



## Baseline sensitivity of Brazilian *Mycosphaerella fijiensis* isolates to protectant and systemic fungicides

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### ABSTRACT

Black Sigatoka caused by *Mycosphaerella fijiensis* is a foliar disease that affects banana plants and large amounts of fungicides are required to prevent crop losses. Intensive applications of single-site fungicides can select for fungicide-resistant isolates. The objective of this study was to assess the sensitivity of 60 isolates of *M. fijiensis* to commonly used fungicides. Using two different protocols, microtiter and Petri plate tests, the effective concentration at which mycelium growth is reduced by 50% (EC<sub>50</sub>) was determined for thiophanate-methyl, tebuconazole, chlorothalonil and mancozeb. Additionally, partial sequences of the cytochrome b gene were obtained for 46 isolates to detect the G143A mutation, commonly associated with strobilurin resistance. The EC<sub>50</sub> values for tebuconazole and thiophanate-methyl ranged from 0.02 to 1.39 and from 0.008 to 8.22 µg mL<sup>-1</sup>, respectively. For chlorothalonil, the lowest and highest EC<sub>50</sub> values were 0.39 µg mL<sup>-1</sup> and 53.7 µg mL<sup>-1</sup>, respectively. For mancozeb, approximately 50% of the isolates had EC<sub>50</sub> values greater than 1000 µg mL<sup>-1</sup>. No mutation was found in the isolates assayed for strobilurin resistance. There was no correlation between sensitivity levels to any fungicide and geographic region. Low EC<sub>50</sub> values were estimated for most fungicides but, some isolates had high EC<sub>50</sub> values for mancozeb.

**Key words:** black leaf streak disease, fungicide resistance, protectant fungicides, strobilurin, tebuconazole, thiophanate-methyl.

Black leaf streak disease (BLSD), also known as black Sigatoka, is caused by *Mycosphaerella fijiensis* M. Morelet (anamorph *Pseudocercospora fijiensis* M. Morelet) Deighton, an Ascomycete species, and is the most devastating foliar disease of bananas (Marín et al., 2003; Churchill, 2011). Yield losses can be high and disease control depends mainly on the application of systemic and protectant fungicide that are alternated as part of an anti-resistance strategy (Marín et al., 2003; Churchill, 2011). Systemic fungicides usually provide better BLSD control than protectant fungicides, but are at a greater risk of control failures due to the development of resistance in the pathogen population (Marín et al., 2003). The main chemical classes of fungicides used to control BLSD are the demethylation inhibitors (DMIs), amines, outer quinone inhibitors (QoI; strobilurins), anilinopyrimidines (APs), benzimidazoles (BCMs), succinate dehydrogenase inhibitors (SDHIs) and guanidines (FRAC, 2010).

Populations of *M. fijiensis* resistant to systemic fungicides are known to occur in several regions. Field isolates of *M. fijiensis* insensitive to benomyl were reported in 1979 (Stover, 1979). Cañas-Gutiérrez et al. (2006) also identified isolates that exhibited high resistance to benomyl. Although benzimidazoles are highly effective on BLSD control, and relatively inexpensive, their use was limited or prohibited in some countries due to the emergence of fungal populations resistant to the fungicides of this group (Marín

et al., 2003). Several studies conducted with different fungal species have identified single nucleotide polymorphisms (SNP) associated with resistance to benzimidazoles. For *M. fijiensis* it was demonstrated that a change from cytosine to adenine in codon 198 of the β-tubulin gene was associated with a change in the phenotype and the mutant isolates acquired medium or high resistance to benomyl (Cañas-Gutiérrez et al., 2006).

Resistance to strobilurin, an outer quinone inhibitor, and sterol demethylation inhibitors are known to occur in several areas. Strobilurin fungicides inhibit the mitochondrial respiration of fungi by binding to the cytochrome bc1 enzyme complex and several studies have been published reporting resistance to these fungicides (Sierotzki et al., 2000; Chin et al., 2001; Amil et al., 2007). Resistance to strobilurins was found in high levels in populations of *M. fijiensis* in banana plantations from Costa Rica. Approximately 80% of resistant isolates showed the G143A mutation in the cytochrome b gene, which confers resistance to the fungicide (Amil et al., 2007). The DMI fungicides inhibit the biosynthesis of sterol C-14 α-demethylation of 24-methylenedihydrolanosterol, a precursor of the cell membrane component ergosterol in fungi (Brent & Hollomon, 2007). Resistance to DMIs is also known and resistant isolates of *M. fijiensis* to propiconazole were reported (Romero & Sutton, 1997).

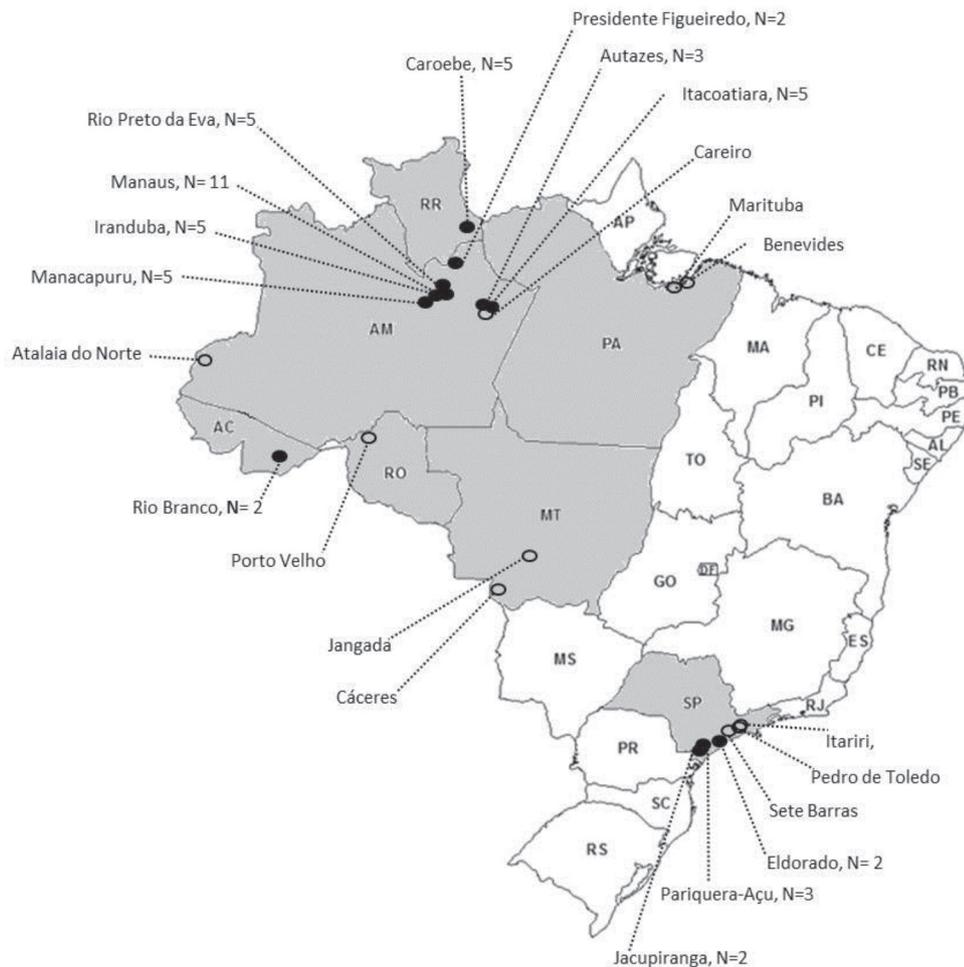
Given that fungicide resistance in *M. fijiensis* seems to be frequent and ubiquitous, monitoring pathogen

populations becomes a key component for the management of BLSD. In Brazil, banana crops are spread throughout the country and BLSD is a major problem in many areas, particularly in the Northern region. This study reports the sensitivity of *M. fijiensis* isolates collected across banana production regions of Brazil to commonly used fungicides for BLSD control.

A total of 60 isolates of *M. fijiensis* collected in 2008 and 2009 in banana fields in seven Brazilian states were stored at Embrapa Amazônia Ocidental – CPAA and utilized in this study (Figure 1). The isolates were obtained from leaves with typical symptoms of BLSD randomly collected from symptomatic plants. The isolation was made with a compound microscope and using a sterile fine needle, conidia present on the surface of symptomatic leaves were picked and transferred to PDA medium. The isolates were incubated at 25°C.

Sensitivity of the isolates were tested for tebuconazole (Folicur 200 CE, Bayer CropScience Ltda), mancozeb (Manzate 800, Du Pont do Brasil S.A.) chlorothalonil (Daconil-BR, Iharabras S.A.) and thiophanate-methyl (Cercobin 700 WP, Iharabras S.A.). The usual methodological procedures for the microtiter

plate test recommended by the Fungicide Resistance Action Committee (FRAC) to monitoring populations of *Mycosphaerella graminicola*, *Phytophthora infestans*, *Fusarium graminearum*, *Botrytis cinerea*, *Pyrenophora tritici-repentis* and other fungi (FRAC, 2012) to assess the sensitivity to tebuconazole and thiophanate-methyl were modified to use fragmented mycelia instead of spore suspension. A total of 45 isolates assayed for these two fungicides were grown in the M4 liquid medium (Junqueira et al., 1984) for 10 days in a rotary shaker at 25°C and 120 rpm. A portion of 0.35g of the fresh mycelium mass was transferred to 10 mL of 2X potato dextrose (PD) liquid medium and the Politron® apparatus was used to grind the mycelial mass (set a speed 4 for 1 min) and to obtain an uniform suspension of hyphal fragments. Fifty microliters of the mycelium suspension was transferred to wells of sterile microtitre plates (ELISA) and mixed with 50 µL of PD amended with fungicides at different concentrations. Tebuconazole was dissolved in dimethyl sulfoxide (DMSO) and mixed with PD as to achieve the final concentrations of the active ingredient (a.i.): 0; 0.01; 0.10; 1.0; 10; and 100 µg mL<sup>-1</sup>. Thiophanate-methyl was dissolved in acetone to the following final concentrations, 0; 0.1; 1.0; 10.0; 100;



**FIGURE 1** - Map of Brazil showing the different states (in gray) and number of isolates collected. Empty circle N=1 and full circle N>1.

and 1000 a.i  $\mu\text{g mL}^{-1}$ . PD with DMSO (1%) or acetone (1%) were used as controls.

Plates were sealed using PVC film to prevent evaporation and contamination, and incubated for 7 days at 25°C and 12 h photoperiod. After incubation, fungal growth was estimated based on mycelium density measured indirectly using a microtiter plate reader at 450 nm (Thermo Scientific). Two readings were carried out to assess fungal mass, before and after the 7-day incubation period and the first reading was subtracted from the second to estimate mycelial growth.

The sensitivity of 41 and 48 isolates to the protectant fungicides chlorothalonil and mancozeb, respectively, was assessed based on the radial growth on culture medium amended with fungicides. The isolates were grown in V8 agar medium for 15 days at 25°C. Four-mm diameter V8 plugs containing growing mycelium of each isolate were excised from colonies and placed upside down in the center of PDA plates amended with different concentrations of fungicides as formulated commercial products dissolved in 1% dimethyl sulfoxide (DMSO). The final concentrations of the fungicides in the medium were 0; 0.1; 1; 10; 100; 1000  $\mu\text{g a.i.mL}^{-1}$ . The control was amended with DMSO (1%) without fungicide. After 15 days of incubation at 25°C, radial mycelial growth of each culture was measured using a ruler in two perpendicular directions and the original plug diameter was subtracted.

The occurrence of the point mutation G143A in the cytochrome b gene was assayed in 46 isolates using the primers MFcytFor\_1 5'CTCAATACTGCCTCAGC-3', MFcytRev\_1 (R1) 5'-CCGTAATGTGGTTCATC-3' and MFcytRev\_S 5' GTTATAACTGTAGCTCC3' (Garcia, 2009). The DNA of each isolate was extracted using a CTAB method as described by Doyle & Doyle (1990). Each PCR reaction was performed in 20  $\mu\text{L}$  total volume, containing 20 ng of template genomic DNA, 2 mM  $\text{MgCl}_2$ , 600  $\mu\text{M}$  dNTPs, 5 $\mu\text{M}$  each primer and 0.4 U of Taq-DNA polymerase (Fermentas). PCR was performed in a thermocycler (PTC-100) with an initial denaturation of 94°C (2min), 40 cycles of 94°C (60s), 70°C (30s) and 72°C (60s), and final elongation at 72°C (10min). Five microliters of each PCR product was subjected to electrophoresis in 2.0% agarose gel in 1x TBE and viewed under UV on gel stained with GelRed (Biotium). Fragments were compared with a 100bp DNA ladder and scored.

Experiments of mycelial growth were set in a completely randomized design with 5 replicates (plates) for each isolate-fungicide concentration combination. For tebuconazole and thiophanate-methyl the effective concentration at which 50% of mycelial growth was inhibited compared to the no-fungicide control ( $\text{EC}_{50}$ ) was determined using linear regression of absorbance reads as a function of the logarithmic-transformed fungicide concentration. This experiment was repeated three times in time. The results had similar trend and data from the third experiment are presented here.

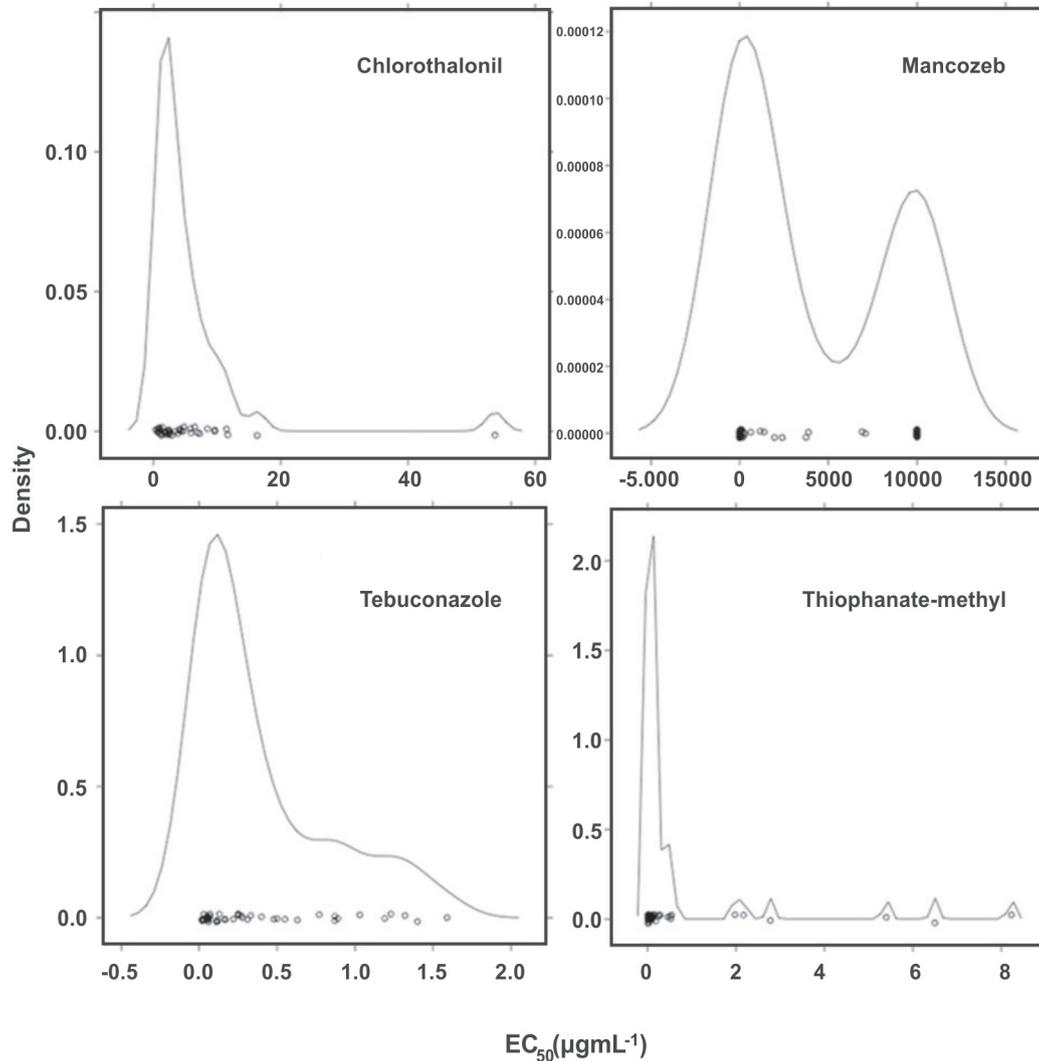
For chlorothalonil and mancozeb, the  $\text{EC}_{50}$  was determined using linear regression analysis of the relative growth reduction on the log-transformed fungicide concentration. A distance matrix of geographic location of the isolates was generated using Euclidean distance. Likewise, a distance matrix of the values of  $\text{EC}_{50}$  was also constructed. The Mantel test was used to assess the correlation between the two matrices. All statistical analyses were conducted with the R software (R Development Core Team 2008).

For tebuconazole only 13% of the isolates had  $\text{EC}_{50}$  greater than 1  $\mu\text{g mL}^{-1}$ . For thiophanate-methyl most isolates (57.8%) had  $\text{EC}_{50}$  lower than 0.1  $\mu\text{g mL}^{-1}$ , 28.9 % of isolates had  $\text{EC}_{50}$  values between 1 and 10  $\mu\text{g mL}^{-1}$  and for 13.3% of the isolates the  $\text{EC}_{50}$  values were greater than 1  $\mu\text{g mL}^{-1}$  (Figure 2).

The  $\text{EC}_{50}$  values for tebuconazole and thiophanate-methyl were based on mycelium growth in microtiter plate test because of the difficulties in obtaining conidia from colonies of *M. fijiensis* grown on artificial culture medium prevented us from using spore suspensions. There are different methods to assess the  $\text{EC}_{50}$  values (Förster et al., 2004), one commonly adopted is the spiral gradient endpoint. This method can be adapted to assess either the inhibition of mycelial growth or conidial germination. The method used in the present study was time-effective and may be more suited for large-scale monitoring of fungicide resistance. One of the drawbacks, however, is that this method requires extra care to avoid contamination.

The lack of baseline sensitivity information for Brazilian isolates of *M. fijiensis* to the fungicides used in this study limits our ability to properly infer the existence and magnitude of potential shifts in fungicide sensitivity. However, there were differences in the sensitivity of *M. fijiensis* isolates regarding the site-specific fungicides. The mean  $\text{EC}_{50}$  value for tebuconazole (0.36  $\mu\text{g mL}^{-1}$ ) was lower than the mean determined for isolates of *M. fijiensis* collected in the Ivory Coast (0.73  $\mu\text{g mL}^{-1}$ ) (Koné et al., 2008). Comparing tebuconazole with other azoles routinely applied to control BLSD, the average  $\text{EC}_{50}$  found in this study was higher than that found in a population of *M. fijiensis* in Costa Rica (0.06  $\mu\text{g mL}^{-1}$ ) (Romero & Sutton, 1997). However, differences in methodological procedures do not allow for direct comparisons. Nevertheless, as resistance to DMI-based fungicides is commonly reported (Knight et al., 2002; Stergiopoulos et al., 2003), monitoring programs for DMI resistance should be conducted on a regular basis in Brazil.

Resistance to thiophanate-methyl has been reported for a number of foliar fungal plant pathogens (Luo et al., 2007; Koch et al., 2009; May-De Mio et al., 2011). However, all *M. fijiensis* isolates tested in this study were sensitive to this fungicide. Even though thiophanate-methyl is not registered to control BLSD in Brazil, it is used for the control of the Yellow Sigatoka, and where these two diseases co-occur, resistant isolates of *M. fijiensis* could



**FIGURE 2** - Density frequency of the  $EC_{50}$  values of isolates of *Mycosphaerella fijiensis* estimated for protectant, chlorothalonil and mancozeb, and site-specific, tebuconazole and thiophanate-methyl, fungicides. The  $EC_{50}$  values ( $\mu\text{g mL}^{-1}$ ) are depicted in the x-axis. The y-axis displays the relative frequency of  $EC_{50}$  values. Each circle represents the average  $EC_{50}$  value for an isolate of *M. fijiensis*.

be selected. Isolates of *Monilinia fructicola* and *Botrytis cinerea* were considered sensitive when  $EC_{50}$  values based on mycelial inhibition data were  $<1.0 \mu\text{g mL}^{-1}$  (Yourman et al., 2000; May-De Mio et al., 2011). In the present work, the  $EC_{50}$  values for most isolates were lower than  $1.0 \mu\text{g mL}^{-1}$ , therefore they were considered as sensitive.

Of the 41 isolates of *M. fijiensis* tested for chlorothalonil sensitivity, 75.6% had  $EC_{50}$  values between 1 and  $10 \mu\text{g mL}^{-1}$ , 14.6% of isolates had  $EC_{50}$  values less than  $1 \mu\text{g mL}^{-1}$  and 9.8% of the isolates had  $EC_{50}$  values greater than  $10 \mu\text{g mL}^{-1}$ . The mean  $EC_{50}$  was  $5.35 \mu\text{g mL}^{-1}$ . The  $EC_{50}$  values for mancozeb had higher range compared with the other fungicides tested. Fifty percent of the isolates had  $EC_{50}$  greater than  $1000 \mu\text{g mL}^{-1}$  (Figure 2). Nevertheless, there is no indication of field resistance of *M. fijiensis* to mancozeb, thus there is no evidence of lack of sensitivity to

this protectant fungicide. Insensitivity in laboratory studies may not necessarily reflect the potential insensitivity under field conditions because of the extreme selection pressure in amended media compared with the selection pressure under field conditions (Chang et al., 2007). It is likely that the insensitive isolates detected can be insensitive mutants selected in the laboratory that are not representative of the naturally occurring population (Koenraadt et al., 1992; Chang et al., 2007). The resistance to mancozeb probably involves mutations within multiples genes that is associated to a gradual decline in efficacy over time. However, up to date, there is no evidence for the occurrence of such process with mancozeb and the more likely potential resistance mechanism is detoxification of the fungicide (Gullino et al., 2010). *In vitro* tests using mycelial growth to assess mancozeb sensitivity allowed the detection of insensitive

**TABLE 1** - Sensitivity of isolates of *Mycosphaerella fijiensis* to the fungicides chlorothalonil, mancozeb, tebuconazole and thiophanate-methyl and the results of the Mantel's test of correlation between geographic distance and mean EC<sub>50</sub> values.

Fungicides	EC <sub>50</sub> <sup>1</sup> values (µg mL <sup>-1</sup> )				Mantel Test (EC <sub>50</sub> Vs Geographic distance)
	N <sup>2</sup>	Minimum	Maximum	Mean±SD	r (P value)
chlorothalonil	41	0.39	53.7	5.35±8.53	-0.02 (0.31)
mancozeb	48	0.09	>1000	>1000±1000	-0.05 (0.32)
tebuconazole	45	0.02	1.39	0.36±0.40	-0.11 (0.82)
thiophanate-methyl	45	0.008	8.22	0.70±1.74	0.26 (0.10)

<sup>1</sup>EC<sub>50</sub> is the effective concentration of the fungicide at which mycelial growth was inhibited by 50%.

<sup>2</sup>N is number of isolates tested for each fungicide.

isolates of *Ascochyta rabiei*, causal agent of ascochyta blight of chickpea (Chang et al., 2007) and *Colletotrichum gloeosporioides* isolates that causes anthracnose in mango (Kumar et al., 2007). Mancozeb inhibits spore germination (Wicks & Lee, 1982) and this kind of test needs to be done to collect further information on the sensitivity of *M. fijiensis* to this fungicide.

There was no correlation between EC<sub>50</sub> values for all fungicides and geographic regions based on Mantel's test (Table 1), meaning that the isolates were not geographically clustered according to their sensitivity to the fungicides.

The G143A point mutation in the cytochrome b was not found in any of the isolates tested. The amplicon of approximately 200 bp was present in all isolates. However, other point mutations conferring resistance can occur in cyt b gene as the substitution of phenylalanine to leucine at position 129 (F129L) reported in resistant isolates of *Alternaria solani* (Pasche et al., 2005). Thus, it is possible that point mutations in other positions can occur in *M. fijiensis* isolates from Brazil and confer resistance to strobilurin.

The Brazilian population of *M. fijiensis* seems to be sensitive at various levels to all tested fungicides. The profile of fungicide sensitivity presented in the present work will allow the appropriate implementation of field monitoring programs to assess the effectiveness of fungicides and to understand the evolution of fungicide resistance in the Brazilian population of *M. fijiensis*. Because most isolates collected in the state of Amazonas were never exposed to fungicides and no clustering of the sensitivity levels according to geographic region was detected, the EC<sub>50</sub> values estimated here may serve as proxy baseline values for future comparison of sensitivity of the population of *M. fijiensis* to fungicides.

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