Fungicide Sensitivity of *Sclerotinia sclerotiorum*: A Thorough Assessment Using Discriminatory Dose, EC₅₀, High-Resolution Melting analysis, and Description of New Point Mutation Associated with Thiophanate-Methyl Resistance

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Abstract

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Thiophanate-methyl (TM), fluazinam, and procymidone are fungicides extensively used for white mold control of common bean in Brazil. We assessed the sensitivity of Brazilian isolates of *Sclerotinia sclerotiorum* to these three fungicides using discriminatory doses and concentration that results in 50% mycelial growth inhibition (EC₅₀) values. In total, 282 isolates from the most important production areas were screened and none was resistant to fluazinam or procymidone. The EC₅₀ values varied from 0.003 to 0.007 and from 0.11 to 0.72 µg/ml for fluazinam and procymidone, respectively. One isolate was resistant to TM. The EC₅₀ of the TM-resistant isolate was greater than 100 µg/ml, whereas the EC₅₀ of the sensitive isolates varied from 0.38 to 2.23 µg/ml. The

TM-resistant isolate had a L240F mutation in the β-tubulin gene. This is the first report of mutation at codon 240 causing resistance to a benzimidazole fungicide in *S. sclerotiorum*. The high-resolution melting analysis allowed the distinction of TM-sensitive and -resistant isolates by specific melting peaks and curves. The TM-resistant isolate had mycelial growth, sclerotia production, and aggressiveness comparable with that of the sensitive isolates, indicating that this genotype will likely compete well against sensitive isolates in the field. This study demonstrates that resistance to TM, fluazinam, and procymidone is nonexistent or rare. Resistance management practices should be implemented, however, to delay the spread of TM-resistant genotypes.

Common bean (*Phaseolus vulgaris* L.) is an important staple food in many countries in Latin America and Africa (Schmutz et al. 2014). Brazil is the world's largest producer of this legume and, in 2012, approximately 3 million tons of dry beans were produced in the country (FAO 2012), distributed in three growing seasons throughout the year: rainy, dry, and fall-winter. One of the economically most important plant pathogens of common bean is *Sclerotinia sclerotiorum*. It is a necrotrophic fungus with a broad host range (Boland and Hall 1994) that causes white mold in common bean. The fungus is widespread in the Brazilian common bean producing areas and its biological features make difficult the control of white mold. The fungus can survive as sclerotia for many years in the soil and employs a wide array of cell-wall-degrading enzymes and oxalic acid to colonize its hosts (Amselem et al. 2011).

Development of white mold in common bean is highly influenced by weather conditions, cultivars, and cultural practices. The pathogen is favored by mild temperatures (18 to 23°C), high humidity, planting density that reduces air flow, over fertilization, and cultivars with a type III prostrate growth habit (Schwartz and Singh 2013). Under these conditions, complete crop losses can occur in fields planted with susceptible common bean cultivars (Schwartz and Singh 2013). Currently, white mold is the major fungal disease of common bean in Brazil and other countries such as the United States, Canada, and Argentina (Schwartz and Singh 2013). In Brazil, common bean crop losses due to white mold are higher in the fall-winter growing season, when climatic conditions are favorable to the pathogen.

Nevertheless, severe epidemics have also been recorded in other growing seasons, especially in fields established above 700 m in altitude.

Fungicide application is the strategy most used to control white mold in common bean. To date, there is no commercial cultivar with high levels of resistance to white mold, and growers rely heavily on intensive fungicide treatment. In highly infested areas and under favorable weather conditions, up to six fungicide sprays are applied to manage the disease. The intensive use of fungicides, especially of site-specific products, can select for resistant isolates and, consequently, may lead to control failures (Brent and Hollomon 2007). Assessing the sensitivity of *S. sclerotiorum* to the most commonly used fungicides is crucial for white mold and resistance management. In Brazil, seven fungicides are registered for white mold control in common bean but three are most frequently used by farmers: thiophanate-methyl (TM), fluazinam, and procymidone. The benzimidazole fungicide TM inhibits mitotic division by disturbing the assemblage of microtubules (Davidse 1986). Fluazinam is a multisite phenylpyridinamine fungicide that inhibits respiration with an uncoupling activity on the mitochondrial oxidative phosphorylation involving protonation or deprotonation of the amino group (Guo et al. 1991). Procymidone is a site-specific dicarboxamide fungicide that affects the osmoregulation of the fungal membranes (Brent and Hollomon 2007).

Resistance to benzimidazoles such as carbendazim or TM has been reported in field populations of many plant pathogens. (Koenraadt et al. 1992). This resistance usually has been associated with point mutations in the β-tubulin gene, which alter amino acid sequences at the benzimidazole-binding site (Koenraadt et al. 1992; Ma and Michailides 2005). In *S. sclerotiorum*, these mutations result in the replacement of glutamine (GAG) by alanine (GCG) at codon 198 (E198A) or of phenylalanine (TTC) by tyrosine (TAC) at codon 200 (F200Y) in the β-tubulin gene (Yang et al. 2004). In other plant pathogens, different mutations have been identified (Banno et al. 2008;

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Koenraadt et al. 1992). Resistance to fungicides with multisite modes of action such as fluazinam is rarely observed and has not been detected in *S. sclerotiorum*; however, reduced sensitivity was reported in this pathogen by Attanayake et al. (2012) and *Botrytis cinerea* populations resistant to this fungicide were identified in bean crops in Japan (Tamura 2000). The resistance mechanisms of plant pathogens to fluazinam have not been investigated. Resistance to dicarboxamide fungicides in plant pathogens is usually associated with mutations in the coiled-coil region of the histidine kinase gene (*BOS-1* gene) (Ma and Michailides 2005). In *S. sclerotiorum*, resistance to dicarboxamide in field isolates was identified only recently to the dimethachlon fungicide (Zhou et al. 2014). Although field resistance to procymidone has not yet been identified, laboratory-induced resistant isolates are relatively easy to obtain (Liu et al. 2010).

High-resolution melting (HRM) analysis is a technique that allows accurate determination of the relationship between temperature and denaturation of DNA fragments (Tong and Giffard 2012), which allows DNA sequences to be distinguished via melting temperature (*T_m*). At this temperature, half of the double-stranded DNA amplicon will dissociate and become single-stranded DNA. The *T_m* is positively correlated with sequence length and GC content, due to the additional hydrogen bond between GC pairs compared with AT pairs (Tong and Giffard 2012). HRM analysis has been used to detect specific single-nucleotide polymorphisms in genes associated with antimicrobial resistance, identification, detection, and the tracking and monitoring of microorganisms (Tong and Giffard 2012). Recent studies have demonstrated the usefulness of HRM for large-scale detection of point mutations associated with fungicide resistance in plant-pathogenic fungi (Banno et al. 2008; Chatzidimopoulos et al. 2014).

The goals of this study were to determine the sensitivity of *S. sclerotiorum* of common bean from various regions in Brazil to TM, fluazinam, and procymidone and to investigate the molecular mechanism in TM-resistant isolates.

Materials and Methods

***S. sclerotiorum* isolates.** In all, 282 *S. sclerotiorum* isolates were obtained from sclerotia collected from common bean plants with white mold symptoms in fields located in the following Brazilian states: Santa Catarina (*n* = 5 isolates), Paraná (*n* = 25), São Paulo (*n* = 30), Minas Gerais (*n* = 99), Espírito Santo (*n* = 25), Goiás (*n* = 49), Bahia (*n* = 15), and Pernambuco (*n* = 34) (Fig. 1). These states

Fig. 1. Map of Brazil showing production areas of common bean and sampling sites (dots): SC = Santa Catarina, PR = Paraná, SP = São Paulo, MG = Minas Gerais, ES = Espírito Santo, GO = Goiás, BA = Bahia, and PE = Pernambuco.

account for approximately 75% of total common bean production in Brazil (CONAB 2013). According to producers, TM, fluazinam, and procymidone have been used for many years to control diseases.

In order to ensure genetic uniformity, only monoascosporic isolates from each selected sclerotium were used in the tests. Monoascosporic isolates were obtained according to methodology described by Lehner et al. (2015). Sclerotia produced by colonies of the monoascosporic isolates were dried and stored at 4°C.

Determination of discriminatory doses and concentration that results in 50% mycelial growth inhibition values. Thirty-one randomly selected isolates were used in this experiment. The following commercial fungicide formulations were used in all tests: TM as Cercobin 700 WP (70% active ingredient [a.i.]), fluazinam as Frowncide 500 SC (50% a.i.), and procymidone as Sumilex 500 WP (50% a.i.) (Iharabras S.A. Indústrias Químicas, Sorocaba, Brazil). The fungicides were dissolved in dimethyl sulfoxide (DMSO) to obtain 100 mg a.i./ml for the stock solution. To obtain the desired concentrations of each fungicide, serial dilutions were prepared from the stock solution. The concentration of DMSO did not exceed 0.1% of the testing solution (fungicide-amended medium). At that concentration, DMSO did not inhibit mycelial growth of *S. sclerotiorum* isolates (data not shown). Fungicides were added to cooled (42 to 50°C) but nonsolidified potato dextrose agar (PDA) medium. The concentrations used for each fungicide were TM at 0 (PDA + DMSO), 0.5, 1.0, 5.0, 10, and 100 µg/ml; fluazinam at 0, 0.0025, 0.005, 0.01, 0.05, and 0.1 µg/ml; and procymidone at 0, 0.05, 0.1, 0.25, 0.5, and 1.0 µg/ml. Mycelial plugs (5 mm in diameter) from a 2-day-old culture of the 31 isolates were placed in the center of petri dishes (6 cm in diameter) containing 10 ml of PDA amended with the fungicides at each of the above concentrations. After 24 h of incubation at 23°C in the dark, the colony diameter (Cd) of each isolate was measured in two perpendicular directions using a digital caliper. The experiment was set in a completely randomized design with four replicates per concentration of fungicide. A petri dish with a mycelium plug was considered as an experimental unit. The experiment was performed twice.

Box plots were used to analyze the mycelial growth data in each fungicide concentration. The discriminatory dose was established as the dose in which sensitive isolates grew less than 25% of the average Cd measured in the control treatment, and resistant isolates grew without inhibition. In addition, for each isolate, a linear regression of the percent inhibition [(Cd in control – Cd fungicide concentrations)/Cd in control] versus the log₁₀ of the fungicide concentration was performed to estimate the concentration that results in 50% mycelial growth inhibition (EC₅₀) using the R program (R Core Team, Vienna, Austria). Because the variances of the two experiments were homogeneous, the mycelial growth and EC₅₀ values for each isolate were averaged.

Assessment of fungicide sensitivity using discriminatory doses. Based on the discriminatory doses determined above, all 282 isolates were screened regarding sensitivity to TM, fluazinam, and procymidone, including the 31 isolates that were previously tested. Mycelial plugs (5 mm in diameter) from a 2-day-old culture of each isolate were placed in the center of petri dishes (6 cm in diameter) containing 10 ml of PDA amended with the discriminatory dose of TM, fluazinam, or procymidone at 5, 0.05, or 0.5 µg/ml, respectively. After 24 h of incubation at 23°C in the dark, Cd of each isolate was measured using a digital caliper. The experiment was set in a completely randomized design. For each isolate, four replicates with and without fungicides were used. The experiment was performed twice. Isolates were considered resistant if they grew on the unamended PDA (control) and if growth was more than 50% compared with the control on the PDA amended with the discriminatory doses. They were considered sensitive if they grew less than 50% compared with the control on the PDA amended with the discriminatory dose of each fungicide.

Analysis of DNA sequence of the β-tubulin gene from isolates sensitive and resistant to TM. Seven sensitive and one resistant isolate were used for DNA analysis. Three biological replicates with independent DNA extractions were conducted for the resistant isolate. Isolates were grown in liquid medium (10 g of sucrose, 2 g of

l-asparagin, 2 g of yeast extract, 1 g of KH_2PO_4 , 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.44 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.48 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and 0.36 mg of $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ in Erlenmeyer flasks at 23°C for 7 days in order to extract DNA. The mycelium was washed with distilled water, transferred to filter paper to dry, and macerated in a mortar with liquid nitrogen. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) following the manufacturer's instructions. DNA integrity was analyzed in agarose gel electrophoresis and its concentration was measured in a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE).

Polymerase chain reaction (PCR) was performed in a final volume of 25 μl with 1 μl of DNA (25 ng/ μl); 1 μl of DMSO; 2.5 μl of bovine serum albumin at 50 mg/ml; 1 μl of each primer at 10 μM ; 6 μl of water; 12.5 μl of Dream Taq PCR Master Mix (2 $\times$) that includes dATP, dCTP, dGTP, and dTTP (0.4 mM each); and 4 mM MgCl_2 (Thermo Fisher Scientific). The primer pair used was TUB-F1 (5'-GCTTTTGATCTCCAAGATCCG-3'; nucleotide positions 1,757 to 1,777) and TUB-R1 (5'-CTGGTCAAAGGAGCAAATCC3'; nucleotide positions, 2134 to 2,115). Each amplification reaction consisted of an initial denaturation at 95°C for 5 min; followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. Amplification was confirmed by using 5 μl of PCR product separated by electrophoresis on a 1% agarose gel. PCR products were purified using ExoSAP-IT cleanup reagent (USB, Cleveland, OH) and sequenced by Macrogen Services (Kumchun-ku, Seoul, Korea) using TUB-F1 and TUB-F2 primers. The nucleotide sequences were edited with Staden Sequence Analysis Package (Staden 1996) and aligned using MEGA 5.0 (Tamura et al. 2011). In the alignment, we included the sequence of the *S. sclerotiorum* 1980 isolate, which had its genome recently sequenced (Amselem et al. 2011).

HRM PCR. DNA of 14 sensitive isolates and DNA of two biological replicates of TM-resistant isolates were used in the PCR assay. HRM analysis was performed in triplicate (three reaction tubes for each template DNA) in a Rotor-Gene Q (Qiagen, Hilden, Germany). The primers TUB-HPF1 and TUB-HPR1 were used for the real-time PCR assay. These primers were designed to specifically amplify a 224-bp region of the β -tubulin gene that includes the 198 and 200 codons (Banno et al. 2008). PCR was performed in a final volume of 10 μl with 1 μl of DNA (25 ng/ μl), 5 μl of 2 $\times$ HRM PCR Master Mix, 0.7 μl of each primer at 10 μM , and 3.3 μl of water RNase-free water (Type-it HRM PCR Handbook; Qiagen). The thermocycling consisted of an initial denaturation of 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 57°C for 30 s, and 72°C for 10 s. The HRM analysis was performed by heating the amplicon DNA gradually from 60 to 95°C in 0.1°C/s increments in order to generate a melt curve. Data were analyzed using the Rotor Gene Q–Pure Detection software (version 2.02; Qiagen).

Mycelial growth, sclerotia production, and aggressiveness of *S. sclerotiorum* isolates sensitive and resistant to TM. Fourteen sensitive isolates, each from a different geographic region, and the resistant isolate were used in all tests. To measure the mycelial growth rate and sclerotia production, mycelial plugs (5 mm in diameter) from a 2-day-old culture were placed in the center of petri dishes (9 cm in diameter) containing 15 ml of PDA with chloramphenicol at 100 mg off/liter. The dishes were kept at 23°C. Cd was assessed after 24 and 29 h of incubation and the average growth rate (millimeters per hour) was estimated as (Cd measured at 29 h of incubation – Cd at 24 h)/5. The number of sclerotia produced in each petri dish was counted after 21 days of incubation. A completely randomized design with four replicates was used. Each replicate was considered as one colony in a plate and the experiment was performed twice.

To estimate aggressiveness, leaflets of the youngest fully expanded trifoliolate leaves of common bean plants at the flowering stage were placed in filter paper moistened with 5 ml of sterilized distilled water inside of plastic boxes (11 by 11 by 3 cm, width by length by height; gerbox). One 2-day-old mycelial disc (5 mm in diameter) from the first subculture of each isolate was placed between the main vein and the leaflet edge. Boxes containing inoculated leaves were kept at 23°C in darkness. The lesion diameter was assessed 48 h after

inoculation using a digital caliper. Treatments were replicated four times in a completely randomized design. The experiment was performed twice.

Data of each variable were analyzed for homogeneity of variance using Hartley's F max test. For each variable, data from two experiments were pooled for statistical analysis if variances were homogeneous; experiments were analyzed separately if variances were not homogeneous. A joint analysis of variance was performed using the R program. Treatments (isolates) and experiment effects were considered as random factors in the analysis. The linear model fit to the data was $Y_{ijk} = m + G_i + A_j + G_{Aij} + E_{ijk}$, where G_i is the effect

Fig. 2. Box plots showing the percentage of mycelial growth of the 31 *Sclerotinia sclerotiorum* isolates in each fungicide concentration. Each whisker represents 25% and the box region 50% of isolates. The line at the central part of the box represents the median value. All circles above or below each box plot represent outliers. **A.** Thiophanate-methyl. **B.** Fluazinam. **C.** Procyimdone.

of treatments, A_j is the effect of experiments, GA_{ij} is the treatment-experiment interaction term, and E_{ijk} is the error effect.

Results

Discriminatory doses and EC_{50} values. For TM at 0.5 or 1.0 $\mu\text{g/ml}$, more than 75% of the isolates grew more than 50% relative to the control (Fig. 2A). At 5.0 $\mu\text{g/ml}$, the mycelial growth varied from 14.5 to 31.0%. More than three-fourths of the isolates grew less than 25%; therefore, this concentration was chosen as discriminatory for TM. For fluazinam, the higher concentration in which the isolates grew less than 25% was 0.05 $\mu\text{g/ml}$. Therefore, this concentration was chosen as discriminatory for fluazinam (Fig. 2B). For procymidone, the isolates grew over 50% relative to the control in the three highest concentrations tested. At 0.5 $\mu\text{g/ml}$, 90% of the isolates grew less than 25% relative to the control. Therefore, this concentration was chosen as discriminatory for procymidone (Fig. 2C).

The EC_{50} values for TM were 0.38 to 2.23 $\mu\text{g/ml}$ (Fig. 3A) and the overall mean was 1.16 $\mu\text{g/ml}$. For fluazinam, the EC_{50} values were 0.003 to 0.007 $\mu\text{g/ml}$ (Fig. 3B) and the overall mean was 0.005 $\mu\text{g/ml}$. For procymidone, the EC_{50} values were 0.11 to 0.72 $\mu\text{g/ml}$ (Fig. 3C) and the overall mean was 0.35 $\mu\text{g/ml}$.

Sensitivity of *S. sclerotiorum* isolates to different fungicides. Using the discriminatory dose of 5.0 $\mu\text{g/ml}$, 1 of 282 tested isolates was resistant to TM. In order to confirm this result, the resistant

isolate (coded as Ss-7) was exposed to a range of TM concentrations. Compared with the control, Ss-7 grew 100% at 0.5 and 1.0 $\mu\text{g/ml}$ and 98, 94, and 47% at 5.0, 10, and 100 $\mu\text{g/ml}$, respectively (Fig. 1A, inset). The mycelial growth of the other 282 isolates varied from 15 to 40% compared with the control (data not shown). Analysis of the partial sequence of the β -tubulin gene revealed no mutations in seven sensitive isolates. The resistant isolate, however, had one mutation replacing leucine to phenylalanine at codon 240, an L240F mutation (Fig. 4).

No isolates were resistant to fluazinam or procymidone. Using the discriminatory doses, the mycelial growth varied from 16 to 35 and 14 to 28% compared with the control for fluazinam and procymidone, respectively (data not shown).

Assessment of TM resistance using HRM. The TM-resistant isolate showed a specific melting peak ($T_m = 82.9^\circ\text{C}$) and curve based on the HRM analysis, allowing differentiation from the sensitive isolates ($T_m = 83.2^\circ\text{C}$) (Fig. 5A and B). Thus, it was possible to distinguish resistant and sensitive isolates of *S. sclerotiorum* via HRM PCR method.

Mycelial growth, sclerotia production, and aggressiveness of *S. sclerotiorum* isolates sensitive and resistant to TM. The variances of the two experiments for all variables were homogeneous. There was no variation among isolates regarding mycelial growth rate ($F = 1.69$, $P = 0.17$), number of sclerotia ($F = 1.92$, $P = 0.14$), and

Fig. 3. Density frequency of the concentration that results in 50% mycelial growth inhibition (EC_{50}) values of the 31 *Sclerotinia sclerotiorum* isolates estimated for **A**, thiophanate-methyl, **B**, fluazinam, and **C**, procymidone. The EC_{50} values ($\mu\text{g a.i. ml}^{-1}$) 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 *S. sclerotiorum* isolate.

	154		198		233
			↑		
<i>Sclerotinia_sclerotiorum</i> _1980	KIREEFDRMMATFSVVPSPKVS	DTVVEPYNATLSVHQLVENS	DETFCIDNEALYDICMR	TLKLSHPSYGD	LNHLVSAVM
Ss-188					
Ss-190					
Ss-191					
Ss-203					
Ss-323					
Ss-332					
Ss-377					
Ss-7					
Ss-7_BR1					
Ss-7_BR2					
	234	240		270	
		*			
<i>Sclerotinia_sclerotiorum</i> _1980	SGVTTC	LRFP	QGLNSDLR	KLAVNMVFP	RLHFFMVGF
Ss-188					
Ss-190					
Ss-191					
Ss-203					
Ss-323					
Ss-332					
Ss-377					
Ss-7		F			
Ss-7_BR1		F			
Ss-7_BR2		F			

Fig. 4. Alignment of β -tubulin amino acid sequences from *Sclerotinia sclerotiorum* isolates. The codes Ss-7_BR1 and Ss-7_BR2 correspond to biological replicates of the thiophanate-methyl-resistant Ss-7 isolate.

aggressiveness ($F = 2.47$, $P = 0.05$) estimated by lesion diameter in leaflets of common bean plants. The average mycelial growth rate varied from 2.19 ± 0.10 mm/h (Ss-7) to 2.68 ± 0.38 mm/h (Ss-122), the average number of sclerotia from 18 ± 10 (Ss-313) to 39 ± 8 (Ss-195), and the average lesion diameter from 23.0 ± 3.0 mm (Ss-379) to 34.2 ± 3.0 mm (Ss-298) (Table 1).

Discussion

White mold management in common bean crops in Brazil is based on the application of fungicides, particularly TM (benzimidazole), procymidone (dicarboxamide), or fluazinam (phenylpyridinamine). Thus, the monitoring of the sensitivity of *S. sclerotiorum* is the key to disease management in the field. In this study, we identified discriminatory doses for three commonly used fungicides that will simplify sensitivity monitoring in *S. sclerotiorum* isolates.

The emergence of fungicide-resistant populations of *S. sclerotiorum* has been reported less frequently compared with other related species of the family Sclerotiniaceae such as *B. cinerea* and *Monilinia fructicola*. Unlike these species, *S. sclerotiorum* is a homothallic fungus that has an asexual lifestyle and does not produce conidia (Amselem et al. 2011). According to McDonald and Linde (2002), plant pathogens with these biological characteristics have lower

evolutionary potential and, therefore, lower risk of developing fungicide resistance. Our survey confirms the slow adaptation of *S. sclerotiorum* to commonly used fungicides but also indicates the potential of resistance development.

Various methods have been used to assess fungicide sensitivity in plant pathogens. Most methods involve the transfer of spores or mycelial plugs of fungal isolates to culture medium amended with various concentrations of fungicide to assess the inhibition of growth or spore germination (Russell 2004). A discriminatory dose is a single dose rate at which, depending upon the reaction of the fungal isolate, it is possible to classify an isolate as sensitive or resistant to a fungicide. Resistant isolates often grow 50% or more in the presence of the fungicide (Russell 2004). Thus, to avoid future misclassification, we chose the lowest threshold concentration possible in which most of the *S. sclerotiorum* isolates grew no more than 25%. Therefore, if any isolate with less sensitivity but still sensitive was not sampled in the present study, it will probably grow less than 50% and will be considered sensitive. In this study, we did not find isolates resistant to fluazinam and procymidone, which prevented testing the discriminatory doses of these fungicides in resistant isolates.

Although resistance to benzimidazoles is relatively frequent in plant-pathogenic fungi (Koenraadt et al. 1992), in the present study,

Fig. 5. Differentiation of the *Sclerotinia sclerotiorum* isolates sensitive and resistant to thiophanate-methyl via high-resolution melting analysis. **A**, Melting peaks and **B**, melting curves.

Table 1. Geographical origin and comparison of mycelial growth, sclerotia production, and pathogenicity among the thiophanate-methyl-resistant and -sensitive isolates of *Sclerotinia sclerotiorum* from eight Brazilian States^a

Isolate code	State of origin (region)	Phenotype	Mycelial growth rate (mm h ⁻¹) ^b	Number of sclerotia ^c	Lesion diameter (mm) ^d
Ss-7	Minas Gerais (Northwest)	Resistant	2.19 ± 0.10	35 ± 15	29.8 ± 5.0
Ss-42	Minas Gerais (Zona da Mata)	Sensitive	2.33 ± 0.20	33 ± 8	30.2 ± 2.6
Ss-47	Minas Gerais (Northwest)	Sensitive	2.37 ± 0.31	23 ± 5	30.9 ± 3.4
Ss-91	Minas Gerais (South)	Sensitive	2.38 ± 0.16	32 ± 8	33.2 ± 3.4
Ss-122	Minas Gerais (Alto Paranaíba)	Sensitive	2.68 ± 0.38	22 ± 9	31.1 ± 5.2
Ss-166	Espírito Santo	Sensitive	2.51 ± 0.17	38 ± 8	25.4 ± 5.0
Ss-195	Paraná (South)	Sensitive	2.17 ± 0.13	18 ± 10	30.9 ± 5.9
Ss-221	São Paulo	Sensitive	2.49 ± 0.17	29 ± 13	33.3 ± 3.9
Ss-250	Minas Gerais (North)	Sensitive	2.50 ± 0.22	25 ± 7	28.8 ± 5.9
Ss-298	Bahia	Sensitive	2.59 ± 0.24	23 ± 7	34.2 ± 3.0
Ss-305	Paraná (North)	Sensitive	2.61 ± 0.18	31 ± 4	29.7 ± 2.8
Ss-313	Santa Catarina	Sensitive	2.64 ± 0.13	39 ± 8	30.1 ± 5.4
Ss-360	Goiás (South)	Sensitive	2.45 ± 0.22	27 ± 3	32.9 ± 7.0
Ss-373	Pernambuco	Sensitive	2.51 ± 0.28	29 ± 9	32.0 ± 3.7
Ss-379	Goiás (Central region)	Sensitive	2.61 ± 0.21	34 ± 8	23.0 ± 3.0

^a Data shown are mean ± standard deviation.

^b Mycelial radial growth was measured after 24 and 29 h of incubation on potato dextrose agar (PDA) at 23°C in the dark. The mycelial growth rate (mm/h) was estimated as (colony diameter measured at 29 h – colony diameter at 24 h)/5.

^c Sclerotia were counted after 21 days of incubation of isolates on PDA at 23°C in the dark.

^d Lesion diameter measured on leaflets of bean plants after inoculation of isolates with one 2-day-old mycelial disc. Inoculated leaflets were kept at 23°C in the dark for 48 h.

only one *S. sclerotiorum* isolate was resistant to TM. In a sample of 31 isolates, the estimated EC₅₀ of sensitive isolates ranged from 0.38 to 2.23 µg/ml, whereas the estimated EC₅₀ of resistant isolates was 106 µg/ml. These results are consistent with those of other studies that found EC₅₀ values of benzimidazole-resistant isolates higher than 5.0 µg/ml in *S. sclerotiorum* (Ma et al. 2009) and higher than 50 µg/ml in *M. fruticola* and *B. cinerea* (Ma et al. 2003; Sun et al. 2010). Other sclerotia from the same field from which the Ss-7 isolate was obtained were also tested for TM sensitivity, and all were resistant to TM.

This is the first report of TM resistance in a Brazilian isolate of *S. sclerotiorum*. Two mutations in the β-tubulin gene are known to occur in *S. sclerotiorum* isolates resistant to benzimidazoles: E198A and F200Y (Yang et al. 2004). Surprisingly, the resistant isolate identified in the present study has a mutation that replaces a leucine (CTC) by a phenylalanine (TTC) at codon 240 in the β-tubulin gene. This amino acid substitution may be phenotypically silent, because both are bulky hydrophobic amino acids, and may not cause major changes in the protein structure and function (Baraldi et al. 2003). However, this was not the case in the present study and in other reports. The L240F mutation has been reported to be associated with a low benzimidazole resistance phenotype in isolates of *Tapesia yallundae* (Albertini et al. 1999) and *M. laxa* (Ma et al. 2005). The low benzimidazole resistance phenotype was defined based on EC₅₀ values between 0.5 and 3.0 µg/ml (*T. yallundae*) or on the capacity of isolates to grow with TM at 1 and 5 but not 50 µg/ml (*M. laxa*). The L240F mutation in the β-tubulin gene was also found in benzimidazole-resistant isolates of *Penicillium expansum* (Baraldi et al. 2003; Cabañas et al. 2009). However, these isolates also had an additional mutation that replaces glutamic acid by valine at codon 198. The presence of the E198V mutation prevented a better understanding of the true effect of the L240F mutation in *P. expansum*.

Fluazinam is one of the most effective fungicides for controlling Sclerotinia diseases (Lemay et al. 2002; Mahoney et al. 2014; Matheron and Porchas 2004; Vieira et al. 2012). This fungicide has activity at multiple sites and, therefore, risk of resistance development is low (Ma and Michailides 2005). In the present study, all isolates were sensitive to fluazinam in a relatively low concentration (0.05 µg/ml). Fluazinam has been referred to as the most effective fungicide to control white mold in Brazil (Vieira et al. 2012) and the low EC₅₀ value reported here supports this claim. In other studies, *S. sclerotiorum* isolates from canola fields in China and the United States (Attanayake et al. 2013) and from one alfalfa field in United States (Attanayake et al. 2012) were assessed using the discriminatory dose of fluazinam at 0.005 µg/ml. The authors found mycelial growth ranging from 20 to 40 and 20 to 60% in canola and alfalfa isolates, respectively. The results reported in the present study support previous observations because, when grown in the same fungicide concentration, growth of Brazilian common bean isolates of *S. sclerotiorum* varied from 31 to 55%.

The dicarboxamide fungicides most commonly used for controlling Sclerotinia diseases are iprodione and procymidone. Although effective (Bradley et al. 2006; Ma et al. 2009), these fungicides have high risk of resistance development because of the site-specific mode of action (Ma and Michailides 2005). In species genetically related to *S. sclerotiorum*, resistance to iprodione or procymidone has been reported in field isolates of *B. cinerea* (Lamondia and Douglas 1997; Sun et al. 2010), *M. fruticola* (Lim and Cha 2003), and *S. minor* (Hubbard et al. 1997). For *S. sclerotiorum*, however, resistance to these fungicides was observed only in laboratory-induced mutants (Liu et al. 2009; Liu et al. 2010). The current study did not find evidence of resistance to procymidone in field isolates of *S. sclerotiorum*. Interestingly, resistant isolates can easily be obtained under laboratory conditions (Liu et al. 2010), which implies a risk of resistance development under field conditions. Thus, farmers should avoid sequential applications of procymidone and alternate fungicides with different modes of action.

In recent years, molecular methods have provided rapid and reliable assessment of fungicide sensitivity in plant pathogens (Banno et al. 2008; Chatzidimopoulos et al. 2014; Suga et al. 2011). One goal

of this study was to test the HRM technique for assessing TM resistance in *S. sclerotiorum*. HRM analysis allowed us to separate the TM-resistant isolate from the sensitive isolates. The resistant *S. sclerotiorum* isolate Ss-7 had *Tm* lower from that of sensitive isolates due to a single nucleotide change. Unfortunately, only one resistant isolate was available and, thus, we were unable to verify the technique with multiple resistant isolates. Furthermore, it is unknown what the *Tm* would look like if isolates were screened with the more common mutations leading to E198A or F200Y. Therefore, at this point, we do not recommend the use of this technique as a sole screening method.

The methodology used in this work may help guide future studies aimed at monitoring sensitivity to fungicides in *S. sclerotiorum* using discriminatory doses or the HRM technique. The agar plate test, however, has the advantage of detecting any resistant isolate without assuming the existence of a specific mutation. The TM-resistant isolate had mycelial growth, sclerotia production, and aggressiveness comparable with that the sensitive isolates. This suggests that it has sufficient parasitic fitness to compete with sensitive isolates in field. However, only one isolate was assessed and extrapolation to other resistant isolates should be approached with caution. It would be wise to reduce the number of TM sprays and to adopt rotation of fungicides compounds with different modes of actions; for example, fluazinam and procymidone, for which there is no evidence of loss of sensitivity to date.

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