A laboratory method to evaluate *Pseudocercospora musae*'s (teleomorph: *Mycosphaerella musicola*) sensitivity to fungicides

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A laboratory method to evaluate *Pseudocercospora musae*'s sensitivity to fungicides.

Abstract — Introduction. This protocol aims at detecting and evaluating sensitivity shifts in Pseudocercospora musae (teleomorph: *Mycosphaerella musicola*) populations towards fungicides that are currently sprayed to control Sigatoka disease of banana. The principle, key advantages, starting plant material, time required and expected results are presented. **Materials and methods**. Necessary laboratory materials, and details of the seven steps of the protocol achieved during four days of experiments are described. **Results**. The protocol results in the observation of conidial germination of *P. musae* (pattern or germ tube elongation) according to the sensitivity to fungicides.

France (Guadeloupe) / *Musa* sp. / disease control / methods / laboratory equipment / *Pseudocercospora musae* / pesticide resistance

Une méthode de laboratoire pour évaluer la sensibilité de *Pseudocercospora musae aux* fongicides.

Résumé — Introduction. Le protocole vise à détecter et évaluer des variations de sensibilité dans des populations de *Pseudocercospora musae* (téléomorphe : Mycosphaerella musicola) vis-à-vis de fongicides qui sont utilisés pour contrôler la maladie de Sigatoka chez le bananier. Le principe, les principaux avantages, le matériel végétal de départ, le temps nécessaire et les résultats attendus de la méthode sont présentés. **Matériel et méthodes**. Le matériel de laboratoire nécessaire et le détail des sept étapes du protocole réalisées sur quatre jours d'expérimentation sont décrits. **Résultats**. Le protocole aboutit à l'observation de la germination de conidies (aspect ou croissance du tube germinatif) de *P. musae* selon la sensibilité aux fongicides.

France (Guadeloupe) / *Musa* sp. / contrôle de maladies / méthode / matériel de laboratoire / *Pseudocercospora musae* / résistance aux pesticides

1. Introduction

Application

This protocol aims at detecting and evaluating sensitivity shifts in *Pseudocercospora musae* (teleomorph: *Mycosphaerella musicola*) populations towards fungicides that are currently sprayed to control Sigatoka disease of banana.

Principle

Heavy conidiogenesis is induced on individual banana leaf spots. Conidia within a same lesion are considered genetically similar since they issue from a single strain. These conidia are transferred to different agar plates amended or not with a specific fungicide concentration. Conidial germination (or subsequent germ tube growth) on the different media is evaluated to determine *in vitro* sensitivity of each strain. The sensitivity of a conidial population from a fungicide-sprayed area is compared with the sensitivity of a wild conidial population originating from an unsprayed area.

Key advantages

This method is derived from that described by Cronshaw [1]. Other methods focus on

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ascospores and are based on a hyphal or colony growth test [2]. However, key advantages of the described method include:

– in contrast with ascospore methods, no risks of confusion with other morphologically similar *Mycosphaerella* species that are present on banana leaves;

– for a given strain, it is possible to carry out simultaneous tests on different media amended with different fungicides. This is particularly important to study cross-sensitivity among several fungicides or to analyse conidial germination on several fungicide concentrations;

– this method is easier to carry out than methods based on colony radial growth, since radial growth is very slow for this pathogen.

However, conidia observation is time-consuming and requires well-trained technicians.

Starting material

The method requires banana leaves bearing individual stage 4 lesions of Sigatoka disease.

Time estimation for one test

Four days are necessary before observing germination or germ tube elongation.

– Day 1:
• leaf sampling: 1 h (transportation to field not included),
• leaf preparation: 45 min,
• agar plate preparation: 1 h preparation, 3 h autoclave, 1 h fungicide stock solutions and addition, and pouring media into petri dishes.
– Day 2: selection of sporulating leaf lesions under a stereomicroscope and transfer of conidia to agar plates: 3–4 h.

– Day 4: germination or germ tube elongation evaluation: (1 to 5) h according to the fungicide [evaluation is faster for antimitotics than for Sterol Biosynthesis Inhibitor (SBI)]

Expected results

We obtain (a) for antimitotic products, the percentage of resistant strains in the population analysed; (b) for SBI fungicides, variation and distribution of the fungicide sensitivity of the isolates, within the population analysed, according to their level of hyphal growth reduction.

2. Materials and methods

Laboratory materials

The protocol requires: plastic bags (30 cm × 40 cm) and clean paper for incubation; agar, sterile plastic petri dishes (90 mm), glassware for media preparation, distilled water, autoclave, fungicide preparations with known concentration, ethanol, 0.45-µm filters, micropipettes; stereomicroscope, glass needles; light microscope.

Protocol

Day 1

• Step 1
Leaf sampling: collect, within the same banana plot, at least 15 leaves (from 15 different plants) bearing numerous Sigatoka disease lesions at the stage 4 according to Brun's scale [3].

Note: selected sample leaves should also bear young active stages of the disease, to ensure that fungal sporulation will not be affected by the last fungicide sprays. For this reason, sampling should not be done less than 4 weeks after the last fungicide application.

• Step 2
Incubation of samples to induce conidial production:
– cut large [(10–20)-cm] leaf portions bearing stage 4 lesions and group all the pieces from the same leaf in a plastic bag humidified with a wet clean paper,
– incubate bags at 22–25 °C for 10–16 h.

• Step 3
Agar plate preparation:

– prepare 90-mm agar plates (30 g agar·L^{-1}),
– prepare fungicide-amended agar plates (30 g agar·L^{-1} + the necessary amount of the fungicide to reach the correct concentration).

Note: fungicides should be added after autoclaving the agar. Stock solutions are prepared in order to add a volume of 500–

1000 µL of fungicide solution for 1 L of medium. If the fungicide is miscible in alcohol, fungicide solution can be added to the medium directly. If the fungicide is miscible in water, sterilise the solution through filtration (0.45 µm) before adding the fungicide to the medium. Commonly used concentrations are: benomyl (5 mg·L^{-1}), propiconazole and most triazoles (0.1 mg·L^{-1}). Agar plates amended with fungicide should not be conserved for more than 1 week.

Day 2

• Step 4
Selection of sporulating lesions: select, under a stereomicroscope, lesions bearing large quantities of conidia.

Note: conidial production should be heavy and distributed on the whole surface of the selected spots in order to facilitate the transfer to the agar plates. Select four lesions per leaf, so that the total sample size is 60 strains per plot.

• Step 5
Transfer of conidia to agar plates:

– draw with a felt-tip pen 16 diametrical lines on the back of each plate. Number the 32 sectors thus obtained,
– using a glass needle, pick conidia off a selected sporulating spot and gently streak them over a marked sector of the non-amended agar plate (control). Repeat this, from the same lesion, for the successive fungicide-amended plates.

Note: in order to facilitate the observations, for the different fungicide and concentration studies, always streak the same strain on the same corresponding sector of the plates (same number),

– incubate plates for 48 h at 25 °C.

Day 4

• Step 6
Microscopic observations:

– after 48 h, observe sectors of the different plates under a light microscope,
– for antimitotic products, conidia with distorted germ tubes are considered as susceptible; conidia with normal germ tubes are considered as resistant (*figure 1*),

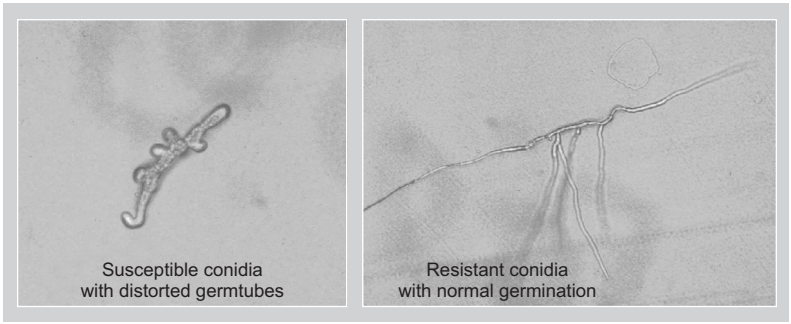


Figure 1.
Example of *in vitro* germination patterns observed on conidia of *Pseudocercospora musae* exposed to a concentration of 5 mg benomyl·L^{-1}.

– for SBI products, measure the germ tube length of conidia with a micrometer on control (Lc) and fungicide-amended medium (Lf).

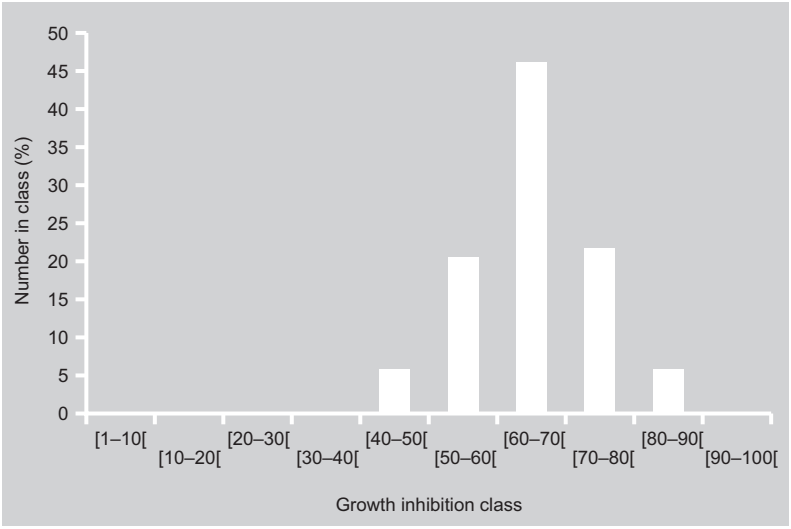
• Step 7
Data analysis:

– for antimitotic products, calculate the percentage of resistant strains in the whole sample (60 strains),

– for SBI products, calculate the growth inhibition (GI) as {GI = [1 – (Lf / Lc)] × 100},

– for each fungicide concentration, determine the distribution of sensitivity of each strain using the following growth inhibition classes [0–10[, [10–20[, [20–30[, [30–40[, [40–50[, [50–60[, [60–70[, [70–80[, [80–90[, [90–100]. Calculate the frequency of strains in each growth inhibition class and make a graphic representation of this distribution (*figure 2*, *3*). Compare this distribution with that obtained for the wild conidial population originating from the unsprayed

Figure 2.
Example of growth inhibition distribution obtained in a sensitivity test conducted on a conidial population of *Pseudocercospora musae* sampled in an untreated area, for a concentration of 0.1 mg propiconazole·L^{-1}.



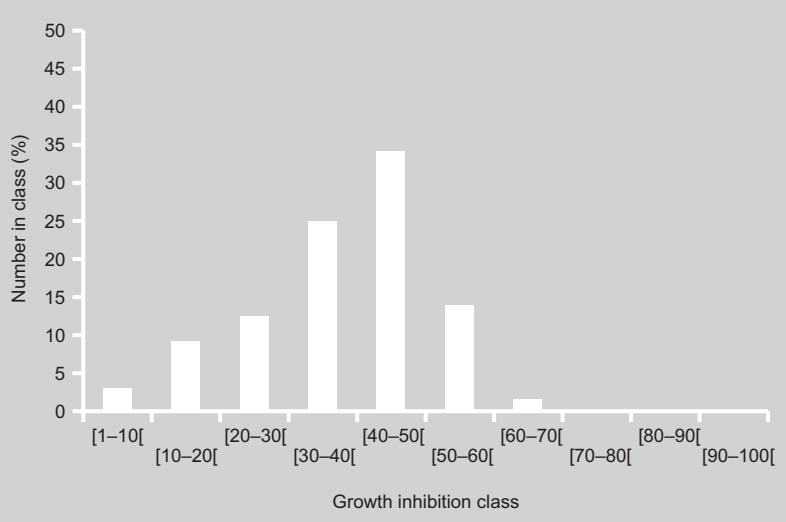


Figure 3.
Example of growth inhibition distribution obtained in a sensitivity test conducted on a conidial population of *Pseudocercospora musae* sampled in a treated area, for a concentration of 0.1 mg propiconazole·L^{-1}.

reference area. Particularly, calculate (NRC), the percentage of strains distributed in classes not represented in the untreated area population (% of strains with a GI < 50%), as well as the mean growth inhibition (MGI). A statistical analysis of the distribution of the two populations will show whether the distribution in the fungal population in contact with the fungicide is different from that of the reference population.

Troubleshooting

Two main problems can occur:

(a) There is little sporulation on lesions: time between the last fungicide application and leaf sampling is too short.

Solution: take care when sampling that active lesions (stages 2–3) are still present on the leaf.

(b) No conidia or too few conidia are present on the sectors when viewing under the microscope. This can result from:

– an incorrect selection of the spots: conidia should cover the whole surface of the spot,

– all conidia were transferred onto the same plate.

Solutions: do not cover all the spots with the glass needle for the same transfer onto one sector; select larger lesions.

3. Typical results obtained

The protocol results in the observation of conidial germination of *P. musae* according to the sentivity fongicides (*figures 1 to 3*).

References

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