

Diagnosis of *Mycosphaerella* spp., responsible for *Mycosphaerella* leaf spot diseases of bananas and plantains, through morphotaxonomic observations

Marie-Françoise ZAPATER¹, Catherine ABADIE^{2*}, Luc PIGNOLET¹, Jean CARLIER¹, Xavier MOURICHON¹

¹ CIRAD-Bios, UMR BGPI,
TA A 54 / K,
34398, Montpellier Cedex 5,
France
marie-francoise.zapater@cirad.fr

² CIRAD-Bios, UPR Mult. Vég.,
Stn. Neufchâteau, 97130,
Capesterre Belle-Eau,
Guadeloupe, France
catherine.abadie@cirad.fr

Diagnosis of *Mycosphaerella* spp., responsible for *Mycosphaerella* leaf spot diseases of bananas and plantains, through morphotaxonomic observations.

Abstract — Introduction. This protocol aims to diagnose under laboratory conditions the main *Mycosphaerella* spp. pathogens of bananas and plantains. The three pathogens *Mycosphaerella fijiensis* (anamorph *Paracercospora fijiensis*), *M. musicola* (anamorph *Pseudocercospora musae*) and *M. eumusae* (anamorph *Pseudocercospora eumusae*) are, respectively, the causal agents of Black Leaf Streak disease, Sigatoka disease and Eumusae Leaf Spot disease. The principle, key advantages, starting plant material and time required for the method are presented. **Materials and methods.** The laboratory materials required and details of the thirteen steps of the protocols (tissue clearing and *in situ* microscopic observations, isolation on artificial medium and cloning of single-spore isolate, *in vitro* sporulation and microscopic observations of conidia, and long-term storage of isolates) are described. **Results.** Diagnosis is based on the observations of anamorphs (conidiophores and conidia) which can be observed directly from banana leaves or after sporulation of cultivated isolates if sporulating lesions are not present on banana samples.

France / *Musa* sp. / *Mycosphaerella fijiensis* / *Mycosphaerella musicola* / *Mycosphaerella eumusae* / foliar diagnosis / microscopy

Identification des espèces de *Mycosphaerella* responsables des cercosporiose des bananiers et plantains, par des observations morphotaxonomiques.

Résumé — Introduction. Le protocole vise à diagnostiquer, en laboratoire, les principales espèces de *Mycosphaerella* pathogènes des bananiers et plantains. Les trois agents pathogènes *Mycosphaerella fijiensis* (anamorphe de *Paracercospora fijiensis*), *M. musicola* (anamorphe de *Pseudocercospora musae*) et *M. eumusae* (anamorphe de *Pseudocercospora eumusae*) sont respectivement responsables de la maladie des raies noires, de la maladie de Sigatoka et de la cercosporiose due à *eumusae*. Le principe, les principaux avantages, le matériel végétal et le temps nécessaires à la méthode sont présentés. **Matériel et méthodes.** Le matériel de laboratoire nécessaire et le détail des treize étapes de réalisation du protocole (prélèvement des tissus et observations microscopiques *in situ*, isolement sur milieu artificiel et clonage d'isolats monosporés, sporulation *in vitro* et observations microscopiques des conidies, conservation à long terme des isolats) sont décrits. **Résultats.** Le diagnostic est basé sur les observations des anamorphes (conidiophores et conidia) qui peuvent être observés directement à partir des feuilles de bananes ou après sporulation des isolats mis en culture s'il n'y a pas de lésions sporulantes sur les échantillons de bananiers.

France / *Musa* sp. / *Mycosphaerella fijiensis* / *Mycosphaerella musicola* / *Mycosphaerella eumusae* / diagnostic foliaire / microscopy

* Correspondence and reprints

1. Introduction

oratory conditions the main *Mycosphaerella* spp. pathogens of bananas and plantains.

Application

The three pathogens *Mycosphaerella fijiensis* (anamorph *Paracercospora fijiensis*), *M. musicola* (anamorph *Pseudocercospora*

This protocol aims to diagnose under lab-

Figure 1.
Protocol for identification of *Mycosphaerella* leaf spot pathogens.

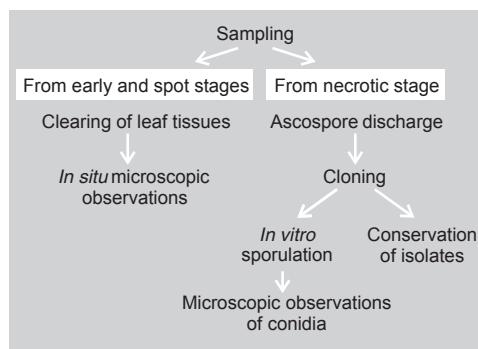


Figure 2.
Early streak stages of Black Leaf Streak Disease caused by *Mycosphaerella fijiensis*.

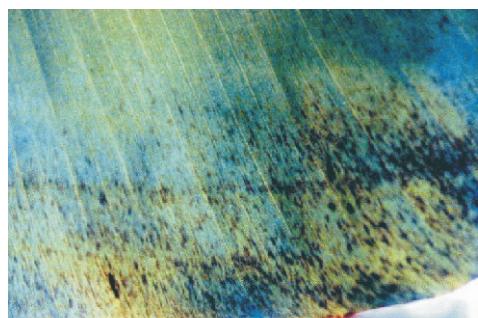


Figure 3.
Spot stages caused by *Mycosphaerella musicola*.

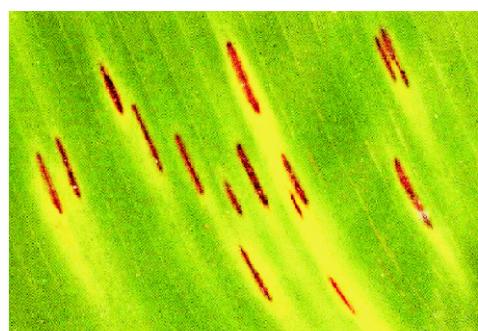
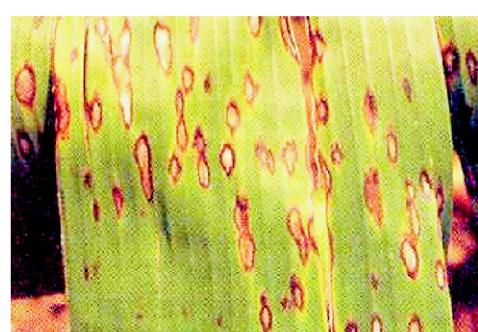


Figure 4.
Spot stages caused by *Mycosphaerella eumusae*.



musae) and *M. eumusae* (anamorph *Pseudocercospora eumusae*) are, respectively, the causal agents of Black Leaf Streak disease, Sigatoka disease and Eumusae Leaf Spot disease.

Principle

The three pathogens *M. fijiensis*, *M. musicola* and *M. eumusae* are difficult to distinguish on the basis of symptom expression, and also on their sexual stages (teleomorphs). However, the species can be identified on the basis of microscopic differences between their asexual stages (anamorphs), whether they are taken directly from diseased leaves or cultured after being isolated, depending on the quality of initial banana materials and on the stage of lesions (figure 1).

Starting material

- For *in situ* microscopic observations, the specimens should be taken from leaves at the early streak stages for *M. fijiensis* (figure 2), and at the spot stages for *M. musicola* (figure 3) and *M. eumusae* (figure 4). Leaves should be kept in plastic bags.
- For fungal isolation and *in vitro* microscopic observations, the specimens should come from completely necrotic leaves regardless of the species (figures 5, 6). Leaves should be thoroughly dried between sheets of paper.

Time required

The total time required is approximately 24 h for clearing of leaf tissues and *in situ* microscopic observations; 48 h for ascospore discharge and cloning; 2 weeks for fungal culture and 2 weeks for *in vitro* sporulation.

2. Materials and methods

2.1. Laboratory materials

To be achieved, the protocol requires water agar (30 g agar-L^{-1}), potato dextrose agar (PDA) medium (39 g PDA-L^{-1}), modified V8-juice medium (100 mL of V8 juice, 0.2 g of CaCO_3 , 20 g agar-L^{-1} of medium, pH 6), a stereomicroscope (light from under the plate) and a microscope for routine laboratory applications (objectives $4\times$, $10\times$, $40\times$); all media include antibiotics added after sterilisation (streptomycin sulphate at $100 \mu\text{g}\cdot\text{mL}^{-1}$ and penicillin at $100 \text{ UI}\cdot\text{mL}^{-1}$).

2.2. Protocols for identification of *Mycosphaerella* spp.

Tissue clearing and *in situ* microscopic observations

- Step 1

Clear leaf tissue as follows:

- Cut leaf tissue with appropriate lesions into 1.5-cm-long pieces.
- Place samples in a solution of KOH (10%) overnight (at least).
- Wash samples five times in water for 10 min each time.

- Step 2

Observe conidiophores associated with lesions: conidiophores can be directly observed on slides, without staining, on lower or upper leaf surfaces, depending on the expected species (*table I*).

- Step 3

Observe conidia:

- Stain cleared tissues for 1 min with a solution of 0.5% blue cotton in a mixture of lactic acid/glycerol at 3v/v.



Figure 5.

Necrotic leaves caused by *Mycosphaerella fijiensis*.



Figure 6.

Necrotic leaves caused by *Mycosphaerella eumusae*.

Table I.

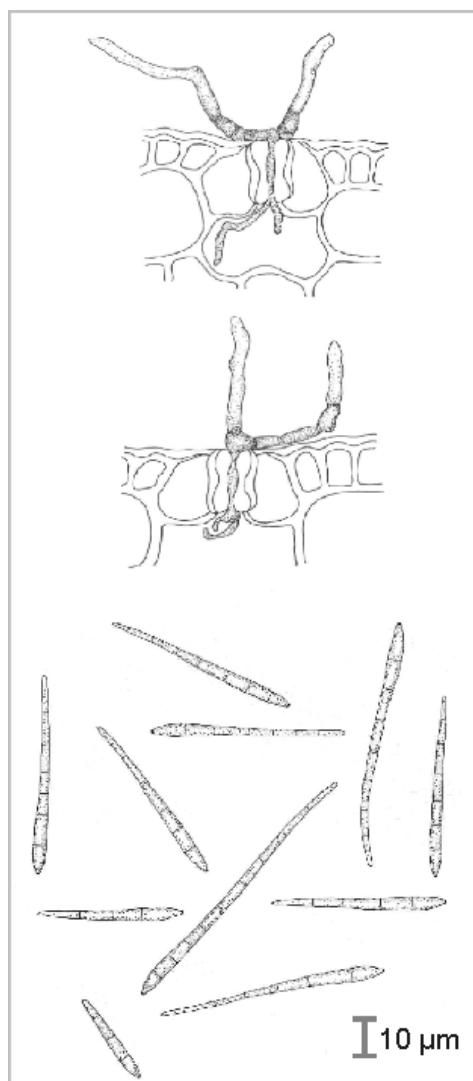
Morphological characteristics of the anamorphs *Paracercospora fijiensis*, *Pseudocercospora musae* and *Pseudocercospora eumusae*, pathogens of bananas and plantains.

Species (anamorph)	Conidiophores	Conidia
<i>Paracercospora fijiensis</i> (figure 7)	<ul style="list-style-type: none"> • First appearance at early streak stage According to Fouré's stages 2 to 3 [1] • Mainly on the lower leaf surface • Emerge singly or in small groups (2 to 6), sporodochia and stromata absent • Straight or bent geniculate, pale to light brown, 0–5 septate occasionally branched, slightly thickened spore-scars • 16.5–62.5 µm × 4–7 µm 	<ul style="list-style-type: none"> • Obclavate to cylindro-obclavate, straight or curved, hyaline to very pale olivaceous, 1–10 septate, distinct basal hilum (scar) • 30–132 µm × 2.5–5 µm
<i>Pseudocercospora musae</i> (figure 8)	<ul style="list-style-type: none"> • First appearance at spot stage (Brun's stage 4, [2]) • Abundant on both leaf surfaces • In dense fascicles (sporodochia) on dark stroma • Straight, hyaline, mostly without septation and geniculation; no spore scars • 5–25 µm × 2–5 µm 	<ul style="list-style-type: none"> • Cylindric to obclavato-cylindric, straight or curved, pale to very pale olivaceous, 0–8 septate, no distinct basal hilum • 10–109 µm × 2–6 µm
<i>Pseudocercospora eumusae</i> (figure 9)	<ul style="list-style-type: none"> • First appearance at spot stage • Mainly on the upper leaf surface • In dense fascicles on brown stroma • Subcylindrical, hyaline or pale brown below, 0–3 septate, straight to geniculate-sinuous • 10–25 µm × 3–5 µm 	<ul style="list-style-type: none"> • Subhyaline to pale olivaceous, thick-walled, smooth, subcylindrical, apex obtuse, base subtruncate, straight to variously curved, 3–8 septate • 30–50 µm × 2.5–3 µm

Adapted from Wardlaw [3], and Crous and Mourichon [4].

Figure 7.

Morphological characteristics of the anamorph *Paracercospora fijiensis* [5].



- Wash samples in water and mount them in glycerin (20% in water).
- Conidia can be observed on slide staining on lower or upper leaf surfaces, depending on the expected species (*table I*).

Isolation on artificial medium and cloning of single-spore isolate

- Step 4
Dry necrotic banana leaves at room temperature for 48 h.
- Step 5
Cut pieces from necrotic leaves (about 30 cm²) and then soak them in distilled water for 15 min.

- Step 6

Secure leaf sections to the underside of the lids of petri dishes containing water agar at 3%.

- Step 7

Ascospores are discharged overnight onto the agar surface. Note: the ascospores of the three *Mycosphaerella* species have two cells and measure between 12–18 µm × 2.5–4.5 µm.

- Step 8

Transfer single germinating ascospores to fresh PDA medium the next morning (using the stereomicroscope). Note: If no ascospore is obtained, incubate leaf sections for 48 h on wet filter paper put in petri dishes, soak them in distilled water for 5 min, then transfer them onto the lids of petri dishes as described above.

- Step 9

Incubate cultures at 25 °C for at least 10 days.

***In vitro* sporulation and microscopic observations of conidia**

Note: conidial sporulation (asexual reproduction) is induced by culturing small pieces of mycelia on modified V8-juice medium.

- Step 10

Transfer three to five small PDA culture plugs onto modified V8-juice medium.

- Step 11

Incubate cultures at 20 °C for (10 to 14) d under continuous and cool-white fluorescent light.

- Step 12

Scrape cultures with a scalpel and suspend conidia in a staining solution (see step 3) directly on the slide before microscopic observation of conidia. Note: if more than one ascospore has been taken during isolation, it can be useful to clone the *Mycosphaerella* strain:

- spread conidial suspension on the agar medium (30 g L⁻¹) and check the density of conidia with a stereomicroscope,
- incubate petri dishes at 25 °C for 48 h,
- take conidia individually and put them on PDA medium.

Long-term storage of isolates

This step is achieved for collection purposes.

- Step 13

Place mycelium fragments from PDA colonies [(10 to 15) d old] in 15% glycerol for 2 h at 4 °C, then transfer them to a freezer for long-term storage at – 80 °C.

3. Typical results obtained

Diagnosis is based on the observations of anamorphs (conidiophores and conidia) which can be observed directly from banana leaves (step 1 to step 3) or after sporulation of cultivated isolates (successively, step 4 to step 9, and step 10 to step 12) if sporulating lesions are not present on banana samples. The morphological characteristics of anamorphs are presented in *table I*, and *figures 7–9*.

Caution: care should be taken to not confuse these pathogens with other fungal species that also attack banana leaves [6].

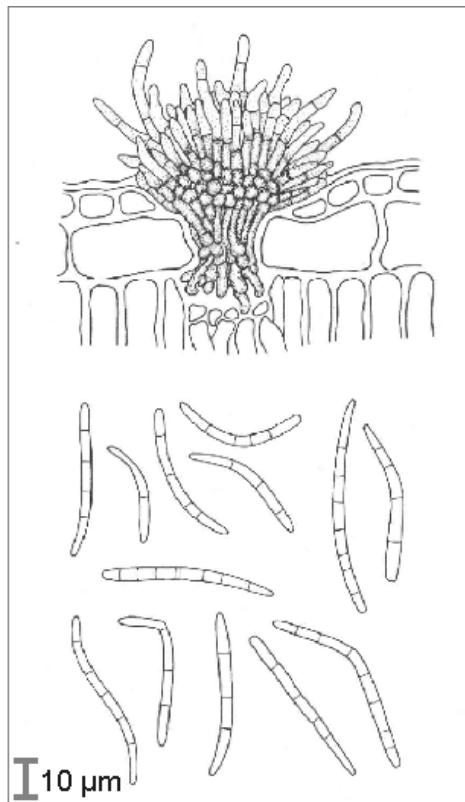


Figure 8.

Morphological characteristics of the anamorph *Pseudocercospora musae* [5].

References

- [1] Fouré É., Les cercosporiose du bananier et leurs traitements. Comportement des variétés. Études de la sensibilité variétale des bananiers et plantains à *Mycosphaerella fijiensis* Morelet au Gabon (maladie des raies noires). I. Incubation et évolution de la maladie, *Fruits* 37 (1982) 749–759.
- [2] Brun J., La cercosporiose du bananier en Guinée. Étude de la phase ascosporée du *Mycosphaerella musicola* Leach., Univ. Orsay, Thèse, Paris, France, 1963, 196 p.
- [3] Wardlaw C.W., Banana diseases including plantains and abaca, 2nd ed., Longman, London, UK, 1972, 648 p.
- [4] Crous P.W., Mourichon X., *Mycosphaerella eumusae* and its anamorph *Pseudocercospora eumusae* spp. nov.: causal agent of eumusae leaf spot disease of banana, *Sydowia* 54 (2002) 35–43.
- [5] Meredith D.S., Banana leaf spot disease (Sigatoka) caused by *Mycosphaerella musicola* Leach., *Phytopathol. Pap.* 11, 1970, 147 p.
- [6] Jones D.R., Diseases of banana, abaca and enset, CABI Publ., Wallingford, UK, 2000, 544 p.

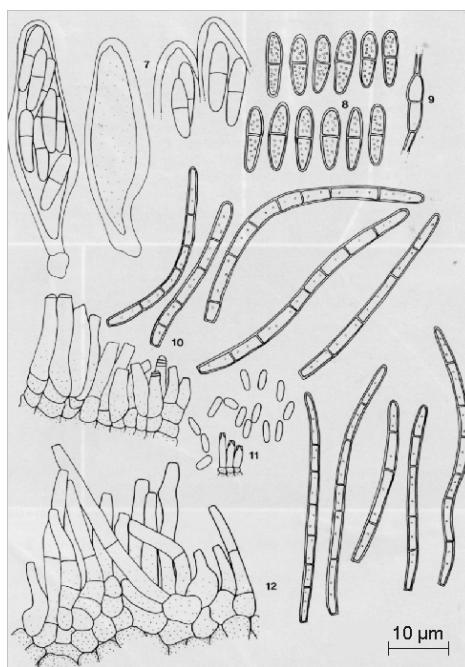


Figure 9.

Morphological characteristics of the anamorph *Pseudocercospora eumusae* (conidia and conidiophores) *in vivo* (No. 10) and *in vitro* (No. 12) [4].