A novel bioassay to monitor fungicide sensitivity in *Mycosphaerella fijiensis*

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Abstract

BACKGROUND: Black leaf streak disease (BLSD) is the most important disease of bananas for export. The successful control of BLSD requires an intensive use of systemic fungicides, leading to the build-up of resistance and failure of control. Early detection of fungicide resistance is crucial to drive rational chemical strategies. Present methods relying on ascospore germination bioassays have several drawbacks that could be overcome using conidia.

RESULTS: Generally, a single genotype is present on the conidial population derived from one lesion. Conidial germination tests with thiabendazole (5 mg L⁻¹) enable a clear detection of strains resistant to methyl benzimidazole carbamates. Germination bioassays on azoxystrobin (10 mg L⁻¹) enable the detection of most QoI-resistant strains, but their proportion might be underestimated with cut-off limits of germ tube length (L > 120 μm) or growth inhibition (GI < 50%). The level of fungicide resistance differs at different canopy levels of a banana tree, which should be considered for sampling. The ascospore germination bioassay provided more variable estimations of the level of resistance by comparison with the new conidial germination bioassay.

CONCLUSION: Germination bioassays performed with conidia obtained from young lesions overcome most drawbacks encountered with ascospore germination bioassays and could be considered as a new reference method for fungicide resistance monitoring in this species. Different steps are proposed, from sampling to microscopic examinations, for the implementation of this technique.

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Keywords: *Mycosphaerella fijiensis*; monitoring; fungicide resistance; mutation; phenotype; conidia

1 INTRODUCTION

The fungus *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease (BLSD) of bananas, exhibits a high adaptation capacity.¹ The rapid, widespread development of fungicide-resistant strains has been responsible for major changes in strategies to manage BLSD, which remains the main constraint to growing bananas for export.² Commercial banana varieties are highly sensitive to the pathogen, and export bananas are managed with intensive chemical control using contact or systemic fungicides, or a combination of both.²³ Antimiotic fungicides, classified as methyl benzimidazole carbamates (MBCs), have been used since the early 1970s, and *M. fijiensis* strains bearing the β-tubulin E198A gene mutation initially emerged throughout Latin America, Africa and the West Indies 2–10 years thereafter.²³–⁵ Demethylation inhibitor (DMI) fungicides were introduced in the mid-1980s, and changes in sensitivity were first observed 10 years later.⁵–⁷ Nevertheless, DMIs are still applied in most banana-growing regions because different compounds of this group are not affected by resistance to the same extent.⁷⁻⁸ Quinone outside inhibitor (QoI) fungicides were introduced in Latin America between 1997 and 2000, and strains carrying the target-site mutation G143A causing strong disruptive resistance appeared after only 2–3 years of their use.⁵⁻⁹⁻¹² The development of fungicide resistance questions the efficacy of sustainable forecasting-based disease control systems, which strongly rely on highly curative systemic fungicides.³ Contact pesticides have therefore been used more extensively because resistant strains to these fungicides have not been detected owing to their multisite mode of action. This has led to a substantial increase in quantities of active ingredients applied per hectare.² As systemic fungicides exert a strong selection pressure on fungal populations, variations in pathogen fungicide resistance must be closely monitored using reliable assessment methods, so as to
be able to tailor more sustainable systemic fungicide treatment strategies.

The method routinely used for monitoring of resistance in *M. fijiensis*, and recognised by the Fungicide Resistance Action Committee (FRAC), is based on ascospore germination tests. This involves mixing composite samples of leaf necrotic mass-infected tissue producing ascospores, and then incubating the samples to induce ascospore projection directly on fungicide-supplemented and control (no fungicide) media (Jacome LH, www.frac.info/Monitoring_Methods). However, this method has some drawbacks. Firstly, the extent of ascospore production is often deficient, so the tests often fail. Leaf necrosis is also hard to sample in commercial banana plantations because lesions are systematically removed for prophylactic reasons. Secondly, there is always a high discrepancy between the analysed ascospore population and the initial sample. Effectively, ascospore production is generally very scarce, and from the initial 25 leaves collected in the field, only some few leaves will produce ascospores in some rare lesions, so the final analysed population is not representative of the initial sampling plan. Thirdly, there is a high risk of confusing *M. fijiensis* ascospores with those of other *Mycosphaerella* species. Indeed, over 20 species of this genus have actually been identified on necrotic banana leaves, and ascospores of these species are very hard to differentiate, especially in the presence of fungicides. Fourthly, in more precise bioassays, single or specific strains are compared in the presence and absence of fungicide, whereas this is not possible with the ascospore method, because the fungicide sensitivity of different ascospore populations is compared between untreated control and fungicide-supplemented media.

In *M. musicola*, the causal agent of another leaf spot disease of banana, conidium production is abundant, and conidial germination tests have been developed for monitoring of fungicide resistance in this species. Conidium production is not as abundant in *M. fijiensis*, but conidia can be harvested by placing lesions over an agar medium.

The authors have hypothesised that conidia could be used for germination inhibition tests in *M. fijiensis* to help solve certain problems that may arise when using ascospores. Indeed, the use of conidia makes it possible: (i) to carry out germination tests in any situation, as conidial sporulation is especially early in this species and therefore always present, even in treated areas; (ii) to comply with a sampling plan because of the sporulation regularity; (iii) to avoid confusion between different species, as only conidia of *M. fijiensis* are present on stage 2 and stage 3 lesions, and also by virtue of their specific morphological features; (iv) to compare the same population of strains on untreated and fungicide-supplemented media.

Thus, the aim of the present study was to develop a new biological test based on conidial germination to be used for routine analysis of fungicide resistance in *M. fijiensis*. Firstly, the validity of this method is based on the assumption that all conidia from the same lesion belong to the same genotype. This hypothesis was tested by analysing genetic diversity between conidia derived from the same lesion. Moreover, the germination tests are based on the hypothesis that resistant strains can be detected through germination phenotypes when a fungicide is present. However, a range of phenotypes may be observed in the presence of a fungicide, and it is sometimes hard to associate them with the presence of mutations conferring a specific level of resistance. In this fungus, the results of germination tests were therefore compared with the molecular detection of mutations that confer resistance to MBC or QoI fungicides.

An attempt was also made to optimise different steps for implementing this method. Firstly, one of the advantages of using conidia is that the same lesion can be compared in terms of the response to a range of different fungicides. Tests were therefore conducted to establish whether successive application of one lesion over several media containing different fungicides would affect conidial germination owing to potential fungicide contamination between media. Moreover, the possibility of certain factors having an effect on the sampling plan was studied. As apical leaf expansion continues until flowering in banana, it was hypothesised that several successive cohort populations could be spatially structured at different banana leaf levels in the canopy. This hypothesis was checked by comparing the level of fungicide resistance at different leaf levels. Finally, apart from the various benefits associated with the use of conidia, a check was also carried out to establish whether this new method could enhance reliability by comparison with the ascospore-based reference method.

2 MATERIALS AND METHODS

2.1 Isolate collection

Isolates were collected from different commercial banana plantations in Cameroon where there was a high likelihood of isolating strains resistant to various fungicides. Monospores were collected from resistant isolates able to germinate in the presence of fungicides, as well as highly sensitive strains. Mycelial fragments of colonies formed by each isolate were then frozen (−80°C) in glycerol (150 mL L⁻¹).

2.2 Fungicides

The fungicides used were technical grade and consisted of the following active ingredients: azoxystrobin and propiconazole (Syngenta, Basel, Switzerland), thiophanate-methyl (Arysta LifeScience, Nogueres, France) and thiabendazole (Sigma, St Gallen, Switzerland). Use was also made of salicylhydroxamic acid (SHAM) (Alfa Aesar, Karlsruhe, Germany), an inhibitor of alternative respiration (AoX). Stock solutions of these fungicides were prepared in pure methanol before being added to various media at the desired concentrations. Stock solution of SHAM was prepared in pure dimethyl sulfoxide (DMSO).

2.3 Spore germination bioassays

2.3.1 Spore germination bioassay using ascospores from field lesions

Ascospores were analysed using the method recommended by the Fungicide Resistance Action Committee (Jacome LH, www.frac.info/Monitoring_Methods).

2.3.1.1 Sampling. For each sample, dry necrotic leaf tissues at stage 6, according to Fournel’s scale, were collected on 25 banana trees.

2.3.1.2 Culturing. necrotic tissue samples were bulked in a plastic bag, moistened with water-soaked cotton and incubated for 48 h at 22°C to enable perithecium maturation. These necrotic tissues were then cut into fragments (2 cm²), and five tissue fragments were randomly selected, stapled onto a filter paper
disc (90 mm diameter), soaked in water for 10 min to induce ascospore discharge and then placed in the bottom of a petri dish. Ascospores were left to discharge for 1–2 h on solid medium (agar 20 g L⁻¹), supplemented or not supplemented with fungicide, and incubated at 25 °C for 48 h. For each sample, 30 fragments, placed in six petri dishes, were monitored for each analysed medium.

2.3.2 Spore germination bioassay using conidia from field lesions

2.3.2.1 Sampling. For each sample, pieces of fresh leaf blades bearing young lesions (stages 2–3 on Fouré’s scale) were collected on 25 banana trees.

2.3.2.2 Culturing. Individual lesions were removed, and their underside was applied on the different solid media (agar 20 g L⁻¹) for 10 days. Conidial sporulation was induced in V8 100 solid medium containing V8 juice (100 mL), CaCO₃ (3 g), agar (20 g L⁻¹) and H₂O (700 mL) at 25 °C for 10 days. Conidial sporulation was induced in V8 100 solid medium containing V8 juice (100 mL), CaCO₃ (3 g), agar (20 g L⁻¹), and H₂O (900 mL) at pH 6 and 20 °C under continuous fluorescent light for 8–10 days. A conidial suspension was then obtained by stirring a fragment of the colony in sterile distilled water (0.5 mL), and 10 μL of this suspension was deposited on different solid media (agar 20 g L⁻¹), supplemented or not supplemented with fungicide. The petri dishes were then incubated in a culture room at 25 °C for 48 h.

2.3.3 Spore germination bioassay using conidia from pure M. fijiensis cultures

Isolates of the collection maintained at −80 °C were first cultured on V8 300 solid medium containing V8 juice (300 mL), CaCO₃ (3 g), agar (20 g L⁻¹) and H₂O (700 mL) at 25 °C for 10 days. Conidial sporulation was induced in V8 100 solid medium containing V8 juice (100 mL), CaCO₃ (0.2 g), agar (10 g L⁻¹) and H₂O (900 mL) at pH 6 and 20 °C under continuous fluorescent light for 8–10 days. A conidial suspension was then obtained by stirring a fragment of the colony in sterile distilled water (0.5 mL), and 10 μL of this suspension was deposited on different solid media (agar 20 g L⁻¹), supplemented or not supplemented with fungicide. The petri dishes were then incubated in a culture room at 25 °C for 48 h.

2.3.4 Germination assessment

For the ascospore bioassays, germination of a population of 100 ascospores was monitored in the fungicide-free control medium and compared with the germination pattern of another population of 100 individuals monitored in fungicide-supplemented medium. These ascospore populations were randomly selected from sporulating samples derived from 30 fragments cultured for the test. For the conidium bioassays, it was assumed that conidia from the same lesion are genetically identical and representative for the same strain, as they are asexually reproduced. For each test, conidia from a 50-lesion (strain) sample were analysed, as conidia from the same lesion were distributed on the different media.

2.3.4.1 MBC fungicides. In the presence of these fungicides, four germination phenotypes are generally observed: (i) distorted or twisted germ tubes (T); (ii) no germination (NG); (iii) germ tubes with normal growth (N), close to that of the fungicide-free control; (iv) non-deformed germ tubes with low growth, i.e. <100 μm (C). It is known that spores with twisted and deformed germ tubes are sensitive, while those whose germination is comparable with that of the fungicide-free control are resistant. For spores showing growth much lower than in untreated (C), it is not known whether these strains carry the E198A mutation, leading usually to complete resistance. The percentage of resistance (% R) was calculated as follows:

\[ \% R = \frac{\text{number of strains whose germination phenotypes corresponded undoubtedly (according to results of experiment 1 in Section 2.4) to the presence of the mutation conferring resistance (E198A)}}{\text{total number of strains}} \times 100 \]

2.3.4.2 QoI fungicides. In the presence of this type of fungicide, the spores germinate but the germ tube length is variable. Therefore, the length (L) of the apical germ tube of each strain was measured, and the percentage growth inhibition (GI) was calculated as follows:

\[ GI = 100 - \left( \frac{L_i}{L_0} \times 100 \right) \]

where \( L_i \) is the germ tube length on fungicide-supplemented medium of strain \( i \), and \( L_0 \) is the germ tube length on fungicide-free control medium. For the ascospore bioassays, \( L_0 \) was the mean germ tube growth measured over the entire ascospore population. For the conidium bioassays, \( L_0 \) was the value of strain on the fungicide-free control medium. In this pathogen it has been suggested that, in the presence of QoI fungicide, resistant strains carrying the G143A mutation have an \( L_0 \) germ tube that is longer than 150 μm, while the sensitive strains without the mutation are much lower than in untreated (C), it is not known whether these strains carry the E198A mutation, leading usually to complete resistance. The percentage of resistance (% R) was calculated as follows:

\[ \% R = \frac{\text{number of strains whose germination phenotypes corresponded undoubtedly (according to results of experiment 1 in Section 2.4) to the presence of the G143A mutation conferring resistance}}{\text{total number of strains}} \times 100 \]

2.4 Experiment 1: comparison of spore germination bioassays and molecular detection of target mutations conferring resistance to MBCs and QoIs

For this comparison, use was made of a collection of 52 strains consisting of a mixture of strains potentially resistant or sensitive to MBCs, and another collection of 60 strains potentially resistant or sensitive to QoIs (Table 1).

2.4.1 Molecular detection of target mutations

For each strain, the authors screened for the presence of the G143A mutation conferring resistance to QoIs and for mutation E198A conferring resistance to MBCs in this pathogen. For each strain, a new culture was initiated from the collection stored in glycerol at −80 °C. Mycelium was transferred to V8 300 solid medium for 2 weeks at 25 °C. DNA extraction from growing mycelia was carried out as described by Halkett et al.
Table 1. Isolates collected for the comparison of spore germination bioassays and molecular detection of mutations E198A and G143A, respectively, for detection of MBC and Qol fungicide resistance

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>Location</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC</td>
<td>Mbanga/Singa</td>
<td>52</td>
</tr>
<tr>
<td>Qol</td>
<td>Mbanga/Mideviv</td>
<td>1</td>
</tr>
<tr>
<td>Qol</td>
<td>Mbanga/Kumbe</td>
<td>2</td>
</tr>
<tr>
<td>Qol</td>
<td>Mbanga/Singa</td>
<td>2</td>
</tr>
<tr>
<td>Qol</td>
<td>Njombé/Njombé</td>
<td>1</td>
</tr>
<tr>
<td>Qol</td>
<td>Manjo/Lala</td>
<td>53</td>
</tr>
<tr>
<td>Qol</td>
<td>Penja/Penja Ouest</td>
<td>1</td>
</tr>
</tbody>
</table>

2.4.1.1 PCR amplifications for molecular detection of the mutation conferring resistance to MBCs. PCR was carried out to amplify a 374 bp portion of the β-tubulin gene containing the codon 198 bearing the E198A mutation. A 20-nucleotide biotin-labelled reverse primer [5′(biotin)GTGCCGAGCTGTTGACAG3′] MYCOFI-XX-E198A-F1B and a forward 24-nucleotide primer [5′GCCAGACATGACAGCGGAGACGAG3′] MYCOFI-XX-E198AR1 were used to amplify this region. PCR was performed using a thermal cycler (MJ Research, St Bruno, Quebec, Canada) with a total 35 μL mixture containing DNA (2 μL), MYCOFI-XX-E198A-F1B (0.7 μL at 10 μM) and MYCOFI-XX-E198AR1 (0.7 μL at 10 μM) primers, sterile water (14.1 μL) and QIAGEN Multiplex PCR Master Mix (QIAGEN, Valencia, CA) (17.5 μL) containing HotStarTaq DNA polymerase, KCl (NH4)2SO4, Tris–HCl, MgCl2 (3 μM) and each dNTP (0.2 μM). The reaction was conducted under the following conditions: 15 min at 95 °C, 30 s at 94 °C, 30 s at 58 °C, 1 min 30 s + 1 s per cycle at 72 °C, 44 cycles of 30 s at 94 °C and a final 10 min extension at 72 °C.

2.4.1.2 PCR amplifications for molecular detection of the mutation conferring resistance to Qol. PCR was used to amplify a 77 bp portion of the cytochrome b gene containing the 143 codon bearing the G143A mutation that confers resistance to Qols. A 17-nucleotide biotin-labelled reverse primer [5′(biotin)AGGGTATAGCGCTCATT3′] MF02_rev_bio and a forward 23-nucleotide primer [5′GTGCCGAGCTGTTGACAG3′] MF01_for were used to amplify this region. PCR was conducted using a thermocycler (MJ Research) with a total 42 μL mixture containing DNA (2 μL), MF01 forward primers (1 μL at 10 μM) and MF02 reverse primers (1 μL at 10 μM), sterile water (17 μL) and QIAGEN Multiplex PCR Master Mix (21 μL) containing HotStarTaq DNA polymerase, KCl (NH4)2SO4, Tris–HCl, MgCl2 (3 μM) and each dNTP (0.2 μM). The reaction was performed under the following conditions: 15 min at 94 °C, 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, 35 cycles of 30 s at 94 °C and a final 10 min extension at 72 °C.

2.4.2 Spore germination bioassay

The 52 and 60 isolates were cultured as described in Section 2.3.3. Germination of 52 isolates was monitored in order to detect MBC resistance in the presence of thiophanate-methyl and thiabendazole at rates of 5 and 10 mg L⁻¹ respectively, as indicated in Section 2.3.4. For the purpose of detecting Qol resistance in 60 isolates, germination was monitored in the presence of azoxystrobin (10 mg L⁻¹), as indicated in Section 2.3.4.

In order to evaluate further the influence of alternative respiration (AoX) on the sensitivity to Qol fungicides, three isolates carrying the G143A mutation (resistant) and three isolates that did not carry the G143A mutation (sensitive) were subsampled. Each strain was grown on different media, as indicated in Section 2.3.4. T1 = agar + 1 mg L⁻¹ of dimethyl sulfoxide (DMSO); T2 = agar + 1 mg L⁻¹ of DMSO + 7.66 mg L⁻¹ of SHAM; T3 = agar + 1 mg L⁻¹ of DMSO + 7.66 mg L⁻¹ of SHAM + 10 mg L⁻¹ of azoxystrobin, T4 = agar + 10 mg L⁻¹ of azoxystrobin. This experiment was carried out in triplicate. The germ tube growth data were submitted to a one-way ANOVA using the Minitab statistical software package (v.16). The mean germ tube growth values were separated with a Tukey test using the same software.

2.5 Experiment 2: analysis of fungal diversity within all the conidia recovered from individual lesions

The aim of this experiment was to determine whether conidia derived from the same lesion were all genetically identical via two approaches: (1) analysis of the entire conidial population within each lesion via germination tests; (2) molecular marker analysis of the neutral genetic diversity in a subsample of this population. Lesions were sampled in a commercial plantation in which the MBC resistance level was around 50%.

2.5.1 Homogeneity of resistance to thiophanate-methyl among all conidia recovered from individual lesions

A total of 100 lesions showing conidial sporulation were selected. Each lesion was then successively applied 12 times on different sectors within a petri dish containing agar medium (20 g L⁻¹) supplemented with thiophanate-methyl (5 mg L⁻¹) in order to recover all conidia of the lesion. Germination of all conidia of each lesion was monitored as described in Section 2.3.4.

2.5.2 Genetic diversity among conidia from individual lesions assessed using microsatellite markers

From the 100 lesions used in Section 2.5.1, a subsample of 33 lesions was randomly selected. For each of these lesions, 6–16 conidia per lesion were isolated (see Table 5). Total DNA was extracted from each isolate, as described in Section 2.4.1. Among all microsatellite markers described in M. fijiensis, eight markers were screened for which high genetic diversity (He > 0.4) had been reported in Cameroonian populations. In these conditions, the probability P that two randomly selected individuals in the population will have the same combination of alleles at these eight loci is very low. The selected loci were dinucleotides MfSSR137 (He = 0.52) and MfSSR203 (He = 0.57), trinucleotides MfSSR322 (He = 0.61), MfSSR305 (He = 0.47) and MfSSR324 (He = 0.44) and tetranucleotides MfSSR405 (He = 0.49), MfSSR413 (He = 0.45) and MfSSR424 (He = 0.5). These conditions, P = (He1 × He2 × He3 × He4 × He5 × He6 × He7 × He8) = 0.0041. PCR was performed according to the following multiplex protocol (adapted from Zapater et al.): 15 min at 95 °C, 45 cycles of 30 s at 94 °C, 90 s at 57 °C and 90 s at 72 °C and a 10 min final step at 65 °C. The reaction mixture (19 μL) included DNA (3 μL), QIAGEN Multiplex PCR Master Mix (5 μL x 2), Q-solution (1 μL x 5) and each primer (0.5 μL). Forward and reverse primers were labelled with one of the following fluorescent dyes: NED, HEX, FAM or PET (Applied Biosystems, Foster City, CA). Primers were tested in panels.
of four markers labelled with these different dyes. Diluted, amplified PCR products were mixed with GeneScan-500LIZ size markers (Applied Biosystems) and separated on a 16-capillary sequencer (Prism 3130XL; Applied Biosystems). They were sized and analysed using GeneMapper software (Applied Biosystems).

### 2.6 Experiment 3: effect of possible fungicide contamination on the germ tube growth of conidia recovered after successive transfer of the same lesion over different fungicide media

A group of 50 sporulating lesions was selected for this study. Each lesion was applied on fungicide-free agar medium (initial control), and then successively on three different media supplemented with the different fungicides: azoxystrobin (10 mg L\(^{-1}\)), propiconazole (0.1 mg L\(^{-1}\)) and thiophanate-methyl (5 mg L\(^{-1}\)), and finally on a fungicide-free medium (final control). The growth of germ tubes of conidia deposited on the different media was measured in the two controls (initial and final) to screen for a possible effect of successive applications on the fungicide-supplemented media on conidial germination. This experiment was carried out in triplicate. The germ tube growth data were submitted to a one-way ANOVA using the Minitab statistical software package (v.16) in order to evaluate the influence of the order of the control in the sequence of the different agar media used. The mean germ tube growth on the initial and final fungicide-free control media were separated with a Tukey test using the same software.

### 2.7 Experiment 4: effect of leaf age on the frequency of resistant strains

Two populations of conidia sampled from the top (7th–8th leaf) and bottom (11th–13th leaf) of the canopy were compared to assess the effect of leaf age on the frequency of resistant strains. Ten lesions were sampled at each canopy level, and the experiment was repeated on 18 randomly chosen banana trees in a plot in which the proportion of strains resistant to thiophanate-methyl was around 50%. Conidia were germinated in the presence of thiophanate-methyl, as indicated in Section 2.3.2, and the percentage resistance was calculated as described in Section 2.3.4.

After arcsine transformation of the square root of the percentage of resistance, means were separated with a two-sample t-test using the Minitab statistical software package (v.16).

### 2.8 Experiment 5: comparison of germination bioassays using ascospores and conidia from field lesions

The aim was to assess the reliability of the resistance measurements via conidial germination tests in comparison with the reference method using ascospores. Stage 2–3 sporulating lesions and leaf necrosis were sampled as described in Sections 2.3.2 and 2.3.1 respectively, on the same day and site. Samples were collected at different commercial plantations: Mantem, Penja-Est, Mussaka and Singa, with different resistance levels. Lesion and leaf necrosis samples from each of the four sites were divided into three subsamples for three replicates. At a different date, a sample was collected at a single site (Senga) and divided into five subsamples for five replicates. Conidia and ascospores were cultured as described in Sections 2.3.2 and 2.3.1 respectively, and the percentage resistance to thiophanate-methyl (5 mg L\(^{-1}\)) was calculated as indicated in Section 2.3.4.

## 3 RESULTS

### 3.1 Comparison of spore germination bioassays and molecular detection of target mutations conferring resistance to MBCs and QoI

#### 3.1.1 Detection of MBC-resistant strains

The germination test showed that, in the presence of thiabendazole and thiophanate-methyl, at 5 and 10 mg L\(^{-1}\), no strains with twisted germ tubes (T) carried the E198A mutation, and all strains with normal germination (N) or short germination (C) were carrying the E198A mutation that confers resistance (Table 2). The distribution of N, C and NG phenotypes in resistant strains varied according to the type of fungicide and rate. A greater proportion of strains with low germination (C) and no germination (NG) was noted in the presence of thiophanate-methyl at 10 mg L\(^{-1}\) (Table 2). Two strains did not germinate (NG) on this medium, and one was carrying the E198A mutation but not the other. On

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**Table 2. Comparison of conidial germination phenotypes in the presence of various MBC fungicides and detection of the E198A mutation conferring resistance to MBC products**

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Thiabendazole (5 mg L(^{-1}))</th>
<th>Thiabendazole (10 mg L(^{-1}))</th>
<th>Methyl-thiophanate (5 mg L(^{-1}))</th>
<th>Methyl-thiophanate (10 mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of isolates</td>
<td>Number of isolates</td>
<td>Number of isolates</td>
<td>Number of isolates</td>
</tr>
<tr>
<td>Carrying</td>
<td>showing the phenotype</td>
<td>showing the phenotype</td>
<td>showing the phenotype</td>
<td>showing the phenotype</td>
</tr>
<tr>
<td>E198A mutation</td>
<td></td>
<td>E198A mutation</td>
<td>E198A mutation</td>
<td>E198A mutation</td>
</tr>
<tr>
<td>T</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>27</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>N+C</td>
<td>1</td>
<td>4</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C+NG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^4\) T = distorted or twisted germ tubes; N = germ tubes with normal growth close to that of the fungicide-free control; C = non-distorted germ tubes but with short growth; NG = no germination; N + C = normal and short germ tube growth; C + NG = short germ tube growth and no germination.
medium containing thiabendazole at 5 mg L\(^{-1}\), strains carrying the E198A mutation mostly had a normal growth pattern (N).

3.1.2 Detection of QoI-resistant strains

The results showed that, in the presence of azoxystrobin (10 mg L\(^{-1}\)), strains bearing the G143A mutation and sensitive strains without the mutation could not be entirely differentiated on the basis of germ tube length \((L_i)\) or on the percentage of growth inhibition \((GI_i)\). However, all slightly inhibited strains \((L_i > 120 \mu m \text{ and } GI_i < 50\%)\) were found to be carrying the G143A mutation, whereas highly inhibited strains \((L_i < 25 \mu m \text{ and } GI_i > 70\%)\) did not have this mutation (Fig. 1). Within this range, some strains carrying the mutation were as strongly inhibited as strains without it.

When AoX was impeded by SHAM, azoxystrobin inhibited germ tube growth of the three strains that did not carry the G143A mutation, but did not inhibit the growth of the three strains carrying this G143A mutation (Tables 3 and 4). However, growth of strain SS1 (not carrying the G143A mutation) was poorly inhibited by azoxystrobin, as when AoX was not impeded (Fig. 1, Tables 3 and 4). On the other hand, SHAM inhibited germ tube growth of the three strains carrying the G143A mutation.

3.2 Analysis of fungal diversity within all the conidia recovered from individual lesions

3.2.1 Homogeneity of resistance to thiophanate-methyl within all conidia recovered from the same lesion

Each analysed lesion was successively applied 12 times on the same medium. The number of conidia decreased markedly between the first and twelfth application (Fig. 2), suggesting that these 12 successive applications made it possible to harvest almost all the conidia formed on the lesions. Figure 2 also shows clearly that, for highly sporulating lesions, sufficient conidia can be recovered after at least four successive applications to carry germination tests. Lesions produced 177 conidia on average, with extremes of 12 conidia for the lesion that had the least sporulation and 1800 for the most highly sporulating lesion.

A sensitive genotype (S) was attributed to a lesion when all harvested conidia had twisted or non-germinated germ tubes. A resistant genotype was attributed to a lesion when all harvested conidia had a normal or short germ tube along with some non-germinated conidia (dead conidia). It was considered that the lesion contained a mixture of genotypes when conidia with twisted, normal and short germ tubes could be simultaneously found. Of the 100 analysed lesions, 41% produced conidia that were exclusively resistant and 51% produced conidia that were exclusively susceptible, and only 8% had a mixture of resistant and sensitive genotypes (Table 5). Lesions in which a mixture of genotypes was detected consisted of a high-majority genotype representing over 80% of the conidia and a minority genotype that was often spatially grouped in one sector of the lesion (Table 6).

3.2.2 Genetic diversity among conidia from the same lesion assessed using microsatellite markers

Over the subsample of lesions studied, this analysis revealed that ten lesions (30.3%) had a mixture of two haplotypes (Table 7), whereas the germination tests were able to detect a mixture of genotypes in only four lesions (12.1%). Note that two haplotypes were also found in the four lesions in which the germination tests enabled the detection of a mixture of genotypes. The neutral genetic diversity analyses thus detected 2.5-fold more mixtures than the germination tests.
3.3 Effect of possible fungicide contamination on the germ tube growth of conidia recovered after successive transfer of the same lesion over different fungicide media

The results showed a slight but significant ($P = 0.01$) reduction in germ tube growth after successive transfer of the same lesion over different media, as germ tube growth was 252 $\mu$m on initial fungicide-free agar and 236 $\mu$m on the final fungicide-free agar. However, this effect was minor, with a decrease of only 7% from the initial germination.

3.4 Effect of leaf age on the frequency of resistant strains

The results showed that the level of resistance to fungicides varied at different leaf levels on the same banana tree (Table 8).
Table 5. Detection of multiple infections within single lesions via the phenotypic germination pattern of all the conidia recovered from single lesions in the presence of thiophanate-methyl (5 mg L\(^{-1}\)). All the conidia of 100 lesions were analysed in this study.

<table>
<thead>
<tr>
<th>Type of lesion(^a)</th>
<th>Number of lesions per type of lesion</th>
<th>Number of conidia per type of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>41</td>
<td>6064</td>
</tr>
<tr>
<td>S</td>
<td>51</td>
<td>10 317</td>
</tr>
<tr>
<td>R + S</td>
<td>8</td>
<td>1 694</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>18 075</td>
</tr>
</tbody>
</table>

\(S = \) all conidia recovered from the lesion were twisted (T) or did not germinate (NG) in the presence of thiophanate-methyl (5 mg L\(^{-1}\)); \(R = \) all conidia from the lesion were normal (N) or short germinated (C); \(R + S = \) twisted conidia (T) and conidia with normal (N) or short (C) germ tubes were recovered from the same lesion.

Table 6. Distribution of susceptible and resistant germination phenotypes within the total population of conidia recovered from lesions showing a mixture of susceptible and resistant strains (type of lesion \(R + S\)). Analyses were carried out in the presence of thiophanate-methyl (5 mg L\(^{-1}\)).

<table>
<thead>
<tr>
<th>Number of conidia</th>
<th>Number of conidia showing a resistant phenotype(^a)</th>
<th>Number of conidia showing a susceptible phenotype(^b)</th>
<th>Percentage of mixture(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L38</td>
<td>60</td>
<td>47</td>
<td>13</td>
</tr>
<tr>
<td>L48</td>
<td>94</td>
<td>9</td>
<td>88</td>
</tr>
<tr>
<td>L52</td>
<td>78</td>
<td>8</td>
<td>70</td>
</tr>
<tr>
<td>L78</td>
<td>77</td>
<td>4</td>
<td>73</td>
</tr>
<tr>
<td>L81</td>
<td>177</td>
<td>1</td>
<td>176</td>
</tr>
<tr>
<td>L87</td>
<td>12</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>L96</td>
<td>234</td>
<td>2</td>
<td>232</td>
</tr>
<tr>
<td>L97</td>
<td>1 116</td>
<td>2</td>
<td>1 114</td>
</tr>
</tbody>
</table>

\(^a\) Conidia twisted and without germination.  
\(^b\) Conidia with normal or short germination.  
\(^c\) Proportion of the less frequent phenotype.

3.5 Comparison of germination bioassays using ascospores and conidia from field lesions

In the ascospore germination tests, a marked dispersion in individual repetition values was observed. The confidence interval and coefficient of variation for the level of resistance to MBC fungicides (27.0 < CoV < 113.6) were thus relatively high in these tests by comparison with the conidial germination tests (19.5 < CoV < 49.5) in which the values for each repetition were less variable (Fig. 3).

4 DISCUSSION

A routine bioassay to monitor sensibility of *Mycosphaerella fijiensis* to fungicides was developed using conidia sampled from early lesions (stages 2–3). It was hypothesised that all conidia from a single lesion are identical as they are asexually reproduced. In most cases, only a single genotype was found within individual lesions, but several genotypes still were detected in around 20% of lesions. Genetic diversity between conidia from the same lesion was analysed using two complementary approaches that yielded different information. Firstly, the conidial germination tests conducted in the presence of thiophanate-methyl made it possible to analyse almost all the conidia of the 100 analysed lesions (up to 1800 conidia for the mostly sporulating lesion). This analysis revealed that conidial germination was generally uniform, and that a high-majority germination morphotype (resistant or susceptible) was usually present when there was a mixture of genotypes.
Table 8. Effect of leaf age on the level of resistance to an MBC fungicide (thiophanate-methyl at 5 mg L\(^{-1}\)). Banana trees were selected just before flowering.

<table>
<thead>
<tr>
<th>Age of leaves</th>
<th>Repetition</th>
<th>Percentage of resistance ± standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young leaves(^a)</td>
<td>18</td>
<td>34 ± 14.6</td>
</tr>
<tr>
<td>Old leaves(^b)</td>
<td>18</td>
<td>21 ± 19.8</td>
</tr>
</tbody>
</table>

\(^a\) 7th–8th leaves from the top of the banana tree.  
\(^b\) 11th–13th leaves from the top of the banana tree.  
\(P\)-value 0.028\(^c\)

\(^c\) The t-test was significant at 5%.

The neutral genetic diversity analysis could only be performed on a smaller conidial sample, but the findings revealed twofold more genotype mixtures within individual lesions than detected in the germination tests. This result was expected because the resistance frequency in the farm where the lesions were collected was roughly 50%, and hence, because loci are independent owing to recombination, the probability of not distinguishing with an MBC germination test two isolates having different genotypes is about 50%. The fact that minority and majority genotypes were spatially located in different parts of the lesion suggests that several separate infections might have occurred at adjacent sites on the leaf, but these infections could not be differentiated when the lesion was cut off. Such events are more likely to occur in cases of high infestation, as described for this pathogen. Finally, the observed extent of genotype mixing might not be sufficient to question the relevance of using conidia from lesions in routine tests because: (i) the extent of mixing could probably be reduced by sampling lesions in the part of leaf areas where there is a very low young lesion density; (ii) the present findings showed that, when a mixture of genotypes was present, there was usually a high-majority genotype; (iii) the different genotypes were distributed in different spatial areas within the lesion, which means that observations may be focused in the part of the lesion where there is uniform conidial growth.

The authors then sought to validate the bioassay, which was mainly designed to detect mutations via germination tests by comparing germination phenotypes grown on media supplemented or not supplemented with fungicides with the presence of the mutation conferring disruptive fungicide resistance. This relationship, concerning G143A and E198A mutations conferring resistance to QoIs and MBCs respectively, had not been convincingly demonstrated hitherto. Regarding MBCs, it was clearly shown that the germination phenotypes enabled detection of the E198A mutation in all cases. In the presence of a fungicide (5 mg L\(^{-1}\)), strains bearing the E198A mutation had a normal (N) or short (C) germtube, while strains without this mutation had a twisted germ tube (T) or did not germinate. This is in line with the findings of previous studies in which strains with a normal germ tube (N) were considered to be resistant, whereas those with a twisted germ tube (T) were classified as sensitive. However, the present study revealed that strains with short germination (C) also carried the E198A mutation, contrary to the assumptions of the above authors. The present comparison of germination profiles in the presence of different fungicides (thiophanate-methyl and thiabendazole) and at different rates (5 and 10 mg L\(^{-1}\)) suggested that thiabendazole, at 5 mg L\(^{-1}\), is better for differentiating resistant strains with normal (seldom inhibited) germination and sensitive strains with a twisted germ tube (T).

For QoIs it was shown that the germination inhibition level in the presence of azoxystrobin (10 mg L\(^{-1}\)) did not make it possible to determine the resistant (G143A mutation carrier) or sensitive...
(without the G143A mutation) status for all analysed strains. However, very slightly inhibited strains (L > 120 μm and GI < 50%) were all carrying the G143A mutation, whereas highly inhibited strains (L < 25 μm and GI > 70%) were not. Between these two groups, there was a range of moderately inhibited strains, some of which had the mutation and others not. Germination tests have been previously carried out to detect and quantify the proportion of QoI-resistant strains in *M. graminicola*, *Botritis cinerea* and *M. fijiensis*. Several authors have suggested that *M. fijiensis* strains bearing the G143A mutation have a germ tube longer than 150 μm in the presence of azoxystrobin at rates of 0.1 or 10 mg L⁻¹. In the present work it was shown that this germ tube cut-off limit could be reduced to 120 μm to encompass a greater number of strains bearing the G143A mutation, and also that this limit does not enable detection of all resistant strains and therefore underestimates their number. Germination tests thus enable detection of QoI-resistant strains but not accurate measurement of their proportion. Finally, the present results suggest that the GI could be a more accurate indicator of the strain status, GI being less variable (CoV 0.41) than the germ tube length (CoV 0.93). Among the moderately inhibited strains, those not carrying the G143A mutation could opt for an alternative oxidation pathway in order to continue growing when a QoI fungicide is present; however, this was not the case for the strain SS1, suggesting that this strain might carry another mutation than the G143A. Indeed, over 11 mutations or mutation combinations in the *cyt b* gene have been described as confering resistance to QoIs in lab mutants, but only G137R, F129L and G143A mutations have been found up to now in field isolates of certain pathogens, and from which G143A mutants clearly lead to the highest resistance factors. In the case of *M. fijiensis*, the mutation G143A also leads to the highest resistance factor, but other mutations could lead to moderate resistance factors, as in the case of strain SS1. Finally, for strains bearing the G143A mutation but that are slightly inhibited, other mutations could also be present, and this mutation combination could affect the strain fitness.

It has been shown that using conidia sampled from early lesions enhanced the bioassay reproducibility. Firstly, with conidia, it was possible to conduct germination tests in all conditions, i.e. with samples originating from fungicide-treated or untreated areas and regardless of the climatic conditions. In a global study aimed to compare the two methods (11 paired comparisons with three replicates during the dry season), 100% of conidial germination tests were successfully performed, whereas 90% of the ascospore germination tests could not be carried out owing to a lack of ascospore production (data not shown). Moreover, only 10–50% of cultured necrotic tissue fragments produced ascospores, whereas 94–100% of cultured early lesions produced conidia, so tests performed with ascospores were less representative of the sampled population. Consequently, the proportion of resistant strains obtained in the ascospore germination test was much more variable than in the conidial germination tests. The conditions required for performing routine conidial germination tests have been specified. Firstly, it was confirmed that conidial production was regular under all climatic conditions, even in commercial treated farms, and a plan was proposed for sampling early lesions. It was shown that resistance levels might differ between populations sampled at different canopy levels. Leaves of the same age (canopy level) should thus be collected to ensure that the population obtained is representative of the same infection period. In practice, it is proposed to collect leaf fragments bearing stage 2–3 lesions on the youngest leaves with lesions (generally rows 7–8) on 25 just-flowering banana trees (flowering stage between shooting and horizontal finger stage) randomly distributed in the plot. It is also suggested that two stage 2–3 lesions should be sampled per banana plant in order to obtain a population of 50 lesions (strains) per sample. Early isolated lesions should be cut from parts of the leaf blade where the lesion density is low in order to reduce the risk of obtaining a mixture of different genotypes within the same lesion. The lesions are then cut, and highly sporulating lesions are selected after being lightly applied on the agar medium. Thereafter, 50 selected sporulating lesions are applied on the control medium and then successively on the different fungicide media to harvest the conidium population. The germ tube growth of conidia of the same genotype can thus be compared in the absence and presence of a fungicide. It was shown that transferring the same lesion on different media could ultimately have a slight effect on germ tube growth. To overcome this problem, it is suggested that, in routine tests and according to the different mode of action of different fungicides, lesions should be successively deposited on the following media: agar; agar + DMI fungicide or agar + Qol fungicide; agar + MBC fungicide. The EC₅₀ (concentration inhibiting 50% of germ tube growth) may also be measured for DMI fungicides by depositing lesions on agar and then on several media containing increasing fungicide rates. If the aim is to assess the same strain with a broader range of fungicides, then the fungus could be cloned and conidia could be produced on artificial media and then deposited on the different media. Finally, in microscopic assessments, the observations should be focused on the same part of the lesion in order to avoid monitoring different genotypes when the lesion contains a mixture of genotypes.

In conclusion, germination tests performed with conidia obtained from early sporulating lesions overcame many problems commonly encountered during ascospore germination tests. Through these tests, it is possible: (i) to perform germination tests in a timely manner; (ii) to comply with a preset sampling plan; (iii) to overcome the problem of confusing *Mycosphaerella* species; (iv) to compare the response of the same population on a control medium and on fungicide-supplemented media. For all of these reasons, it is believed that the conidia-based method is a good alternative for more reliable assessment of resistance in *M. fijiensis*.

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A novel bioassay to monitor fungicide sensitivity in *M. fijiensis*