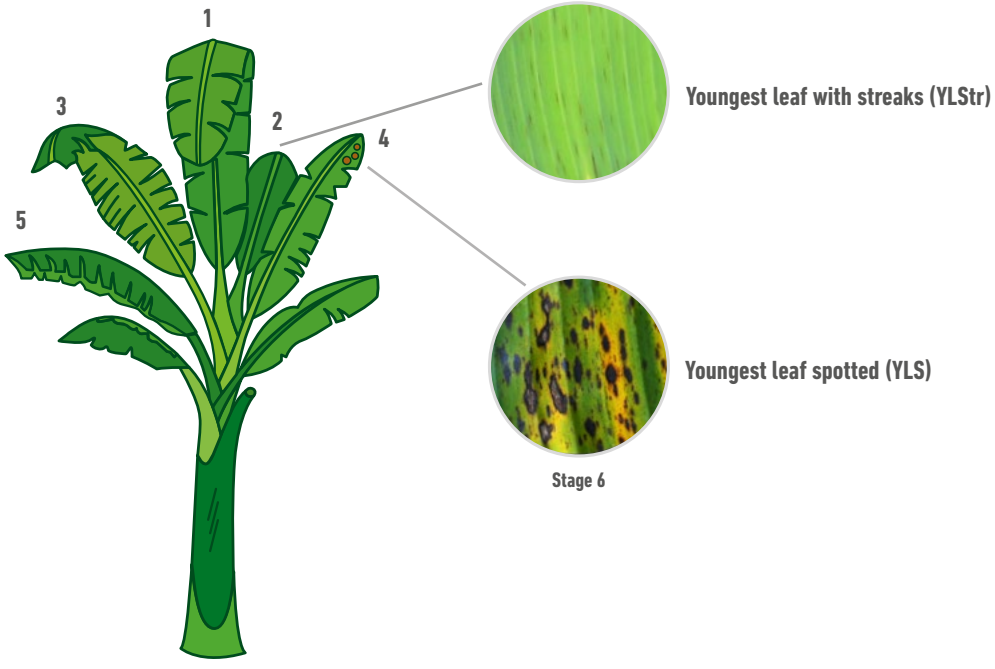


Figure 16. Scheme of the youngest leaf with streaks (YLStr) and youngest leaf spotted (YLS) determination (Adapted from Carlier et al., 2002)

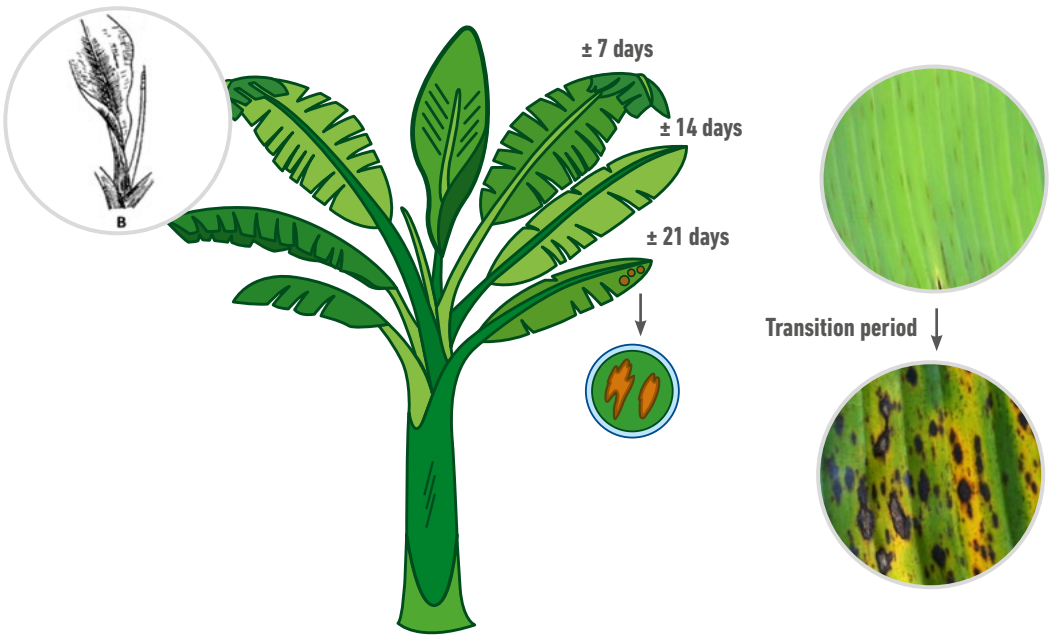


Figure 17. Scheme of disease development time determination (DDT, adapted from Carlier et al., [2002]) and transition period from streaks to spots according to descriptions of Simmonds (1939), Meredith and Lawrence (1970) and Hernández y Pérez (2001).

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.

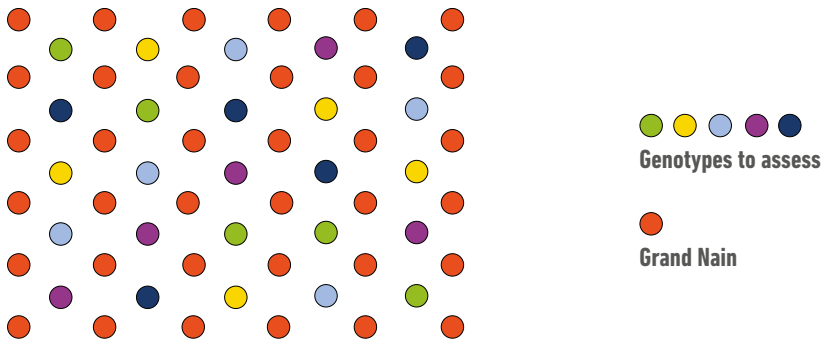


Figure 18. Layout for the early selection phase with many hybrid accessions.

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.

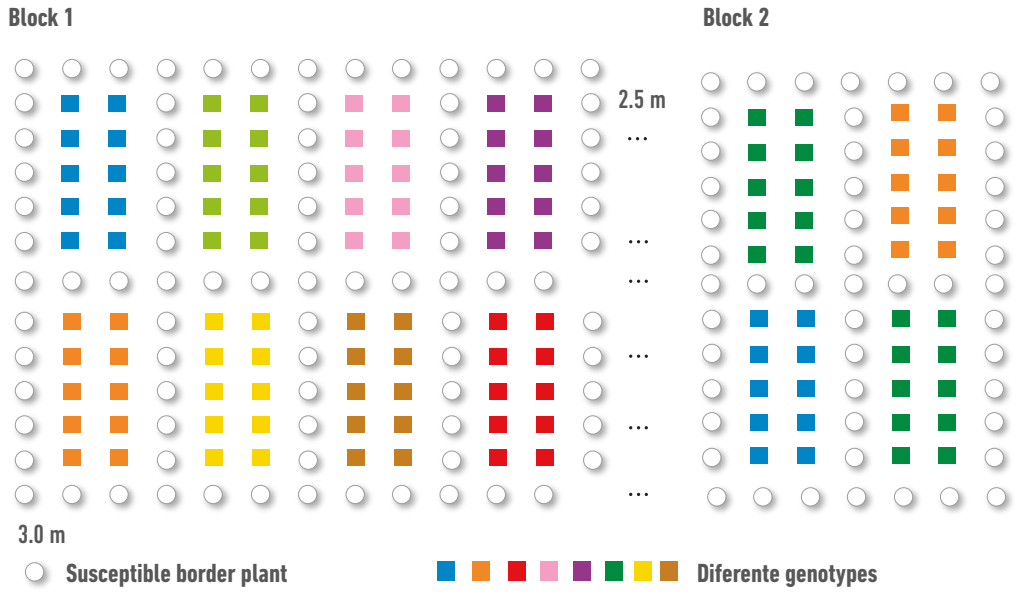


Figure 19. Layout of the randomized complete block design for phase 2.

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									

Phenotyping protocol for drought tolerance in banana

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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

Drought is complex and affects multiple agricultural traits. The timing in the phenology, the duration of the water deficit and the actual environmental factors (Vapor Pressure Differential (VPD), radiation, precipitation) drive the plant responses and greatly influence the manifestation of the symptoms. Absolute classifications towards tolerance are, therefore, not possible. The terms "susceptible" and "tolerant" are relative terms which are dependent on the given set of genotypes evaluated under a given set of environmental conditions. Depending on the magnitude and the duration of the drought period, different genotypes might be required for the targeted environment. Following the definition from Taiz and Zeiger (2002), drought tolerance could be described as being able to "tolerate" and restore the disequilibrium or disadvantageous influence created by the lack of water. In an ecological plant-central approach, the disadvantageous influence is any influence that threatens its existence; therefore, tolerance is solely focused on withstanding the adverse period. The focus lies here on the survival of the plant and does not necessarily consider the growth potential under the unfavourable conditions. In an agricultural approach, the disadvantageous influence is any influence that threatens the yield; thus, the focus lies on safeguarding production and profit if water-limiting conditions occur during the crop cycle. The survival of long-term severe drought is associated with water-saving strategies and growth arrest, which are negatively correlated with safeguarding production. Plant growth is determined by the interaction of the genotype with the environment. Crop growth is determined by the interaction of the genotype with the environment and the farm management. During the vegetative growth stage, root and shoot growth are crucial to ensuring sufficient water and nutrient uptake and energy production through photosynthesis.

However, maintaining a high-growth pace and transpiration under unfavourable conditions could be detrimental if not corrected by agricultural management. The natural habitat of wild bananas is a forest environment. Wild bananas are opportunistic pioneer plants that occur in disturbed areas of forest and thrive at places in the forest where light is abundant. Many clumps of wild bananas survive only for a few generations because they are seral plants, which do not survive when the climax vegetation becomes established (Gibbs and Turner, 2018; Simmonds, 1962). Wild banana species require high temperatures, high humidity and high light intensities. But humans have brought the crop into open fields, where the protective surrounding canopy of the forest is no longer present. An open field has higher radiation and a higher VPD and so a higher evaporative demand than the often man-made-transient habitats in the forest. Selection by humans of edible bananas has most likely taken place for fruit characteristics with less or no attention to water usage. Therefore, there is an urgent need to evaluate our banana diversity in the field.

A terminology was introduced by Berger-Landefeldt to describe the daily patterns of water relations, distinguishing plant species based on their ability to decouple their leaf water potential from atmospheric demand: iso/anisohydric (Berger-Landefeldt, 1936). Isohydric behavior is what we would call "conservative behaviour": there is strong regulation of the leaf water potential and stomata close to ensure a high leaf water potential (Ψ_l). However, different definitions exist and the molecular mechanism controlling the plant/leaf water potential in response to the evaporative demand (isohydric vs anisohydric plant) is still not fully known (Hochberg et al., 2018). The use of the terminology iso/anisohydric is suitable to describe the mechanism evolved to react to a high evaporative demand

but cannot be used for classification of plant species or in our case different cultivars. The hydraulic parameters related to iso/anisohydric terminology are not solely determined by the genotype but also by the environment (relationship between the soil-to-canopy-hydraulic conductance (k), soil water potential (Ψ_{soil}), the leaf water potential (Ψ_l) and the maximal transpiration (E_{max})). Ψ_{l_crit} is variable and is a close interaction between the genotype and the environment. One assumes that the transpiration (E) is regulated by the root conductivity and the stomata to prevent Ψ_l from falling below a critical water potential (Ψ_{l_crit}). The daily drop in water potential ($\Delta\Psi$) is then defined by Ψ_{soil} and Ψ_{l_crit}. ABA is the hormone that is sent by the roots when Ψ_{soil} is too low and can be released by the leaves when Ψ_{l_crit} is reached.[1] Mencuccini et al. (2000) have shown that stomatal responsiveness to Ψ_l depends on the time of the day under constant light. It has also been shown via online transpiration monitoring in the same controlled environment that this stomatal responsiveness is genotype specific in banana (van Wesemael et al., 2019). Some genotypes are "risk takers" and send the signal to reduce E relatively late, while others are "conservative" and reduce or stop E sooner. In general, all banana cultivars close their stomata relatively soon to avoid a large drop in Ψ_l. To determine whether a cultivar is a "conservative water user" or rather "a risk taker," the transpiration in response to the environment needs to be monitored.

Banana is a fast-growing crop with a long crop cycle (Figure 1). A lack of water reduces the yield and prolongs the crop cycle (Robinson and Alberts, 1986). The impact of the drought on yield and the length of the crop cycle depends on the developmental stage when the water deficit is experienced (Figure 1). Depending on the banana-growing area and the cultivar, the crop cycle can vary between 9–20 months (Robinson, 1996). In many areas, the crop cycle includes a dry season of 1–3 months. When the dry season takes place during the vegetative phase, then it will delay flowering and affect sucker emergence and growth. When it takes place at the floral and/or fruit phase, it will affect the fruit development and filling and the sucker growth. Therefore, the time of planting has a considerable effect on the yield loss for the first cycles.

1. Note that in practice a reduction of E can already take place before Ψ_{l_crit} is reached since transpiration is dominantly driven by the radiation.

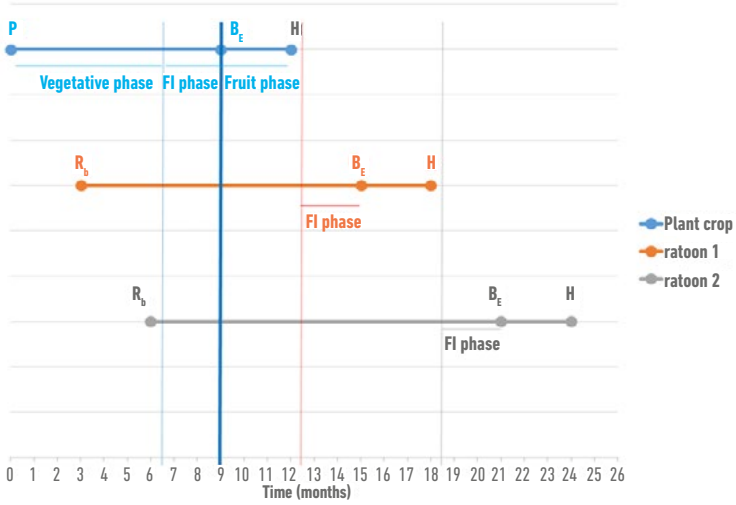


Figure 1. Crop cycle and distribution of harvest of the plant and two ratoon crops. P: Planting date, FI: Flowering phase. R_b: Time at which the ratoon begins (selection of sucker). B_E: time of flowering or bunch emergence, H: Harvest. Figure adapted from Robinson and Alberts (1986) and Turner (1995). The time frame indicated here is indicative. The real time depends on the environment x genotype x management interaction. A water deficit during the vegetative phase will delay the transition from vegetative to floral phases and so will prolong the crop cycle. A water deficit during the floral phase will prolong the crop cycle and will reduce the yield. The number of fruits per hand are especially affected (Gibbs and Turner, 2018). Water deficit during the fruit phase affects the fruit filling and will reduce the bunch and hand size and weight. Flower abortion might also influence the number of fruits per hand.

Trait and variable selection

Yield is the only metric that matters from an agricultural perspective. Since most plantations are harvested over multiple years (Figure 1), the trait yield is generally measured by the variable "plant annual yield" (Kg/Ha*Y). The harvest-to-harvest time varies between 6–13 months (Figure 1) (Robinson, 1996). However, if a field trial cannot be organized and/or yield cannot be measured due to experimental constraints or lack of throughput, other traits/variables need to be measured (Robinson and Alberts, 1986; Nyombi et al., 2010; Ravi et al., 2013). Which other measurable variables describing a trait are correlated to yield? For that, the phenological stage of the plant is important. In ratoon banana crops, the growth cycle has several important milestones indicating key phenological stages: time of emergence and start of ratoon growth, harvest time of the mother plant, time of flowering and harvest time of the ratoon bunch (Figure 1). Unfortunately, few experiments separate these components and so one must be aware that any factor, such as water deficit, may affect one more than the other (David Turner, personal communication).

The transpiration ratio measures the relationship between water loss and net carbon gain. The reciprocal of the transpiration ratio is called the **Water Use Efficiency,** which is a measurement of the efficiency of water used to produce a unit of harvestable product. A major drawback of selection for water use efficiency is that it can guide phenotype selection towards slow growers. This was suggested by Blum (2009), following de Wit's equation (Eq. 1.1).

$$B = \frac{n \times Tr}{E_0} \qquad (1.1)$$

B is the biomass or fresh weight accumulated, *Tr* is the transpiration, *n* is a transpiration independent crop constant and E_0 is the free water evaporation.

Decreasing transpiration will thus increase transpiration efficiency but further decrease biomass accumulation. Therefore, it is equally important to take growth into account. **Non-stressed conditions** are often disregarded when looking for drought tolerance. As stated above, since most banana growing areas have a crop cycle of 9–20 months with a dry season of 1–3 months, the period with no water deficit is the most prevalent situation. For that reason, the selected variables should be tested and compared between contrasting phenotypes under favorable conditions as well. Better performing genotypes under stress conditions are not necessarily the best performers under favorable conditions. Therefore, the usage of genetic diversity should be promoted, especially in extensive agriculture where inputs and particularly water supply are not mastered. From this perspective, we proposed the use of double ranking to evaluate both water use efficiency and growth for drought-tolerant genotype selection (Kissel et al., 2015). In this approach, the growth of all genotypes is ranked relatively under both conditions (drought and control). A suitable phenotype for drought stress is a phenotype that has a better than average growth both under normal and stress conditions.

Ideally all genotypes are screened in the field. But screening numerous accessions from start to harvest would be extremely labour, time and cost intensive. Therefore, we propose a workflow where the biodiversity is first quickly screened through an early screening approach. An early screening approach via, for example, pots in a greenhouse or a growth chamber, enables us to screen the cultivars in a controlled environment and to assess the growth potential in a fast and repeatable manner. The output of the early screening is a relative ranking of the different cultivars to further validate in the field. A greenhouse screening setup offers great control over the water potential, but VPD and light are more difficult to control. In a growth chamber, VPD, light and soil water potential can be perfectly controlled, but the bottleneck is light intensity. In the field, many biotic and abiotic factors also influence plants along with water deficit. The focus of the early screening is throughput. High throughput means screening many plants/cultivars over a limited period as thoroughly as possible. The more variation can be attributed to a difference in genetic makeup of genotypes without being overshadowed by noise due to the environment, the more precisely we can distinguish differential reactions between genotypes. Therefore, we can observe a difference between genotypes much faster, which reduces the time needed for screening.

Using the early screening method, we intend to **evaluate the growth potential of the genotypes both under normal and stress conditions** as vegetative growth influences yield (Taulya et al., 2014). As stated above, we specifically want to consider the growth potential under favorable conditions as those are the conditions that prevail most in the field. A farmer would not benefit from a tolerant cultivar under stress that performs badly under favorable conditions. The cultivars with the best growth potential both under stress and non-stress conditions are regarded as the most suitable in a rainfed system and agro-ecozone with a dry season of 2–3 months.

Testing under controlled conditions allows one also to make correlations towards the molecular physiology and genetics (van Wesemael et al., 2018, 2019; Cenci et al., 2019). However, the banana genotypes which give a normal bunch, fruit development and economically viable yield is the ultimate goal. Therefore, a subsequent field trial in the right target environment of 2–3 cycles is crucial.

2. EARLY SCREENING

Hydroponics screening in a growth chamber: simulating a water deficit at root level

Starting material

The starting material should be *in vitro* plantlets that are ideally obtained from the International *Musa* Germplasm Transit Centre. Material can be ordered through the *Musa* Germplasm Information System (MGIS; www.crop-diversity.org).

Medium[2]

- The plant[3] need to be grown at least 35 days in an autotrophic system before starting the experiment. The composition of the medium is 3.61 g/L KNO_3, 1.21 g/L K_2SO_4, 1.61 g/L $MgSO_4$.7H2O, 1.81 g/L $MgCl_2$.6H2O, 0.6 g/L Sequestrene, 0.0114 g/L H_3BO3, 0.027 g/L $MnSO_4$.H2O, 0.0023 g/L $ZnSO_4$.7H2O, 0.0016 g/L $CuSO_4$.5H2O, 0.0007 g/L $NaMo_4$.2H2O, pH = 6. The plants need to be placed in a controlled environment where the VPD is not a limiting factor e.g. at 25°C, 75% relative humidity, and a 12h/12h light/dark cycle[4].
- The stressed plants need to receive the medium described above supplemented with 5% (W/W) poly-ethylene glycol 8000 (PEG8000) added to it[5].
- Determine the fresh weight and the projected leaf area of each individual plant, discard plants that are too small or too big and make sure that the population that is subjected to both treatments are homogenous.
- Make a cut in the youngest leaf or mark so that you have a reference point of the start of the experiment (Figure 2).
- Determine the area of the whole canopy in top view weekly (CO_325:0000882).[6] Use an algorithm that separates green plant pixels from the blue background by colour segmentation. Use a red reference surface of known size (e.g. 10 x 5 cm).
- At 28 days, determine all the variables described in Table 1.
- Perform a two-way ANOVA[7] to evaluate the genotype, treatment and genotype × treatment effect of all the variables. A p-value lower than 0.05 can be considered as significant.
- Rank the tested cultivars/genotypes accordingly (Figure 3).[8]

2 Whatever system is used, care should be taken that enough oxygen is supplied to the submerged roots so that this is not a stress issue.

3 Each tested group (genotype × treatment) needs to contain at least six biological replicates. Despite the fact that the cultivar plants are clones and have the same age, a considerable amount of variability might be present. So a high number of biological replicates is necessary.

4 The light intensity plays a crucial role in the evapotranspiration. Artificial light is usually limited in its intensity. A minimum of 350 µ mol/m^2 s at canopy level is recommended.

Table 1. Overview of the measured and derived variables.

TRAIT ID	TRAIT DESCRIPTION	METHOD ID	METHOD DESCRIPTION	FORMULA	SCALE
CO_325:0000921	The projected leaf area of a plant (photosynthetic surface)	CO_325:0010537	Top view image		cm^2
CO_325:0000922	The area of a leaf	CO_325:0010538	Top view image of loose leaves		cm^2
CO_325:0000924	The length of a leaf	CO_325:0010541	The longest diagonal of a fitted ellipse		cm
CO_325:0000926	The width of a leaf	CO_325:0010543	The shortest diagonal of a fitted ellipse		cm
CO_325:0000932	The ratio of dry mass of belowground (root) to aboveground (shoot) biomass	CO_325:0010549	Ratio of belowground (root) to aboveground (shoot) dry mass	rootDryMass / shootDryMass	unitless
CO_325:0000933	The relative water content in a leaf	CO_325:0010550	(Leaf Fresh Weight - Leaf Dry Weight) / Leaf Fresh Weight	(FW-DW)/FW	unitless
CO_325:0000934	The relative water content in a pseudostem	CO_325:0010551	(Pseudostem Fresh Weight - Pseudostem Dry Weight) / Pseudostem Fresh Weight	(FW-DW)/FW	unitless
CO_325:0000936	The relative water content in the whole plant	CO_325:0010553	(Plant Fresh Weight - Plant Dry Weight) / Plant Fresh Weight	(FW-DW)/FW	unitless
CO_325:0000941	The amount of water loss through the plant over time	CO_325:0010558	Weighing of water loss through plant between time points (refer to experimental metadata)	measurements on 2 time points required (refer to experimental metadata)	mL
CO_325:0000942	The growth (weight increase) per volume transpired water	CO_325:0010559	Gram accumulated biomass per volume water transpired	growthDry / waterLossPlant	g / mL

5 This is an appropriate osmotic stress level to distinguish different genotypes for drought stress tolerance (~ -50 kPa). A stress that is too mild will result in growth differences that are too small to statistically assess and a stress that is too strong will result in a growth arrest of all genotypes. Care must be taken to grow the plants in clean conditions. Contamination with micro-organisms that metabolize PEG have been reported with upscaling, disturbing the experiment (van Wesemael, PhD thesis KULeuven, 2019).

6 These variables are extracted from the digital images with a self-developed software tool in R based on the EBImage Bioconductor package and ImageMagick.

7 ANOVA is a parametric test that assumes normality and homoscedasticity. This needs to be verified. This can only be tested when enough biological replicates are used. If the requisites are not met, then non-parametric tests need to be used.

8 The normalized ranking views the growth of the cultivars compared to the group median. A growth higher than 0 implies that this genotype grows better than the group median.

TRAIT ID	TRAIT DESCRIPTION	METHOD ID	METHOD DESCRIPTION	FORMULA	SCALE
CO_325:0000950	The average daily water loss	CO_325:0010567	Measurement of system water loss between two time points (refer to experimental metadata)	waterLoss / number of days between consecutive water loss measurements	mL/ day
CO_325:0000951	The amount of water loss in the system normalized by the leaf area	CO_325:0010568	Weighing of water loss through system normalized by leaf area	waterLossSyst / totLeafArea	mL / cm^2
CO_325:0000952	Dry mass accumulation over time	CO_325:0010569	Dry weight difference between time points (refer to experimental metadata)	measurements on 2 time points required (refer to experimental metadata)	g
The full list of banana crop ontology is available at http://www.cropontology.org/terms/CO_325/					

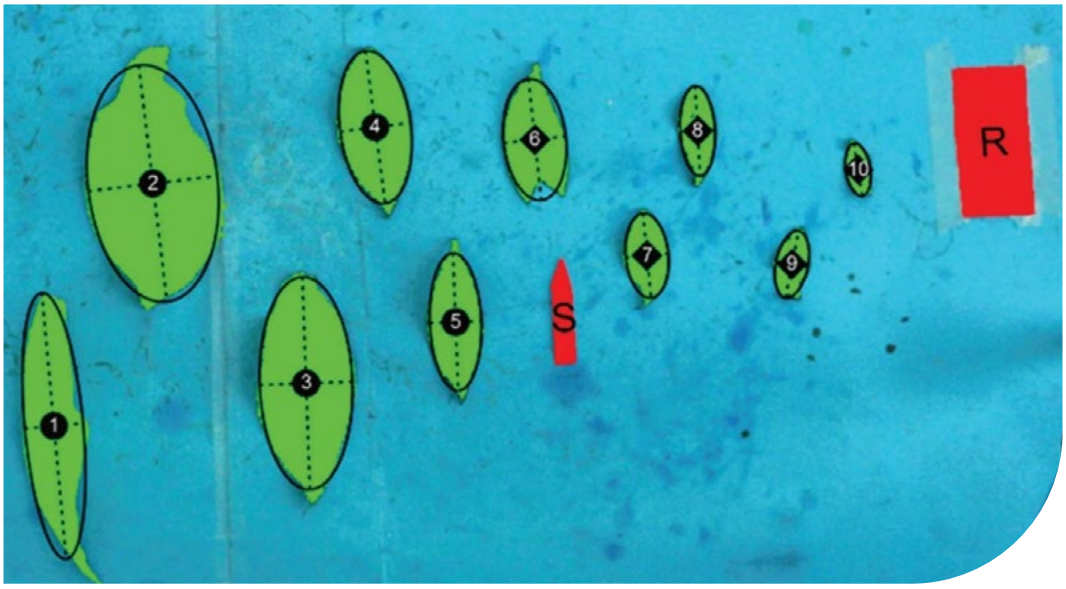


Figure 2. Visualization of the automatic leaf area analysis based on digital images. The green objects represent the detected plant parts by the algorithm. The labels on the green object represent the different objects that were detected. The circle shape of the label means that the leaf was formed after the experiment has started, and the square shapes represent the leaves that were present when the experiment started. Area is calculated by detecting the red reference rectangle as represented by the red object labeled 'R'. The ellipses show the elliptic fit of the leaf objects from which the width and length can be used to approximate leaf width and length. Leaf number 6 has the cut. This means that all leaves below 6 are newly formed during the experiment.

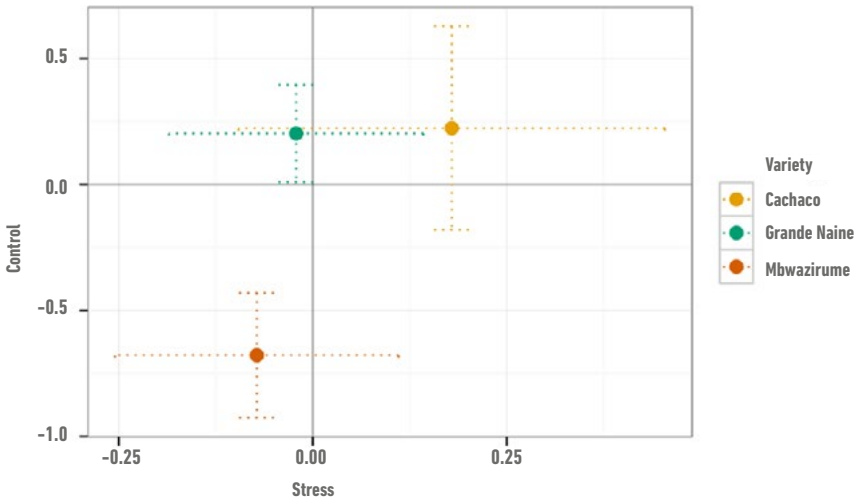


Figure 3. Median normalized plant growth CO_325:0000952 during the experiment under control and stress for each cultivar after 21 days of osmotic stress (n=6). The error bars indicate the standard error.

POT SCREENING

Starting material

The starting material should be *in vitro* plantlets that are ideally obtained from the International *Musa* Germplasm Transit Centre.

Soil

- Characterize the composition of your soil and construct a water retention curve to control the water volume in the pots[10] (Figure 4).
- Sacrifice 50 plants of different sizes per genotype to determine the relation between the area of the whole canopy in top view (CO_325:0000921) and the plant mass (Figure 4).[11]
- Determine the fresh weight and the projected leaf area of each individual plant (Figure 4).
- Make a cut or mark the youngest leaf so that you have a reference point of the start of the experiment.
- Determine the area of the whole canopy in top view weekly (CO_325:0000921) and feed the area into your equation to calculate the plant mass.
- Add water to stay within the limits of the treatments (Table 2).
- At the end of the experiment[12] (at least 28 days), determine all the variables described in Table 1.
- Perform a two-way ANOVA[13] to evaluate the genotype, treatment and genotype × treatment interaction of all the variables. A p-value lower than 0.05 can be considered significant.
- Rank the tested cultivars/genotypes accordingly.

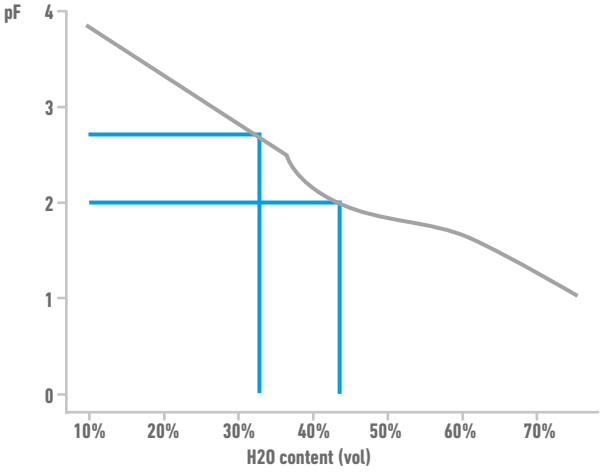
9 If *in vitro* facilities are not available, then the PIF technique should be applied (Kwa M. 2002. New horticultural techniques of mass production of bananas: the PIF technique (plants issued from stem bits). Technical data sheet CARBAP. 2 p.) This technique risks to introduce more variability in the starting population and will be reflected in the number of biological replicates needed.

10 Different curves and so equations might be needed depending on the soil and the water volume. Care should be taken to have always the same bulk density. For an example, see Kissel et al. (2015). A pot volume between 10 to 30 L is appropriate.

11 The area can also be determined by hand - see section on field work.

12 The end of the experiment will be determined by the ratio pot size/plant size and the linearity range of the plant mass/area of the whole canopy in top view.

13 ANOVA is a parametric test that assumes normality and homoscedasticity. This needs to be verified and can only be tested when enough biological replicates are used. If the requisites are not met, then non-parametric tests need to be used.



$$m_{total} = m_{plastic} + m_{drysoil} + m_{plant} + m_{water}$$

f (volume soil) f (leaf area)

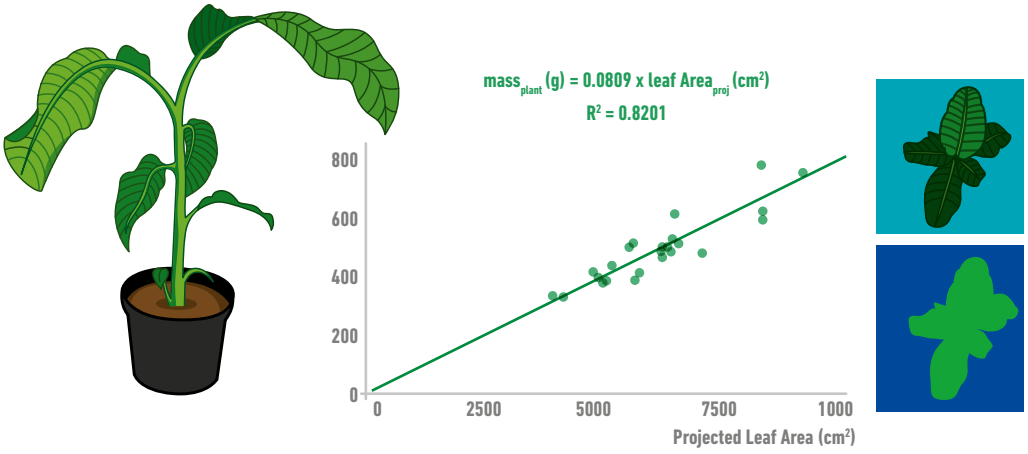


Figure 4. Above: Water retention curve of the soil. The retention curve determines relationship between the soil water volume and the water potential. The soil water volume can be deduced from the pot weight and so the limits of the pot weight can be determined for each treatment. An example is given for pF 2 in blue (control treatment) and for pF 2.7 (water deficit treatment). This relation is soil specific and must be determined for each experiment. Below: Relation of whole canopy in top view and real plant mass. In the experiment, weekly pictures are taken, the new plant mass is estimated based on the whole canopy-plant mass relation and the target pot weight is adjusted in order to ensure the correct water volume in the pot.

Table 2. The different treatments given as an interval of soil water potential Ψ and of its logarithmic scale. The volumetric water content for every Ψ depends on the experimental specific water retention curve. It is important that the pF of control level stays below 2.1 since we have evidence of starting water deficit at that level. The other 2 stress levels are indicative and should be chosen based on real values encountered in the field of choice.

WATER LEVEL	SOIL Ψ (hPa)	LOG |Ψ|= pF
0 (control)	[-63, -126]	[1.8, 2.1]
1 (stress)	[-316, -501]	[2.5, 2.7]
2 (stress)	[-631, -1259]	[2.8, 3.1]

3. FIELD SCREENING

Introduction

Screening bananas in the field is challenging. It involves detailed and careful planning to accommodate different genotypes with varied crop durations. In addition, tolerant traits need to be measured during the critical stages in the ontogeny (Gibbs and Turner, 2018). The following protocol describes screening many banana accessions in the field. As stated above, the life cycle of a banana shoot can be divided into three phases: vegetative, floral and fruiting (Figure 1). At the start of the floral phase, the apex changes from leaf formation towards the inflorescence formation. During this crucial floral phase, the following events take place: formation of nodes (hands) of fruit-forming flowers along the female peduncle, the slow elongation of the aerial true stem and male peduncle, and then the formation of nodes of male flowers. The floral phase ends when the inflorescence emerges from the top of the pseudostem (Figure 1). The fruit phase is from inflorescence emergence until fruit maturity. The sequence of events during bunch formation is best observed with reference to retrospective counting of leaves, starting at bunch emergence. The start of the floral phase (bunch formation) begins at leaf −11 (11 leaves before bunch emergence), the formation of the fruit-bearing hands can be seen microscopically at leaf −9 and is completed at about leaf −7 or −6 (Gibbs and Turner, 2018).

During the vegetative phase, a water deficit will delay the transition from vegetative to floral phases (Fortescue et al., 2011). The interaction between seasonal changes in temperature, photoperiod and soil-water balance will influence the timing of the transition from vegetative to floral phase, contributing to seasonal variation in flowering and bunch harvest (Gibbs and Turner, 2018). The floral phase is the most vulnerable. There is a competition for resources between the developing bunch, growth of emerging leaves and elongation of the aerial true stem. A water deficit during the floral phase will prolong the crop cycle and will reduce the yield, especially affecting the number of fruits per hand (Gibbs and Turner, 2018). Water deficit during the fruit phase affects the fruit filling and will reduce the bunch, hand size and weight. Late flower abortion might also influence the number of fruits per hand.

Characterization of targeted population environment

The experimental setup must target the environment where the improved varieties are to be grown. The target environment varies from region to region. Drought risk analysis needs to be obtained from

the knowledge and experience of farmers and/or weather station data. Monitoring the following water levels / and VPD during crop growth period is essential:

1. Early water deficit that occurs during the vegetative phase
2. An intermittent water deficit that occurs during the floral phase
3. Late water deficit that may occur during fruit phase

In addition to the timing, it is also important to control the severity of the water deficit and compare it to fully irrigated controls. Therefore, it is important to know the water availability for the complete crop growth period.

Field testing

- Determine the water balance model based on weather data[14] and knowledge of soil texture & root depth and estimate the available water during crop growth period.
- Collect soil from the experimental field at 15–30 cm depth at different places.[15]
- Saturate the soil and subject it to different pressures in the pressure plate membrane apparatus to derive the soil moisture release curve.
- From the soil moisture release curve, calculate the soil matric potential.[16]
- Prepare the land accordingly[17, 18] and dig pits of 45 cm x 45 cm x 45 cm at a distance of 2m x 2m and finally add 10–15 kg of well decomposed Farmyard Manure / composted manure per pit.[19]
- Select 'sword suckers'[20] with broad corm and narrow sword-like leaves from plants, which are free from pests and diseases (virus, fungus, bacteria, corm-borer and nematodes).
- Move the suckers to the field when they are 2–3 months old, uniform in size, weighing 1–1.5 kg (varies with genotypes).
- Dip the suckers[21] in 0.2% Carbendazim (2 g/litre of water) and 0.25% triazophos (2.5 ml/litre) solutions for about 30 minutes as a prophylactic measure against Fusarium wilt disease, nematodes and rhizome weevil infestation.

14 If no weather station data are available, make a simulation of the climate using the free FAO software NewLocClim.

15 Take care to conserve the soil structure and density.

16 This is the same as in Figure 4, pF stands for Log |Ψ| in hPa see Table 2.

17 The selection field is important. We must find a good field with uniform soil physical properties which can accommodate all the accessions to screen. The more heterogeneous the field is, the more replicates and blocks that need to be taken into account.

18 The use of ploughing is currently heavily debated since it disturbs the soil structure completely and might interfere with the soil biome.

19 Though the choice of optimum plant density depends upon locality, cultivar, soil type and fertility and management level. For screening purposes, we recommend 2.1 m x 2.1 m, which can accommodate 2260 plants per ha.

20 One should avoid putting suckers directly in the field. Small suckers need to be grown in pots and transferred to the field as homogeneous plantlets with well-deployed leaves. The ideal is uniform *in vitro* plants or if not possible, we can use the uniform PIF plants. We recommend using genotypes that have a similar crop cycle in favorable conditions.

21 In case of tissue culture plants (Figure 5), two days before planting, apply 10 g Carbofuran in the polythene bags and drench the plants with 0.1% Emissan (1 g per litre of water) to protect the plants against nematode infestation and head rot disease.

Figure 5. Uniform tissue cultured plants (Photo: Ravi Iyyakutty).

- Plant the suckers in the centre point of the pit and press the soil around the suckers firmly[22].
- The design for the experiment should be a split plot with at least three replications. Main Plot (M1 = Irrigated; M2 = Drought; Sub Plot = Genotypes). Each replication must have a minimum of five plants. This experiment must be repeated at least twice for each phase (vegetative, floral and fruit).
- Irrigate[23] the plants daily through drip method during non-rainy season until they are ready for the treatment imposition.

22 Soon after planting, it is important to provide shade using leaves, gunny bags, etc., as it protects the plants against hot sunny weather that helps in better initial establishment.

23 Drip irrigation is desirable as it is easy to control water and reduces the possibility of later seepage of water into irrigated treatments. It is better to have individual taps to each plant level to regulate water to get uniformly to all the treatment plants. The requirement of water per plant may vary with season and stage of plant. Generally, water is applied by drip between 16–24 litres of water per day per plant during non-cloudy days.

Figure 6. Drought phenotyping field trial in India (Photo: Sebastien Carpentier).

Figure 7. Drip irrigation facility laid out in the field (Photo: Ravi Iyyakutty).

- Apply the treatment at an appropriate moment (see above). Impose the treatment through closure of the drip irrigation channel [24].

- Monitor the soil moisture (15 cm to 30 cm of the soil depth) at least at weekly intervals until it reaches a level relevant for the environment[25]. This value can be derived from the soil moisture release curve developed from the experimental field. Measure the soil moisture % from the field at regular intervals through gravimetric method or Time Domain Reflectometry (TDR) or any other soil probe.

Plant growth parameters

It is important to keep track of the number of leaves so that one can count back and determine the shift of the vegetative to the floral phase see above and Gibbs and Turner (2018). A list of useful parameters and the link to the crop ontology is given in Table 3.

Vegetative and floral stage

Plant height, pseudostem girth, leaf length, leaf width, leaf emergence rate and leaf senescence rate (start of yellowing of leaves) are the variables to be measured. Based on these variables other parameters can be derived (Ravi et al., 2013).

Instead of image software, the leaf area (A) can also be measured manually with this formula: A= 0.83 (lb) where l = length of lamina in cm and b = breadth of lamina at its widest point (Summerville, 1944).

Leaf Area Index (L): area of leaf (A) per unit area of land (dimensionless).

The leaf emergence rate (LER) is a useful index of the vegetative development rate of a banana plant and is closely related to temperature. The leaves emerged during the experimental period are noted in both control and treated plants. The total number of fully opened leaves produced during the experimental period should be counted on a weekly basis.

LER = Leaf number / week

Fruit stage

At the fruit stage, record the time of flowering, time of harvest, the fruit filling index, the number of hands and number of fruits at harvest and their weight.

Table 3. List of agronomic traits for field testing

PHASE	TRAIT	ONTOLOGY ID[A]	HOW/WHEN MEASURED
VEGETATIVE PHASE	Plant height (cm)	CO_325:0000009	Measure weekly the distance from the collar, or from the pseudostem base at the ground if the collar is not visible, to the intersection of the petioles of the two youngest leaves (leaf ranks 1 and 2), using a measuring pole or sliding ruler
	Weekly leaf emission rate	CO_325:0000726	Rank of previously marked leaf at one point in time, minus 1 divided by the time elapsed between the two "Date of data collection" events
	Pseudostem height increase per leaf formed	CO_325:0000956	Pseudostem height difference between time points (refer to experimental metadata) per number of new leaves formed between time points

24 During treatment, fertilizers should not be applied. Fertilizers can be applied 10-15 days prior to the treatment.

25 For example, in India a value of -0.6 to -0.7 MPa (pF 3.77–3.84) is relevant.

PHASE	TRAIT	ONTOLOGY ID[A]	HOW/WHEN MEASURED
FLORAL PHASE	Plant girth (cm)	CO_325:0000012	Measure the circumference of the pseudostem of the plant at 75 cm from the collar, or from the pseudostem base at the ground if the collar is not visible, using a tape measure
	Height of tallest sucker (cm)	CO_325:0000027	On the tallest sucker, measure the distance from the pseudostem base at the ground to the intersection of the petioles of the two youngest leaves, using a measuring pole or sliding ruler
	Number of suckers[26]	CO_325:0000024	Count how many of all types of suckers are in the mat
	Weekly leaf emission rate	CO_325:0000726	Rank of previously marked leaf at one point in time, minus 1 divided by the time elapsed between the two "Date of data collection" events
FRUIT PHASE	Bunch weight (kg)	CO_325:0000034	Measured at harvest after removing the peduncle and the rachis
	Number of fruits	CO_325:0010353	Total number of fruits on a bunch at harvest
	Number of hands	CO_325:0000478	Total number of hands on a bunch at harvest
	Number of fingers in hand	CO_325:0000042	Count how many fingers are in a hand. Associate the data with the hand rank
	Fruit length	CO_325:0000482	Measure at harvest the length of the internal arc of a fruit, without pedicel. Record on the inner fruit in the middle of the mid-hand of the bunch. If there is an even number of hands, there will be two middle hands so use the upper hand that developed first. Record the range
	Average finger weight	CO_325:0000335	Calculate at harvest the sum of the finger weight measurements, divided by the number of those measurements
WHOLE CYCLE[27]	Plant cycle	CO_325:0000006	Difference between harvest date and planting date (only for cycle 1)
	Planting to flowering	CO_325:0000000	The time elapsed from planting to when the inflorescence emerges from the pseudostem
	Flowering to harvest	CO_325:0000292	
	Expected yield (t/ha/year)	CO_325:0010077	Bunch weight divided by 1,000, multiplied by annual crop cycle proportion, multiplied by number of plants per ha

[a] Crop Ontology identifier for the measured agronomic traits (source: Crop Ontology Curation Tool, http://www.cropontology.org/ontology/CO_325/Banana).

26 In a normal management, the number of suckers is limited to a selection of 2 generations and so de-suckering is done.

27 Although the Crop ontology website states these variables in days, the ideal way to measure them are in Thermal Units (Turner and Lahav, 1983).

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