RHAPHAEL ALVES SILVA

GENETIC STRUCTURE AND FUNGICIDE SENSITIVITY OF THE POPULATION OF *Sclerotinia sclerotiorum* IN BRAZIL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae*.

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APROVADA: 19 de fevereiro de 2018.

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(Orientador)

A minha avó Maria Helena Dos Santos, aos meus pais, Nasario Alves da Silva e Neuza dos Reis da Silva Alves, aos meus irmãos e minha namorada, **dedico.**

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MUITO OBRIGADO!

SUMÁRIO

ABSTRACT

Silva, Rhaphael Alves, M.Sc., Universidade Federal de Viçosa, February, 2018. **Genetic structure and fungicide sensitivity of the population of *Sclerotinia sclerotiorum* in Brazil.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-adviser: Miller da Silva Lehner.

Sclerotinia sclerotiorum causes white mold or sclerotinia stem rot and affects important crops such as grains, fibers and vegetables. This study was conducted to assess the genetic variability; the sensitivity to thiophanate-methyl (TM), fluazinam and procymidone of 238 isolates of *S. sclerotiorum* from different hosts and regions in Brazil; and the phenotypic stability of fungicide resistant isolates. Microsatellites (SSR) and mycelium compatibility groups (MCG) markers were used to assess the genetic structure of the population. Twenty-two MCGs and 53 multilocus genotypes (MLG) were identified. Most isolates (67.7 %) were distributed in either MCG 1 or 2. Seven genetic groups were detected. The population has a clonal structure largely determined by MCGs and there was no evidence of random mating. Fungicide sensitivity was assessed using the discriminatory doses for TM, fluazinam and procymidone. The stability of nine isolates TM-resistant and one sensitive was assessed after 10 transfers in culture medium without fungicide. Mycelial growth rate of six TM-resistant isolates reduced with successive transfers. There was no evidence of resistance to fluazinam or procymidone. Thirteen isolates collected in common bean fields in the municipalities of Cabeceira Grande (8 isolates) and Unaí (5 isolates), Minas Gerais state, were resistant to TM. The resistance was associated with the L240F mutation in the β-tubulin gene. Resistance to TM seems to be localized; and there is evidence for a fitness cost in resistant isolates.

RESUMO

Silva, Rhaphael Alves, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Estrutura genética e sensibilidade a fungicidas da população de *Sclerotinia sclerotiorum* no Brasil.** Orientador: Eduardo Seiti Gomide Mizubuti. Coorientador: Miller da Silva Lehner.

Sclerotinia sclerotiorum, agente causal do mofo branco ou podridão de sclerotinia, infecta espécies de interesse agronômico, tais como aquelas destinadas à produção de grãos, fibras e hortaliças. Este estudo foi conduzido para avaliar: a variabilidade genética do patógeno; a sensibilidade aos fungicidas tiofanato-metílico (TM), fluazinam e procimidone de 238 isolados de *S*. *sclerotiorum* coletados em diferentes hospedeiros e regiões no Brasil; e a estabilidade fenotípica de isolados resistentes. Os marcadores microssatélites (SSR) e os grupos de compatibilidade micelial foram utilizados para acessar a estrutura genética da população. Vinte e dois MCGs e 53 genótipos foram identificados. A maioria dos isolados (67,7 %) foi classificado como MCG 1 ou 2. Sete grupos genéticos foram identificados. A população foi estruturada por MCG e não houve evidência de acasalamento aleatório. A população como um todo possui estrutura genética clonal. A sensibilidade aos fungicidas TM, fluazinam e procimidone foi avaliada com doses discriminatórias. O crescimento micelial de nove isolados resistentes e um isolado sensível ao TM foi avaliado durante 10 gerações. A taxa de crescimento de seis isolados resistentes reduziu com o passar das gerações. Não houve resistência a fluazinam ou procimidone. Treze isolados coletados na cultura do feijoeiro nos municípios de Cabeceira Grande (8 isolados) e Unaí (5 isolados), Minas Gerais, foram resistentes ao TM. A resistência ao TM foi causada pela substituição de leucina por fenilalanina no códon 240 do gene da β-tubulina (L240F). Isolados resistentes a TM foram encontrados apenas em uma região. Isolados resistentes coletados em Cabeceira Grande têm adaptabilidade reduzida. Em síntese, no Brasil predominam clones de *S*. *sclerotiorum* fortemente determinados por MCG, constataram-se isolados resistentes ao TM e para manejo do mofo branco, recomenda-se o uso de fungicidas com diferentes modo de ação.

General Introduction

Brazil is one of the world's largest producers of grains, fibers and vegetables. Total grain production in the 2016-2017 season reached 238 million tonnes (CONAB 2017). The main grain crops serve different purposes such as staple food (common bean and rice), oil sources (soybean, corn, oilseed rape, and sunflower), or export commodities (corn and soybean). In the staple food group, common bean is an important crop in many Latin American countries (Schmutz et al. 2014). In Brazil, 3.4 million tonnes of common bean were produced in 2016-2017 (CONAB 2017). In the group of oilseed crops, soybean is the most commonly grown species in Brazil (114 million t., 47.9 %), while cottonseed, sunflower, and oilseed rape account for 0.97; 0.04; and 0.03 % of the total production of grains, respectively (CONAB 2017). In relation to the production of fibers, Brazil is the fifth largest producer of cotton: 1.5 million tonnes (USDA 2018). In addition to grains and fibers, vegetables are also important agricultural products in Brazil. Vegetables are cultivated in all regions in Brazil and generate thousands of direct and indirect jobs. Potato and tomato are the most commonly grown vegetables. In 2017 a total of 4.3 and 4.4 million tons of potatoes and tomatoes, respectively, were produced in Brazil (SIDRA IBGE 2018).

Fungal diseases can be major limiting factors to the production of grains, fibers and vegetables. White mold or sclerotinia stem rot is a destructive disease caused by the Ascomycete *Sclerotinia sclerotiorum* (Lib.) de Bary. Distinctive features make white mold a destructive disease: the pathogen is a necrotrophic fungus that can infect a wide range of crops, of worldwide distribution (Boland and Hall 1994); it produces cell-wall-degrading enzymes (Bolton et al. 2006); and the pathogen can form resistance structures (sclerotia) that can remain viable for many years in soil (Adams and Ayers 1979). Currently, white mold management relies on cultural practices such as less dense planting stands; crop rotation with non-host crops; controlled irrigation; but more reliably, on fungicide application, presently, the most efficient practice to manage white mold (Vieira et al. 2012; Meyer et al. 2014). In Brazil, the fungicides thiophanate-methyl (TM), fluazinam, and procymidone are commonly used to control white mold (Lehner et al. 2015). Although effective in many

situations, selection for resistant isolates and control failures can occur (Brent and Hollomon 2007), which in turn can negatively affect white mold management.

Integrating several control measures is an option to reduce the dependency on the use of fungicides and to preserve the efficacy of these compounds. The use of resistant varieties is the most suitable method to prevent crop losses due to white mold without relying too much on fungicide application. However, to date, there is no highly resistant cultivar to white mold that has good agronomic features and that is cultivated in the main production areas in Brazil. Nevertheless, there are breeding programs for different host plants aimed at developing resistant varieties. The success of these programs depends on proper knowledge of the evolutionary mechanisms that influence the dynamics of genetic variability of the pathogen population. Thus, it is advisable to assess the genetic variability of *S*. *sclerotiorum* from different hosts and geographic regions and attempt to parameterize the evolutionary mechanisms that shape the pathogen population. Unfortunately, no such study was conducted in Brazil with an adequate number of representative isolates and with a proper set of high-resolution analytical techniques. In addition to the knowledge gap related to evolutionary mechanisms of *S*. *sclerotiorum* in Brazil, and considering that chemical control is still the main management strategy available to reduce yield losses caused by white mold, it is also important to assess the sensitivity of the population to the most commonly used fungicides to control the disease. Considering these demands, the present study was designed to: quantify the genetic variability, determine how the variability is distributed, make inferences about evolutionary mechanisms that shape the population of *S*. *sclerotiorum* in Brazil; and to assess the sensitivity of isolates of *S. sclerotiorum* collected from different hosts and regions to TM, fluazinam, and procymidone.

Chapter 1: The population of *Sclerotinia sclerotiorum* from different hosts and regions in Brazil is structured by mycelial compatibility groups (MCGs).

Abstract

The genetic structure of the population of *Sclerotinia sclerotiorum* may be influenced by different factors such as host plants, regional, ecological and climate features, among others. The objective of this study was to assess the genetic variability of *S*. *sclerotiorum* isolates collected from several hosts and regions. Microsatellite (SSR) and mycelium compatibility groups (MCG) markers were used to analyze 238 isolates from Brazil. Twenty-two MCGs and 53 multilocus genotypes (MLG) were identified. Reference isolate Ss-1980 was incompatible with all other isolates. There was no association between MCG and hosts or regions. Most isolates (67.7 %) were assigned to only two MCGs. Eight new MCGs were found. At least 39 MCGs are present in Brazil. Seven genetic groups were found and they were associated with MCG. Linkage disequilibrium was detected in the total population and in the subpopulations defined by the MCGs 1 and 2. Most genetic variation (89.7 %) was due to differences between MCGs. Therefore, the population was structured by MCGs. Two large MCGs are widely distributed in the country and dominate the population of *S*. *sclerotiorum*. The results also support previous findings of a clonal structure of *S*. *sclerotiorum* in Brazil.

Introduction

White mold or sclerotinia stem rot, caused by the Ascomycete *Sclerotinia sclerotiorum* (Lib.) de Bary, is a highly destructive fungal disease of cultivated and non-cultivated plants worldwide. For cultivated plants, white mold represents one of the main biotic factors that reduces production of grains, fibers, and vegetables. The pathogen has a wide host range, approximately 400 plant species (Boland and Hall 1994), including important crops such as soybean, common bean, cotton, sunflower, oilseed rape, and many vegetables (Bolton et al. 2006). *S*. *sclerotiorum* can also infect many weed species such as *Bidens* sp., *Ranunculus* sp., *Sonchus* spp., *Euphorbia* spp. and others (Boland and Hall 1994). In the past, the pathogen was tentatively used as biological control agent in pastures (Noonan et al. 1996), however, its wide host range limited its use, due to the risks associated with the multiplication and dissemination of inoculum to commercially-grown crops (Boland and Hall 1994).

S. sclerotiorum is a necrotrophic pathogen and may cause significant yield losses under favorable conditions when proper disease management is not adopted. In Brazil, soybean losses due to white mold were estimated to be greater than 1 billion dollars per year if epidemics are not controlled (Lehner et al. 2016). In theory, the integration of several practices should contribute to reduce yield losses due to white mold, but in reality disease control has heavily relied on fungicide application (Vieira et al. 2012; Meyer et al. 2014).

Crop resistance is the best method to manage white mold, but the development of cultivars with durable resistance depends, among other factors, on the knowledge of the population genetics of *S*. *sclerotiorum*. Breeding programs for disease resistance must consider the information about the amount and distribution of the genetic variability in the population of *S*. *sclerotiorum*, or in other words, the genetic structure of the pathogen population (McDonald 2014).

The first genetic studies of the population of *S*. *sclerotiorum* were conducted using restriction fragment length polymorphism (RFLP) and mycelial compatibility groups (MCGs) as markers. Most studies reported the occurrence of clonal populations, even when isolates collected from different host crops were sampled (Kohn et al. 1991; Kohli et al. 1992; Cubeta et al. 1997;

Hambleton et al. 2002). In the past few years, most population genetics studies were conducted with microsatellites (SSR) and MCGs markers, and most reported high variability (Lehner and Mizubuti 2016).

Despite the high yield losses caused by white mold in the crops previously mentioned, few studies were carried out in Brazil to investigate the genetic structure of the population of *S*. *sclerotiorum* using isolates collected from different hosts and with wide geographical representation. The Brazilian population of *S*. *sclerotiorum* has been studied mostly using SSR markers and efforts were largely devoted to investigate isolates collected from common bean (Abreu and Souza 2015; Gomes et al. 2011; Lehner et al. 2015; Lehner 2015; Zancan et al. 2015). Gomes et al. (2011) reported high variability among 79 isolates collected in four dry bean fields in the Brazilian cerrado. Abreu and Souza (2015) also found high variability among 50 isolates from different regions in Minas Gerais state. Lehner et al. (2015) used SSR and MCGs as markers and concluded that the population of *S*. *sclerotiorum* in Minas Gerais state has a clonal genetic structure. More recently, an expanded study of the population of *S*. *sclerotiorum* from common bean was conducted and 300 isolates from different production regions throughout Brazil were analyzed. This study reports that the pathogen population is structured by MCGs (Lehner 2015). Interestingly, different results were obtained when 320 isolates of *S*. *sclerotiorum* from dry bean fields located in different regions of the United States and collected between 2003 and 2010 were examined. The USA population was structured by region, but there was no relationship between MLG and MCG (Kamvar et al. 2017). Sampling time affected population structure to some degree in the analysis of the USA population. It is possible that different haplotypes, with different MCGs, occurred in the population and this contributed to the lack of association between MLG and MCG. Additionally, another difference between the common bean study conducted in Brazil and in the USA is the way individuals were defined. Brazilian isolates were monosporic (monoascosporic), while those in the USA study came directly from mycelial discs from colonies formed after sclerotia germination on culture medium. It has been shown that there may be different haplotypes in a single sclerotium of *S*. *sclerotiorum* (Lehner et al. 2015). In order to improve the assessment of genetic variability in the population of *S*. *sclerotiorum* in Brazil, it would be desirable to

reduce the collection time span of the isolates and to analyze individuals from different hosts.

In Brazil, only one study was conducted with isolates obtained from different hosts (Litholdo Júnior et al. 2011). A set of 40 isolates collected from cabbage, carrot, oilseed rape, common bean, lentil, lettuce, pepper, radish, sunflower, and tomato crops were genotyped using random amplified polymorphic DNA (RAPD) markers. The MCG of the isolates was also determined. The authors found five MCGs and 65 % of the loci were polymorphic, and concluded there was high variability in the population. In the United States the genetic variability of the isolates collected from sunflower, dry bean, soybean, and oilseed rape was assessed using SSR and MCG markers. There was high genotypic diversity and weak evidence of structuring by host (Aldrich-Wolfe et al. 2015). In the United Kingdom the population structure of *S*. *sclerotiorum* was assessed with isolates collected from buttercup, celery, carrot, lettuce, oilseed rape, and pea. The UK population had a clonal genetic structure with one SSR-haplotype distributed in high frequency in the population (Clarkson et al. 2013).

The current scenario about the Brazilian population of *S*. *sclerotiorum* needs to be clarified. Specifically, it is necessary to include monosporic isolates from different hosts and controlled sampling time span. More robust analysis of the population of *S*. *sclerotiorum* in Brazil, using larger sample sizes collected from different hosts and high-resolution multiallelic marker such as SSR need to be conducted in order to generate a better picture of the genetic variability of the white mold pathogen. Thus, the objective of the present study was to quantify and evaluate the distribution of the genetic variation in the population of *S*. *sclerotiorum* using isolates collected from different hosts and regions.

Materials and Methods

Sampling. Sclerotia were collected from fields located in the following Brazilian states: Bahia (32 isolates), Espírito Santo (10 isolates), Goiás (49 isolates), Mato Grosso (one isolate), Mato Grosso do Sul (8 isolates), Minas Gerais (80 isolates), Paraná (39 isolates), Pernambuco (one isolate), Rio Grande do Sul (three isolates), São Paulo (six isolates), Santa Catarina (four isolates), and in

the Distrito Federal (five isolates). The sampled fields were divided in four sections. In each section sclerotia were collected from plants with white mold symptoms in three points. Collection points were spaced at least 10 m from one another. The number of sclerotia collected per field varied from one to 12, depending on the disease incidence and field size.

From the collection of sclerotia from a sampled field, one sclerotium was randomly selected and disinfested as previously described (Lehner et al. 2015) and transferred to 90 mm-diameter Petri dishes containing 20 mL potato dextrose agar (PDA). After 5 days, mycelial discs (0.5 cm - diameter) were transferred to Erlenmeyer flasks with sterilized carrot disks (approx. 0.5 cm thick and 1.5 cm diameter) and maintained at 23°C in the dark for 15 days for multiplication of sclerotia. Monosporic isolates were obtained and sclerotia were produced as previously described (Lehner et al. 2015).

Mycelial compatibility group (MCG). The mycelial compatibility of all isolates was determined as described elsewhere (Lehner et al. 2015). Mycelial discs (0.5 cm - diameter) from 2-day-old cultures were paired in Petri dishes (60 mm - diameter) containing 15 mL of PDA supplemented with 75 µL L^{-1} of McCormick's red food coloring (Schafer and Kohn 2006) and kept at 23 °C in the dark. The compatibility tests were first performed in subgroups of 20 isolates. The isolates from these subgroups were paired with each other in all possible combinations. After defining their MCGs, one isolate representing the MCGs detected in each subgroup was randomly selected to be paired against each other. When the MCGs became more well defined some isolates were randomly selected to be paired with new, unknown, isolates introduced in the population. Four pairings were performed per Petri dish. Each pairing was performed twice. Isolate Ss-1980 was used as external tester (Amselem et al. 2011). The compatibility reaction was assessed after 3 and 6 days of incubation.

Finally, some representative isolates of the MCGs detected in this study were paired with representative isolates (testers) of MCGs (Supplementary Table 1) previously identified in the *S*. *sclerotiorum* population from common bean fields in Brazil (Lehner 2015). This was done so that it would be possible to keep a consistent identification system of the MCGs in Brazil and also to start a collection of standard testers.

DNA extraction. The isolates were cultivated in liquid medium in Erlenmeyer flasks at 23°C in the dark to allow for mycelium growth (Lehner et al. 2015). After 5 days, the mycelium was carefully washed with distilled water, transferred to filter paper to dry for 24 h, and macerated in TissueLyser II (QIAGEN, Germany). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. DNA integrity was analyzed by electrophoresis on a 1% agarose gel and its concentration was estimated by comparison to a 100 bp DNA ladder and adjusted to 25 ng μL^{-1}.

Microsatellite genotyping. The genetic variability of the *S. sclerotiorum* population was quantified using 13 SSR loci (Sirjusingh and Kohn 2001). The forward primers were labeled with the fluorescent dyes, and divided in three multiplex (M1 to M3) to conduct PCR reactions: M1: 55-4 (NED), 13-2 (6-FAM), 110-4 (PET), 36-4 (6-FAM), and 42-4 (VIC); M2: 8-3 (NED), 5-2 (6-FAM), 17-3 (VIC), and 114-4 (PET); and M3: 7-2 (VIC), 12-2 (PET), 9-2 (NED), and 92-4 (6-FAM) (Lehner et al. 2017). Multiplex PCR reactions were performed with the Multiplex PCR 5X Master Mix kit as described by the manufacturer (New England Biolabs, Inc.). Amplifications were confirmed by electrophoresis in 2 % agarose gel. Fragments were compared with a 100 bp DNA ladder. DNA of Ss-1980 isolate was used as positive control (Amselem et al. 2011). Ten isolates were randomly selected to check for consistency of the SSR assay (true biological replicates). Independent DNA extraction was conducted from colonies originated from different sclerotia of the 10 randomly chosen isolates. Genotyping reactions to confirm reproducibility were carried out for the 13 loci previously used when genotyping the population. The PCR products were lyophilized and fragment analysis was done in Macrogen Inc. (Seoul, South Korea) using the GeneScan-500 LIZ size standard (Applied Biosystems) in ABI 3730XLs. The fragment sizes were assigned using GeneMarker v.2.7.0 (SoftGenetics).

Data analyses. The analyses were conducted in R version 3.4.1. using different packages (R Core Team, 2017). The number of observed alleles was obtained per locus. Nei's gene diversity estimate (1978) and allelic evenness (E_5) per locus was assessed with the package 'poppr' (Kamvar et al. 2014). Multilocus genotypes (MLG) were obtained for the whole population after removing

monomorphic loci. The clonal fraction of the population was calculated as 1 - [(number of different genotypes)/(total number of isolates)] (Zhan et al. 2003). The occurrence of random mating in the population and within-MCGs (MCG 1 and 2) was tested with linkage disequilibrium, estimated with the I_a and r_D indices, using 999 permutations (Agapow and Burt 2001). Minimum spanning network was applied to identify the relationship between MLGs using the Bruvo's distance genetic in the package 'poppr' (Kamvar et al. 2014).

P_{sex} was calculated with package 'Rclone' (Bailleul et al. 2016) to assessed the likelihood of the occurrence of the same MLG by different sexual events (Arnaud-Haond et al. 2007).

Population structure. First, the function *pca.dudi* of the 'adegenet' package (Jombart 2008) was used to obtain the principal components (PCAs) that describe the whole population. Principal component analysis was conducted to examine the existence of clusters before defining population structure. Discriminant analysis of principal components (DAPC) was applied to identify the genetic structure of the whole population (Jombart et al. 2010). The optimal number of PCs recommended for the DAPC should be the lowest root mean squared error (RMSE) that was identified during the cross-validation analysis (function *xvalDapc*). The function *find.cluster* was used to find the number of clusters (K) based on the Bayesian Information Criterion (BIC) (Jombart et al. 2010).

Distribution of the variation. Analysis of molecular variance (AMOVA) to infer about the distribution of variation was conducted with SSR haplotypes among and within MCGs using the package 'poppr' (Kamvar et al. 2014). AMOVA was carried out only for MCGs composed by at least five isolates.

Results

Isolates. A total of 238 monoascosporic isolates were obtained from samples collected between 2014 and 2017 from different hosts: soybean (77 isolates), common bean (49 isolates), cotton (28 isolates), sunflower (17 isolates), oilseed rape (eight isolates), tomato (22 isolates), lettuce (eight isolates), pea (nine isolates), vegetable crops (nine isolates), weeds (five isolates) and other hosts (six isolates) with white mold symptoms.

Mycelial compatibility group (MCG). Twenty two MCGs were identified among the isolates analyzed (Figure 1 and Supplementary Table 1). The reference isolate Ss-1980 was incompatible with all Brazilian isolates. Thus, Ss-1980 was assigned as belonging to MCG-0. Most isolates (67.7 %) were distributed in either MCG 1 (92 isolates; 38.7 %) or MCG 2 (69 isolates; 29.0 %). Both MCGs were composed by isolates collected from different hosts and regions in Brazil. Eleven MCGs were composed by a single isolate (Figure 1). Eight new MCGs were identified in the current study and named: MCG 32, 33, 34, 35, 36, 37, 38, and 39 (Supplementary Table 2).

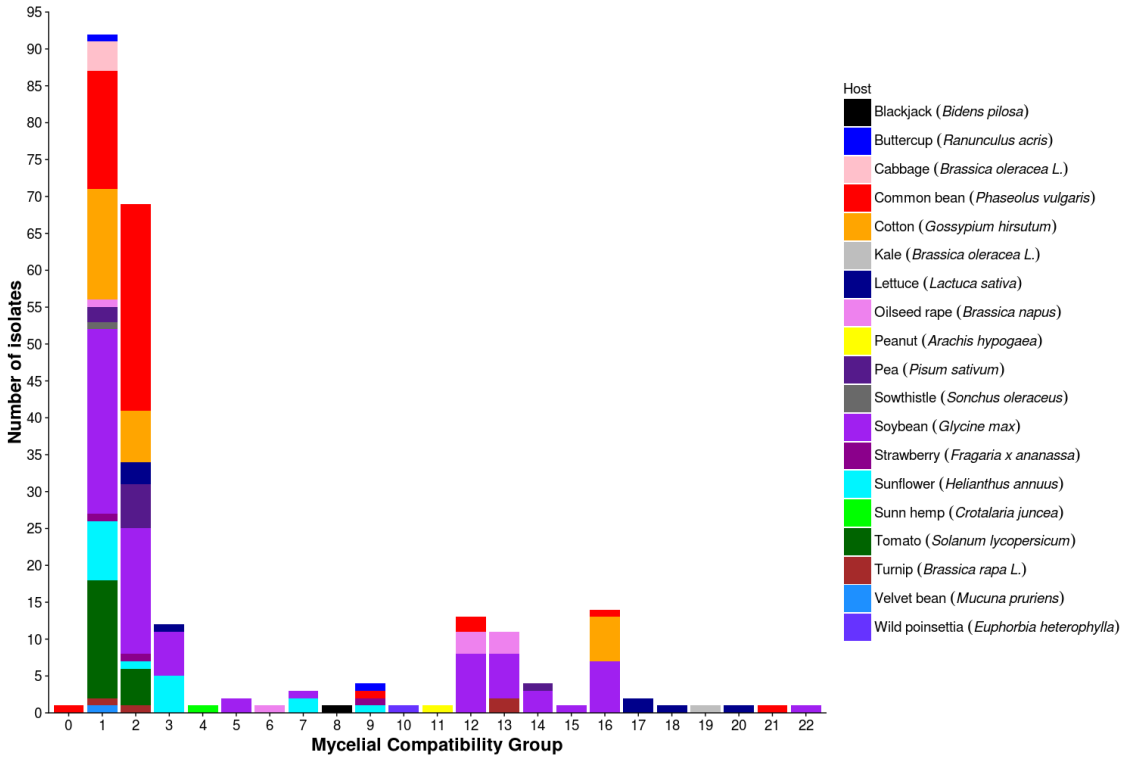

Figure 1. Frequency of 22 mycelial compatibility groups (MCGs) of *Sclerotinia sclerotiorum* sampled from different hosts in Brazil. Isolate Ss-1980 was assigned as belonging to MCG-0.

Genetic variability in the population. The haplotypes of the biological replicates selected to check for reproducibility were identical to the previously recorded ones. The allele sizes of Ss-1980 were confirmed. Eleven microsatellite loci were polymorphic. Two loci (36-4 and 42-4) were monomorphic. The number of alleles varied from 3 (5-2 and 110-4) to 15 (114-4). Nei's gene diversity and evenness varied from 0.17 to 0.68 (mean of 0.44)

and 0.44 to 0.96 (mean of 0.66), respectively (Table 3). Linkage disequilibrium at all loci was detected in the total population.

Table 3. General descriptors of the microsatellite loci used to characterize the population of *Sclerotinia sclerotiorum* from different hosts

Locus[a]	Motifs	Number of alleles	Size range (bp)	Gene diversity[b]	Evenness
55-4	(TACA)10	5	157-189	0.51	0.70
13-2	(GTGGT)6	7	275-325	0.50	0.50
110-4	(TATG)9	3	361-373	0.50	0.96
36-4	(CA)6(CGCA)2(CAT)2	1	411	-	-
42-4	(GA)9	1	406	-	-
8-3	(CA)12	8	244-268	0.55	0.53
5-2	(GT)8	3	315-319	0.17	0.47
17-3	(TTA)9	9	335-374	0.63	0.71
114-4	(AGAT)14(AAGC)4	15	338-440	0.64	0.44
7-2	(GA)14	6	148-174	0.68	0.80
12-2	(CA)9	5	216-224	0.65	0.88
9-2	(CA)9(CT)9	5	358-370	0.31	0.52
92-4	(CT)12	4	372-378	0.59	0.80

[a] Microsatellite loci published by Sirjusingh & Kohn (2001).

[b] Nei's 1978 gene diversity.

Fifty-three MLGs were identified among the isolates, resulting in a clonal fraction of 0.78. Based on the low probabilities inferred in the Psex analysis, there was no evidence that MLGs could have resulted from different reproductive events ($P_{sex} < 0.05$). The most frequent MLGs were 34 and 13. The MLG 34 was composed by 77 isolates that were of MCG 1, and one individual each of MCG 6 and 12. The MLG 13 was composed by 42 isolates of MCG 2. Both MLGs were represented by isolates collected from different hosts and regions.

The MCGs represented by more than 10 isolates, MCG 1, 2, 3, 12, 13, and 16, were composed by 10, 13, 3, 4, 3, and 4 MLGs, respectively. The MCGs 9 and 14 both represented by four isolates, were composed by two and one MLG, respectively. MCGs 5 and 17 both represented by two isolates, were composed by two and one MLGs, respectively. MCG 7 represented by three isolates, was composed by two MLGs. The MCGs 4, 8, 10, 11, 15, 18, 19, 20, and 21 were represented by one isolate each, each with a distinct MLG. As

stated above, two MLGs (MLG 34 and 46) were associated with more than one MCG (Figure 2). The seven isolates of the MLG 46 were classified as MCG 13 (six isolates) and MCG 22 (one isolate).

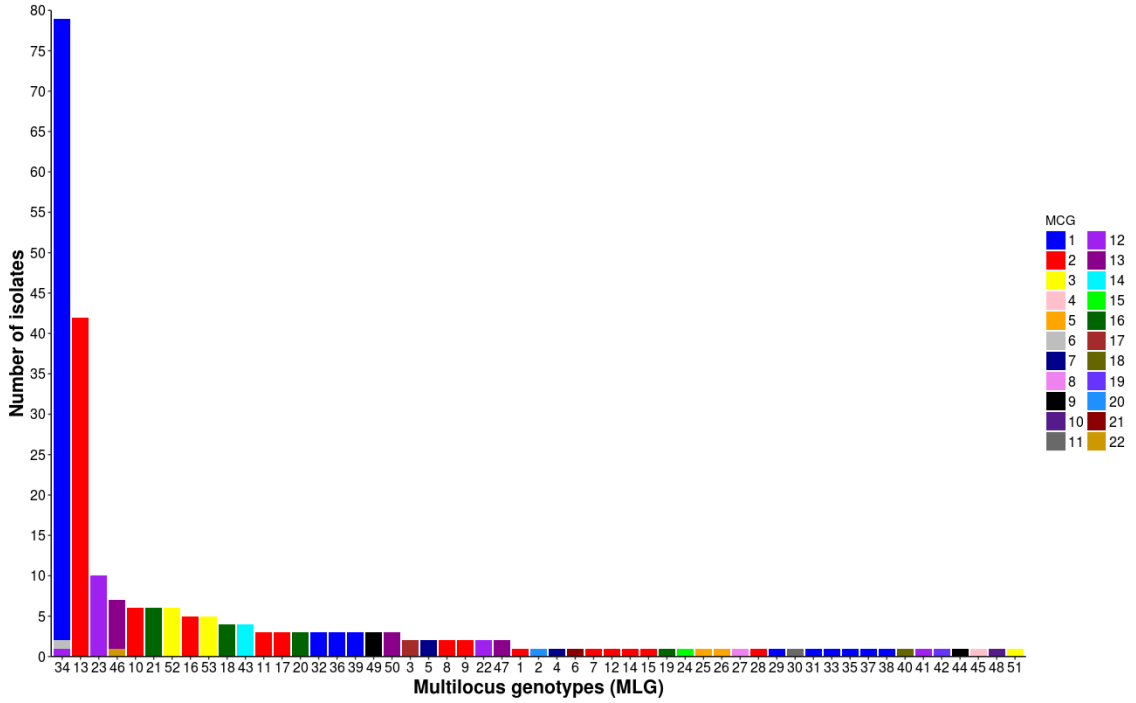

Figure 2. Frequency of 53 multilocus genotypes (MLGs) detected in the population of *Sclerotinia sclerotiorum* from different hosts and regions in Brazil.

Population structure. Based on the lowest RMSE the number of principal components (PCs) suitable for the DAPC analyses was five. The most likely number of clusters was k = 7. Each clusters was composed of isolates from different hosts and regions in Brazil. Group 2 (purple), composed by 69 isolates (MCG 2) from different hosts and regions, also has a high genotypic diversity and 13 distinct MLGs were found. Group 3 (green) was composed by 13 isolates of MCG 12, 12 from southern Brazil (Rio Grande do Sul and Paraná state) and one from Minas Gerais state. Group 6 (blue) was composed of 14 isolates of MCG 16 that form black colonies, they were compatible with black isolates of the population from common bean (Lehner 2015). Interestingly, one isolate of group 5 (MCG 21) that form black colony, was closer in the minimum spanning network to the isolates of group 6. Group 1 (black) was composed by 11 isolates of MCG 13 and one isolate of MCG 22. The isolate of MCG 22 was sampled in Teixeira Soares, Paraná state, with two more isolates of MCG 13. Group 5 (red) was composed by 12 isolates of MCG 3, three isolates of MCG 7,

and one isolate of MCG 21. Group 4 (orange) was more diverse composed by seven MCGs: MCG 4, 8, 10, 15, 18, 19, 20; each represented by one isolate. Additionally, group 4 also has two isolates of MCG 5, four isolates of MCG 9, four isolates of MCG 14, and two isolates of MCG 17. Group 7 (yellow) was composed by 92 isolates of MCG 1, and one each of MCG 6, MCG 11, and MCG 12 (Figure 4). The isolate of MCG 12 was obtained from tomato in Corumbá, Goiás state, with four more isolates of MCG 1. The isolate of MCG 6 and 11 were collected in oilseed rape and peanut, respectively.

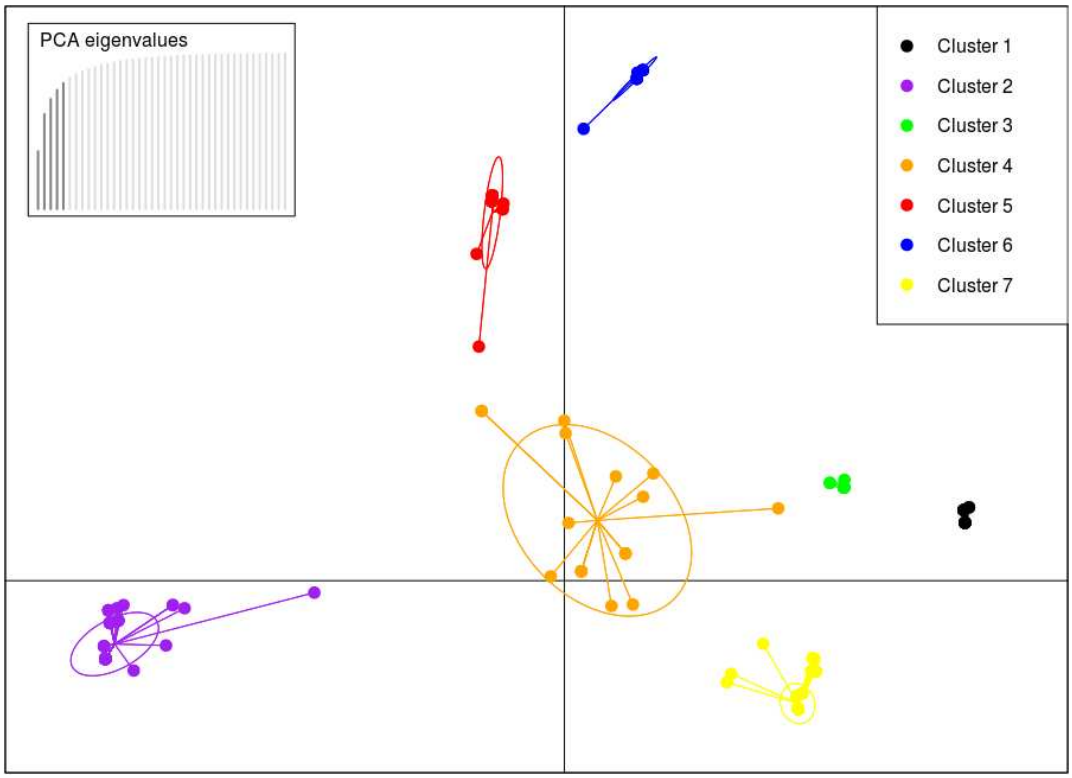

Figure 4. Scatterplot of the discriminant analysis of principal components (DAPC) of 238 isolates of *Sclerotinia sclerotiorum* that were genotyped using 13 microsatellite loci.

Genetic Variability in the subpopulation. Linkage disequilibrium at all loci was detected in the subpopulations defined by the MCGs 1 (92 isolates) and 2 (69 isolates). Both indices I_A and r_D were significantly different from zero ($P < 0.05$). The AMOVA revealed that 89.7 % of the variance could be attributed to differences between MCGs. Only 10.3 % was due to variation within MCGs (Supplementary Table 3).

The Bruvo's genetic distance in the minimum spanning network varied from 0.038 to 0.335 (Figure 5). Isolates of the genetic groups identified in the DAPC analysis were placed close to one another. The group 7 (yellow) was closely related to group 1 (Black), with a Bruvo's distance of 0.057.

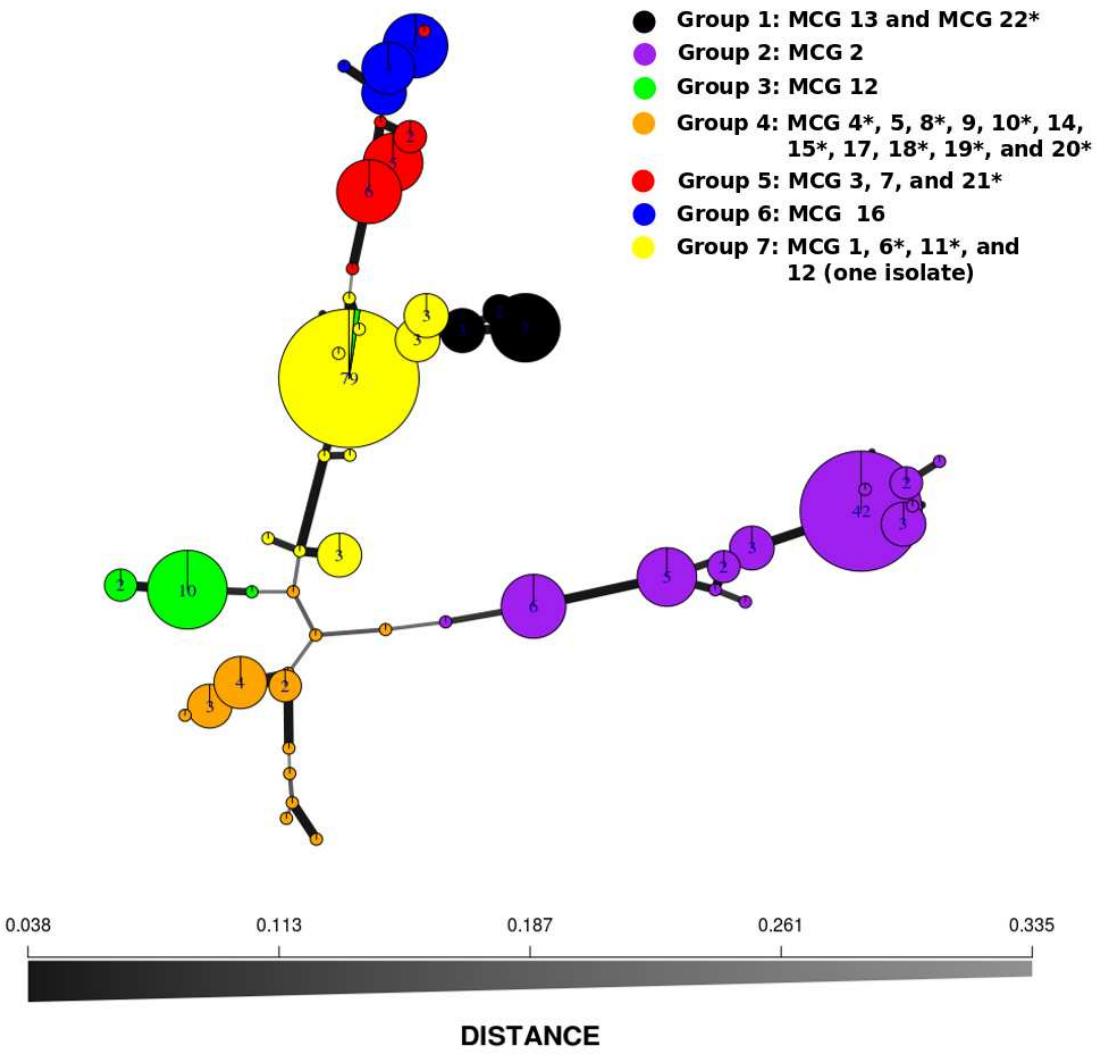

Figure 5. Minimum spanning network of the relationships between the multilocus genotypes (MLG) of 238 isolates of *Sclerotinia sclerotiorum*. Node size corresponds to the number of MLG and were colored according to the genetic groups identified in the DAPC analysis. (*) MCGs composed by a single isolate.

The relationship between genetic groups defined by DAPC, the MLGs and the MCGs revealed that groups are mainly determined by MCGs (Figure 6). The bigger groups are defined by MCGs that are connected to variants that

differ by small Bruvo's distance values. As stated earlier, MLGs 34 and 46 appear connected to different MCGs, but those were the only exceptions.

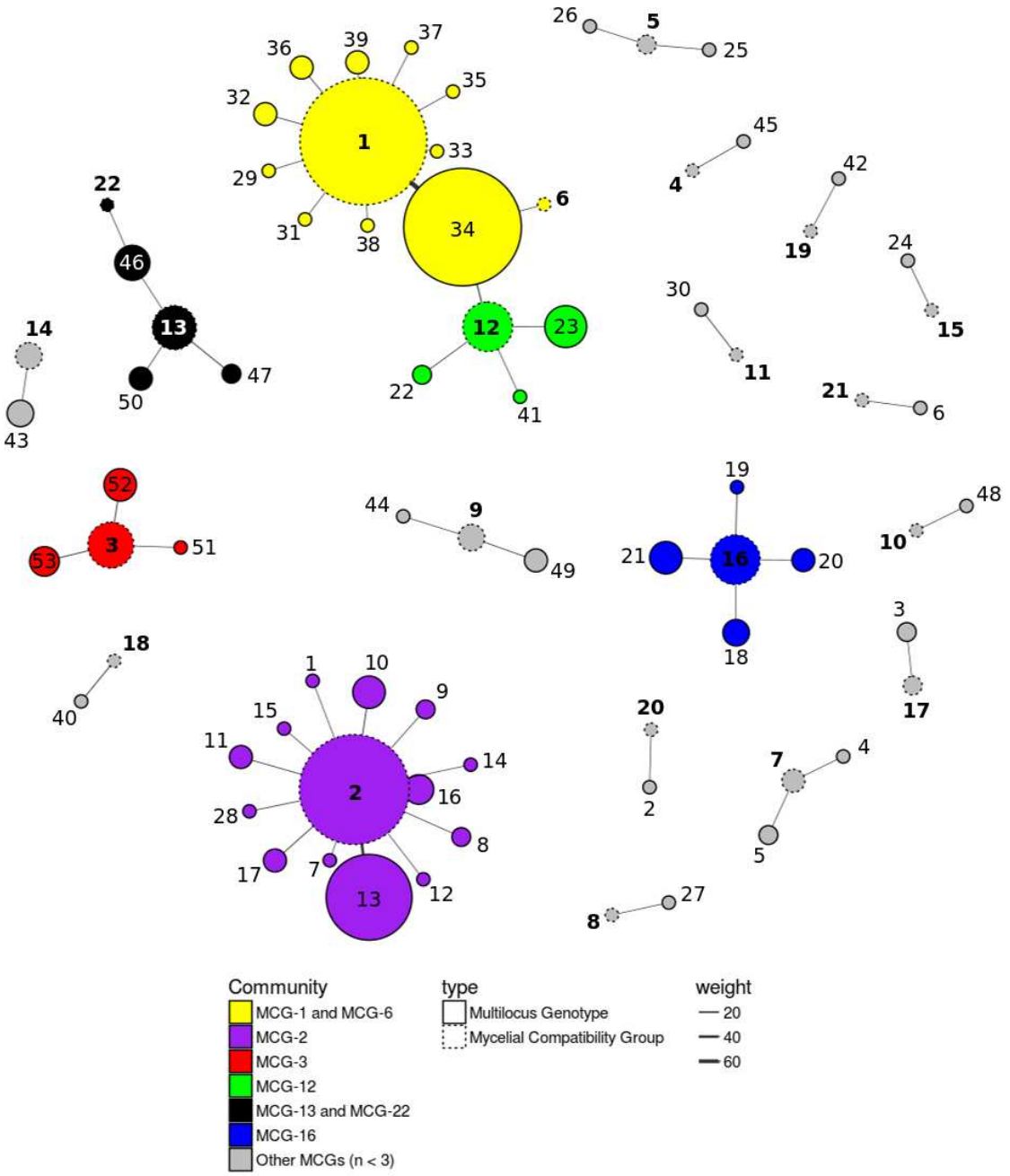

Figure 6. Relationships between the multilocus genotypes (MLGs) and mycelial compatibility groups (MCGs) of *Sclerotinia sclerotiorum* collected from different hosts and regions.

Discussion

This is the first study conducted using monosporic isolates of *S. sclerotiorum* collected from different hosts and regions in the Brazil genotyped with SSR combined with MCG phenotyping. Analyses of the population were conducted to generate a better knowledge of the genetic variability of the pathogen. There was evidence for a clonal genetic structure. Two large MCGs are widely distributed in the country and dominate the population. Linkage disequilibrium at all loci was detected in the total population, and in the subpopulations defined by the MCGs 1 and 2. There was association between the SSR and MCG markers.

The genetic structure of the pathogen population reported here is similar to what was found in previous studies that analyzed isolates of *S. sclerotiorum* collected from common bean fields (Lehner et al. 2015; Lehner 2015). Furthermore, the two most frequently reported MCGs (MCG 1 and 2) were also the two most commonly found in the population of *S. sclerotiorum* from common bean in Brazil. Linkage disequilibrium occurred at all loci when the total population from different hosts and regions was analyzed. Similar results were reported for the population from common bean. However, contrary to what was previously reported (Lehner 2015) there was no evidence of random mating within MCGs 1 or 2.

In Brazil, only one study was conducted with isolates (40) obtained from different hosts. Isolates were genotyped using RAPD and MCG markers (Litholdo Júnior et al. 2011). There was an association between MCG and the genetic distance based on RAPD marker. However, there was no relationship with hosts or geographic origin of the isolates. Additionally, high genetic variability was reported and authors claimed that recombination can account for such variation. Nevertheless, no formal analysis was conducted to infer random mating. A rather distinct scenario is reported in the current study. The population has a relatively high clonal fraction (0.78) and there was no evidence of random mating. Furthermore, an additional characteristic that supports clonal structure is the association between MLGs and MCGs.

The population of *S. sclerotiorum* in this study has a clonal genetic structure and low genotypic diversity. Among 238 isolates assessed only 22

MCGs and 53 MLG were found. MCGs composed by more than one isolate had different MLGs. Only two MLG (MLG 34 and 46) were associated with more than one MCG. There was strong evidence of association between MCG and MLG. A study conducted in the USA with 145 isolates from different hosts reported high genotypic diversity and weak evidence of structuring by host. They found an association between 49 MCGs and 53 haplotypes that were identified in the population, but the structuring according to MCG was not investigated (Aldrich-Wolfe et al. 2015). The population of *S*. *sclerotiorum* in Brazil is well established in the fields with the predominance of clones largely influenced by MCGs. It can be speculated that the presence of two major MCGs acting as a clonal lineage may be due to recent introduction of the pathogen in Brazil, compared to more variable and diversified populations reported in other countries.

The genetic structure of the population of *S*. *sclerotiorum* in Brazil is determined in most part by the MCGs. Each MCG can be defined as a lineage, as has been suggested before (Lehner et al. 2015, Lehner 2015). Therefore, to better understand the genetic variability of *S*. *sclerotiorum*, future works shall use a collection of standard testers made available in this study to identify the MCGs in the population (Supplementary Table 1). To date, at least 39 MCGs are present in the population of *S*. *sclerotiorum* in Brazil.

Breeding programs shall use different isolates, from different MCGs, mainly of the MCG 1 and 2, when screening cultivars for durable resistance to *S*. *sclerotiorum*. However, at this point it is not clear whether it makes sense to orient any efforts towards the development of resistance to MCGs. To date, there is no evidence for differences in virulence or other epidemiological traits that supports separate management strategies.

Supporting Information

Supplementary Table 1. Mycelial compatibility group (MCG), isolate code (UFVSs) and host of 238 *Sclerotinia sclerotiorum* isolates

MCG	Isolate code number (UFVSs) – Host code
1	506 (Bn)*; 401, 413, 414, 491 (Bo); 646 (Br); 460 (Fa); 437, 504, 505, 613, 618, 619, 620, 621, 625, 630, 645, 650, 651, 652, 653 (Gh); 441, 444, 447, 467, 474, 475, 477, 502, 507, 512, 514, 515, 516, 532, 533, 537, 541, 565, 568, 603, 607, 608, 638, 642, 657 (Gm); 404, 405, 409, 410, 411, 637, 643, 661 (Ha); 494 (Mp); 407, 436 (Ps); 420, 479, 480, 486, 487, 489, 490, 492, 493, 509, 517, 522, 529, 597, 605, 635 (Pv); 403 (Ra); 402, 416, 418, 424, 440, 458, 573, 575, 579, 580, 581, 587, 588, 595, 599, 636 (Sl); 412 (So)
2	550 (Br); 445 (Fa); 612, 622, 623, 627, 631, 654, 655 (Gh); 428, 457, 464, 465, 466, 468, 476, 513, 523, 528, 530, 531, 557, 559, 578, 614, 626 (Gm); 470 (Ha); 469, 592, 593 (Ls); 408, 421, 422, 426, 427, 430 (Ps); 443, 448, 449, 450, 451, 452, 453, 454, 463, 478, 481, 482, 483, 484, 485, 495, 496, 497, 498, 508, 510, 511, 518, 519, 520, 524, 659, 660 (Pv); 417, 419, 435, 459, 586 (Sl)
3	399, 406, 423, 425, 429 (Ha); 606 (Ls); 534, 535, 536, 539, 591, 572 (Gm)
4	461 (Cj)
5	438, 442 (Gm)
6	434 (Bn)
7	446, 471 (Ha); 577 (Gm)
8	415 (Bp)
9	647 (Fa); 649 (Ha); 526 (Pv); 400 (Ra)
10	439 (He)
11	472 (Ah)
12	432, 433, 488 (Bn); 549, 556, 558, 562, 564, 567, 600, 644 (Gm); 574 (Sl)
13	431, 455, 456 (Bn); 542, 658 (Br); 499, 500, 601, 633, 639, 641 (Gm);
14	473, 640, 648 (Gm); 462 (Ps)
15	609 (Gm)
16	527, 624, 628, 629, 632, 656 (Gh); 538, 540, 576, 611, 615, 616, 617 (Gm); 521 (Pv)
17	598, 604 (Ls)
18	589 (Ls)
19	590 (Boa)
20	594 (Ls)
21	610 (Pv)
22	501 (Gm)

*Ah = *Arachis hypogaea*; Bp = *Bidens pilosa*; Bn = *Brassica napus*; Bo = *Brassica oleracea* L. var. *capitata*; Boa = *Brassica oleracea* L. var. *acephala*; Br = *Brassica rapa* L. var. *rapa*; Cj = *Crotalaria juncea*; Eh = *Euphorbia heterophylla*; Fa = *Fragaria x ananassa*; Gh = *Gossypium*

hirsutum; Gm = *Glycine max*; Ha = *Helianthus annuus*; Ls = *Lactuca sativa*; Mp = *Mucuna pruriens*; Ps = *Pisum sativum*; Pv = *Phaseolus vulgaris*; Ra = *Ranunculus acris*; Sl = *Solanum lycopersicum*; So = *Sonchus oleraceus.*

Supplementary Table 2. Isolates representing MCGs in the population of *Sclerotinia sclerotiorum* from common bean fields in Brazil (Lehner 2015)

MCG	Isolate code number (UFVSs)
1	1; 52; 395
2	5; 292
3	85
4	51
5	90
6	15
7	360
8	18
9	123
10	91
11	102
12	4
13	59
14	69
15	176
16	377
17	195
18	167
19	190
20	317
21	138
22	240
23	200
24	173
25	321
26	320
27	390
28	203
29	216
30	312
31	332
32*	438*

33*	434*
34*	472*
35*	589*
36*	590*
37*	604*
38*	610*
39*	501*

*Testers for the eight MCGs newly found (in bold).

Supplementary Table 3. Analysis of molecular variance (AMOVA) for six mycelial compatibility groups (MCGs) of isolates of *Sclerotinia sclerotiorum* from different hosts in Brazil

Source of variation	df	Sum of squares	Variation (%)
Between MCGs	5	482.64	89.73
Within MCGs	206	77.35	10.27
Total	211		

Chapter 2: Fungicide sensitivity of *Sclerotinia sclerotiorum* from different crops and regions and phenotypic instability of thiophanate-methyl resistant isolates

Abstract

Fungicide application is the main method used to control white mold epidemics in several crops. The objective of this study was to assess the sensitivity of *Sclerotinia sclerotiorum* to thiophanate-methyl (TM), fluazinam and procymidone; and to assess the phenotypic stability of resistant isolates. Fungicide sensitivity of 238 isolates collected from different hosts and regions was assessed using discriminatory doses for TM (5 µg/ml), fluazinam (0.05 µg/ml), and procymidone (0.5 µg/ml). The phenotypic stability was assessed during and after 10 transfers in culture medium without fungicide. Resistance was assessed after 10 generations. There was no evidence of resistance to fluazinam or procymidone. The mycelial growth of the sensitive isolates varied from 13% to 44%; from 16% to 36%; and from 15% to 41%, compared to the control for TM, fluazinam and procymidone, respectively. Thirteen isolates from common bean fields were resistant to TM. Mycelial growth rate of six (UVFSs-007, 450, 451, 452, 453, and 454) TM-resistant isolates reduced with successive transfers. However, there was no loss of resistance. Resistance to TM seems to be localized; and there is evidence for a fitness cost in resistant isolates. Management of resistant populations must employ fungicides with different modes of actions.

Introduction

White mold or sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is a highly destructive fungal diseases of cultivated and non-cultivated plants. Under favorable environmental conditions, mild temperatures (18 to 24 °C) and high humidity, the pathogen may infect all aerial plant tissues of hundreds of host plants, including important crops such as soybean, common bean, cotton, sunflower, oilseed rape, and vegetables (Boland and Hall 1994; Bolton et al. 2006).

One strategy to reduce yield losses caused by white mold is integrating several control measures that include cultural practices such as reducing planting density and controlled irrigation, as well as biological and chemical control. However, the success of white mold management has almost exclusively relied on fungicide application (Vieira et al. 2012; Meyer et al. 2014). In Brazil, the fungicides thiophanate-methyl (TM), fluazinam, and procymidone are commonly used to control white mold (Lehner et al. 2015). Although effective in many situations, selection for resistant individuals and control failures can occur. These failures are more often associated with inadequate usage of site-specific fungicides (Brent and Hollomon 2007).

Procymidone (site-specific dicarboximide fungicide) and fluazinam (multisite phenylpyridinamine fungicide) were the most efficient products in the National Tests of Fungicide Efficiency for management of white mold in soybean (Meyer et al. 2014). Recently, a collection of 282 isolates of *S*. *sclerotiorum* obtained from common bean was assessed regarding sensitivity to TM, procymidone, and fluazinam (Lehner et al. 2015). There was no evidence of resistant isolates of *S*. *sclerotiorum* to procymidone and fluazinam, but one resistant isolate to TM, collected in dry bean fields in the municipality of Cabeceira Grande, Minas Gerais state, was reported for the fist time in Brazil. The isolate had a L240F (leucine to phenylalanine at codon 240) mutation in the β-tubulin gene. This mutation is different from others previously reported to be associated with reduced sensitivity to TM, i.e. E198A and F200Y, glutamine to alanine at codon 198 and phenylalanine to tyrosine at codon 200, respectively (Yang et al. 2004). Field isolates resistant to carbendazim, a benzimidazole fungicide similar to TM, were reported in China (Zhu et al. 2016). The absence

of reports of resistance of *S*. *sclerotiorum* to fluazinam, after 20 years in the market, is likely due to its multisite mode of action. On the other hand, field isolates of *S*. *sclerotiorum* resistant to dimethachlone, a dicarboximide fungicide, showed cross-resistance to iprodione and procymidone, two other fungicides of the same group (dicarboximides) (Zhou et al. 2014; Firoz et al. 2015).

Fungicide-resistant isolates can lack phenotypic stability and hence reduced fitness. Stability of resistance of the pathogen can be influenced by different factors such as the intrinsic biological and genetic aspects of the species, chemical features of the fungicide and storage method (Li et al. 2015). Resistance to benzimidazole fungicides can be maintained for many years even without being exposed to the active ingredient, however, persistence is dependent on the initial amount of resistant isolates (Ishii and William 2015).

Resistance to fungicides can be associated with fitness cost, which in some cases are reflected in phenotypic instability, i.e. the incapacity of resistant isolates to persist insensitive to the target fungicide after successive generations (Brent and Hollomon 2007) or the changes in other traits that can reduce the persistence of resistant isolates in the field. Phenotypic instability has been reported in fungicide-resistant isolates of *Monilinia fructicola*, another species of the Sclerotiniaceae family (Cox et al. 2007). Isolates resistant to propiconazole were subjected to consecutive transfers in potato dextrose agar (PDA) and there was an increase in sensitivity to the fungicide, a reversal effect. Spore production and germination were also reduced after transfers when compared to sensitive isolates (Cox et al. 2007). The mycelial growth of a dimethachlone-resistant field isolate was slower than that of a sensitive isolate on PDA (Firoz et al. 2015). Laboratory-induced resistant isolates of *S*. *sclerotiorum* to dimethachlone had their insensitivity reduced after four years in cold storage and also after 20 successive mycelial transfers on PDA (Li et al. 2015). However, boscalid-resistant laboratory mutants of *S*. *sclerotiorum* were stable after eight consecutive transfers in unamended PDA (Wang et al. 2014).

For *S*. *sclerotiorum*, phenotypic stability can vary and a careful assessment of this phenomenon should be investigated to predict the consequences for white mold management. The objectives of this study were to assess the sensitivity of isolates of *S. sclerotiorum* collected from different hosts

and regions to thiophanate-methyl (TM), fluazinam and procymidone; and to assess the phenotypic stability of the resistant isolates.

Material and Methods

Isolates of *Sclerotinia sclerotiorum*. A total of 238 isolates were evaluated in this study. Isolates were obtained from sclerotia collected between 2014 and 2017 from different infected hosts: common bean (*Phaseolus vulgaris*), oilseed rape (*Brassica napus*), peanut (*Arachis hypogaea*), soybean (*Glycine max*), sunflower (*Helianthus annuus*), sunn hemp (*Crotalaria juncea*), velvet bean (*Mucuna pruriens*); cotton (*Gossypium hirsutum*); cabbage (*Brassica oleracea* L. var. capitata), kale (*Brassica oleracea* L. var. acephala), lettuce (*Lactuca sativa*), pea (*Pisum sativum*), tomato (*Solanum lycopersicum*), turnip (*Brassica rapa L.* var. *rapa*); blackjack (*Bidens pilosa*), buttercup (*Ranunculus acris*), sowthistle (*Sonchus oleraceus*), wild poinsettia (*Euphorbia heterophylla*); and strawberry (*Fragaria* x *ananassa*); and from different states in Brazil: Bahia, Espírito Santo, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Pernambuco, Rio Grande do Sul, São Paulo, Santa Catarina, and in the Distrito Federal (Figure 1). There are important production areas of grains, fibers and vegetables in these states and intensive chemical control programs are routinely adopted to manage white mold.

Only monosporic (monoascosporic) isolates, obtained as previously described (Lehner et al. 2015), were used in the experiments. Isolates were maintained as sclerotia formed in colonies developed from a single ascospore germination. Sclerotia were placed in paper envelopes stored in a refrigerator (8 °C) and retrieved whenever required.

Figure 1. Origin of the isolates of *Sclerotinia sclerotiorum* collected between 2014 and 2017 from different hosts and regions in the Brazil.

Fungicide sensitivity assay with discriminatory doses. The sensitivity of the 238 isolates from Brazil and reference isolate 1980 (Ss-1980) (Amselem et al. 2011) was assessed using discriminatory doses to TM, fluazinam, and procymidone as previously described (Lehner et al. 2015). Potato dextrose agar medium amended with fungicide at the discriminatory doses was prepared after serial dilutions of fungicide stock solutions (100 mg a.i./ml) made from commercial products: Cercobin 700 WP (70% TM), Frowncide 500 SC (50% fluazinam), and Sumilex 500 WP (50% procymidone). Fungicides were dissolved in 0.1 % dimethyl sulfoxide (DMSO). Mycelial discs (5 mm - diameter) of the edge of 2-day-old colonies were taken with a cork borer and transferred to the center of 60 mm - diameter Petri dishes containing 10 mL of PDA supplemented with fungicides at 5; 0.05; or 0.5 µg/mL, the discriminatory doses of TM, fluazinan and procymidone, respectively. Control plates comprised of PDA with DMSO at 0.1 %. Plates were maintained at 23°C in the dark. After 24

h of incubation, the colony diameter of the each isolate was measured with a digital caliper. Isolates UFVSs-007 and UFVSs-360, resistant and sensitive to TM (Lehner et al. 2015), respectively, were used as internal controls in all runs of the experiment. The sensitivity of each isolate was assessed twice, in independent experiments. In each experiment, set in a completely randomized design, four experimental units (one experimental unit = one Petri dish with mycelial disc) with and without fungicides were used. Resistant isolates were considered as those that were able to develop a colony on fungicide amended medium with a diameter at least 50% of the diameter on unamended PDA (control). Sensitive isolates were considered as those that the colony diameter in fungicide amended plates was less than 50% of the diameter recorded in the control.

Stability of TM resistant isolates. The phenotypic stability of eight TM-resistant isolates UFVSs-443, 448, 449, 450, 451, 452, 453, and 454, collected in 2015 in common bean fields located in the municipality of Cabeceira Grande, in Minas Gerais state, was analyzed by periodic transfers. Colony diameter was assessed after each transfer. The growth rate was assessed in 10 successive mycelial transfers (“generations 1 to 10”) on PDA medium without fungicide. In addition to the eight TM-resistant isolates, UFVSs-007 and UFVSs-360 isolates, resistant and sensitive to TM (Lehner et al. 2015), respectively, were used as controls. To measure the growth rate, one sclerotium was randomly selected and disinfested by immersion in ethanol 70% for 1 min followed by immersion in sodium hypochlorite (1%) for 3 min, rinsed in sterilized water and transferred to Petri dishes (90-mm-diameter) containing 15 mL PDA. The first generation (G01) was represented by a single colony originated from this sclerotium. Plates were maintained at 23°C in the dark. The colony diameter of each isolate at G01 was measured in two perpendicular directions with a digital caliper, at every 12 h of incubation, until the colony reached the edge of the Petri plate. In addition to G01, growth rate was assessed in every generation up to the 10^{th}. To start a new generation, a 5 mm-diameter disc from the edge of the colony from the previous generation was taken with a cork borer and transferred to a new Petri plate.

To assess phenotypic stability the values of colony diameter at each generation was plotted against time (hours). Scatter plots were constructed and

overall there were linear trends and a linear model was fit to the data using a regression approach. The *F* test for lack of fit was used to examine how well the model described variation in the data set (Neter et al. 1990). The slopes obtained in the regression analysis were used to calculate the confidence interval for the difference between the slopes of the resistant isolates and the sensitive isolate. When the mycelial growth rate of sensitive isolate was significantly different to TM-resistant isolate, this comparison received a score 1 and when no significant difference was found the comparison received a score 0. The scores were used to construct a heat map with the ggplot2 package. All statistical analyses were conducted using the R package version 3.4.1 (R Core Team, 2017).

Confirmation of fungicide sensitivity. One sclerotium was randomly selected from the colony formed at G10. The sclerotium was disinfested as described above and transferred to Petri dishes (90-mm-diameter) containing 15 mL PDA. Plates were maintained at 23°C in the dark. After 4 to 7 days of incubation, but before the colony reached the edge of the Petri plate, mycelial discs (5 mm - diameter) of the edge of the colonies were taken with a cork borer and transferred to the center of 60 mm - diameter Petri dishes containing 10 mL of PDA supplemented with the discriminatory doses of TM (5 µg/mL). The control plates comprised of PDA with DMSO (0.1 %). Assessment of mycelial growth and design were as previously described in the fungicide sensitivity experiment.

Partial sequence of the β-tubulin gene. The partial sequence of the β-tubulin gene was obtained for: two sensitive isolates, Ss-1980 and UFVSs-360, in generations 1, 3, 6, and 10; TM-resistant isolates and UFVSs-007, 451, and 452 in the generations 1, 3, 6, and 10.

Isolates were cultivated in liquid medium in Erlenmeyer flasks at 23°C in the dark (Lehner et al. 2015). After 5 days, the mycelium was carefully washed with distilled water, transferred to filter paper to dry for 24 h, and macerated in TissueLyser II (QIAGEN, Germany). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. DNA integrity was analyzed by electrophoresis on a 1% agarose gel and its concentration was estimated by comparison to a 100 bp DNA ladder. Final concentration of the polymerase chain reaction (PCR) amplification product was adjusted to 25 ng μL^{-1}.

PCR was performed in a final volume of 12.5 µL with 1 µL of DNA; 0.75 µL of each primer at 10 mM (TUB-F1: 5'-GCTTTTGATCTCCAAGATCCG-3' and TUB-R1: 5'-CTGGTCAAAGGAGCAAATCC-3'); 7.12 µL of water; 0.25 µL of dNTPs (includes dATP, dCTP, dGTP, and dTTP); 2.5 µL of 5X Q5 Reaction Buffer; and 0.13 µL of Q5 High-Fidelity DNA Polymerase (New England Biolabs, Inc.). The PCR conditions were: initial denaturation at 98°C for 30 s; followed by 35 cycles at 98°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 2 min. Reaction was confirmed by electrophoresis on a 1% agarose gel. PCR products were purified using ExoSAP-IT cleanup reagent (USB, Cleveland, OH), lyophilized and sequenced by Macrogen Services (Seoul, Korea). The contigs were obtained with DNA Baser v4.36.0 and aligned using MEGA 6.0 (Tamura et al. 2013).

Genotyping of isolates. Three TM resistant (UFVSs-007, 451, and 452) and one sensitive (UFVSs-360) isolate of the generations 1, 3, 6, and 10 were genotyped with 13 microsatellite (SSR) loci (Sirjusingh and Kohn 2001). The forward primers were labeled with the fluorescent dyes 6-FAM, VIC, NED or PET. Multiplex PCR reactions were performed with the Multiplex PCR 5X Master Mix kit as recommended by the manufacturer (New England Biolabs, Inc.). The PCR products were lyophilized and fragment analysis was done in Macrogen Inc. (Seoul, South Korea) using the GeneScan-500 LIZ size standard (Applied Biosystems) on ABI 3730XLs. The fragment sizes were assigned using GeneMarker version 2.7.0 (SoftGenetics).

Results

Fungicide sensitivity assay with discriminatory doses. The mycelial growth of the sensitive isolates at discriminatory concentrations varied from 13% to 44%; from 16% to 36%; and from 15% to 41%, compared to the control for TM, fluazinam and procymidone, respectively. Eight (UFVSs-443, 448, 449, 450, 451, 452, 453, and 454) and five isolates (UFVSs-483, 484, 485, 496, and 497) collected in dry bean fields in the municipalities of Cabeceira Grande and Unaí, Minas Gerais state, respectively, were resistant to TM. Similar growth patterns were observed for resistant isolates growing in both fungicide-amended and

control plates. There was no evidence of resistance to fluazinam or procymidone.

Stability of TM resistance. The mycelial growth rate varied from 1.15 mm/h to 1.86 mm/h (G01); 0.53 mm/h to 1.63 mm/h (G02); 0.49 mm/h to 1.66 mm/h (G03); 0.50 mm/h to 1.62 mm/h (G04); 0.38 mm/h to 1.68 mm/h (G05); 0.32 mm/h to 1.63 mm/h (G06); 0.29 mm/h to 1.63 mm/h (G07); 0.30 mm/h to 1.63 mm/h (G08); 0.25 mm/h to 1.63 mm/h (G09); and 0.25 mm/h to 1.63 mm/h (G10) (Figure 2).

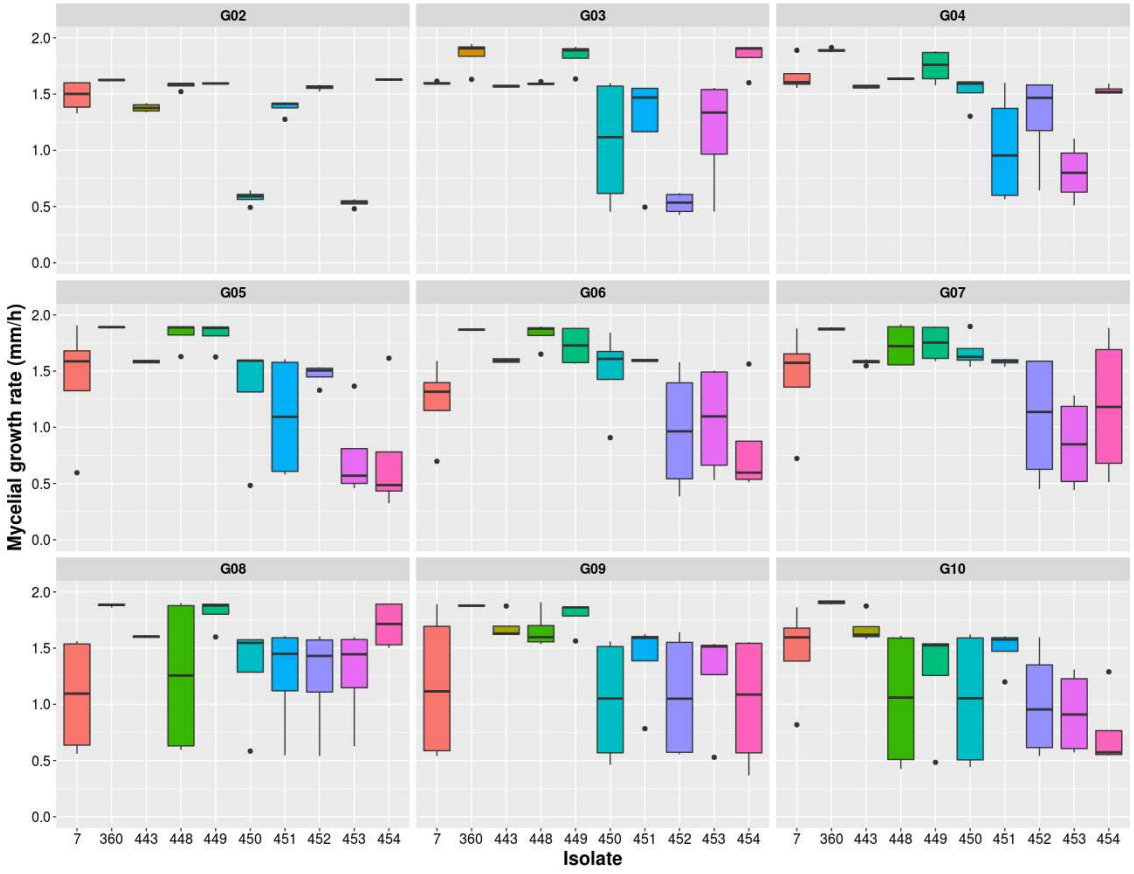

Figure 2. Boxplots of the mycelial growth rate of TM-resistant and sensitive (UFVSs-360) isolates of *Sclerotinia sclerotiorum* in nine generations (G02 to G10).

The mycelial growth rate of isolates UFVSs-007, 450, 451, 452, 453, and 454 reduced significantly with successive transfers compared with the sensitive isolate and longer time was required for the colony of these isolates to reach the edge of the Petri dish (Figure 3 and 5). The sensitive isolate required 60 h in all generations for the colony to reach the edge of the Petri dish, with exception at G01 that required 72 h (Figure 4 and 5). TM-resistant isolates UFVSs-443, 448,

and 449 were the more stable ones compared to UFVSs-360 (Figures 3, 4 and 5). Resistant isolates remained insensitive to the fungicide after 10 successive generations in unamended PDA plate.

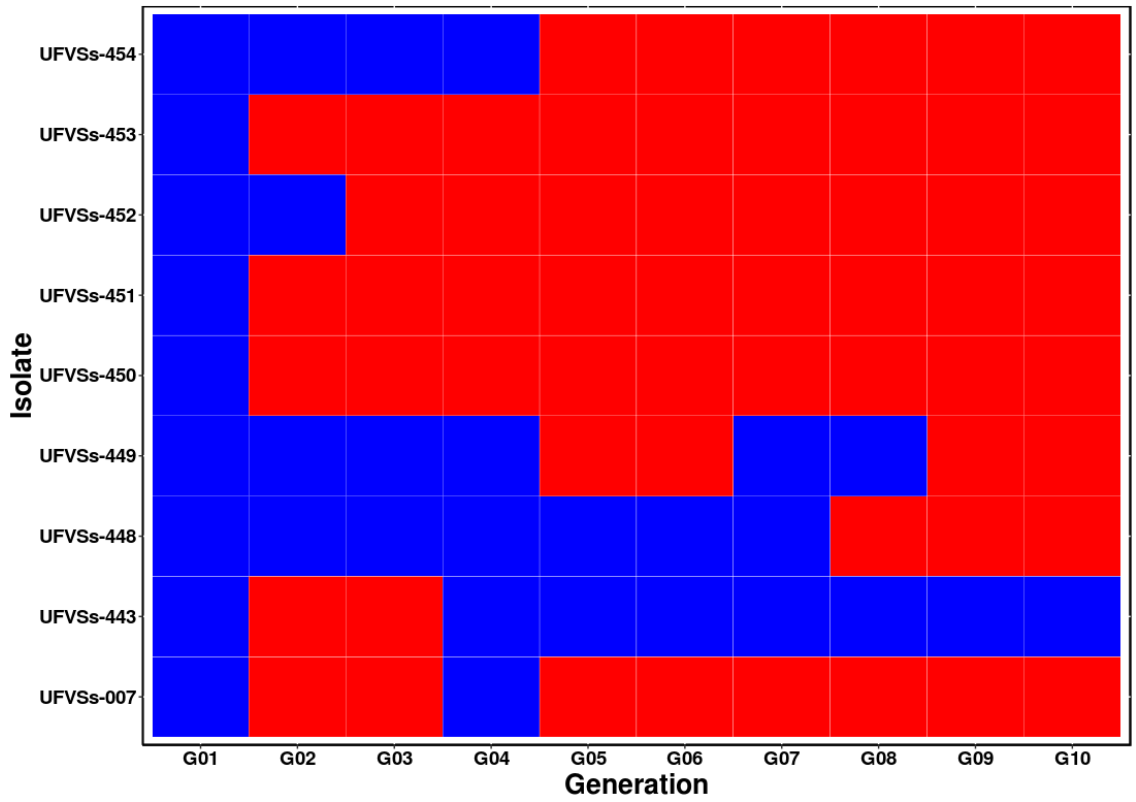

Figure 3. Heat map of the mycelial growth rate of TM-resistant isolates of *Sclerotinia sclerotiorum* at different generations compared to the sensitive isolate (UFVSs-360). When the growth rate of UFVSs-360 was significantly different to the TM-resistant isolate, the result was scored as 1 (red); and when there was no difference, the result was scored as 0 (blue).

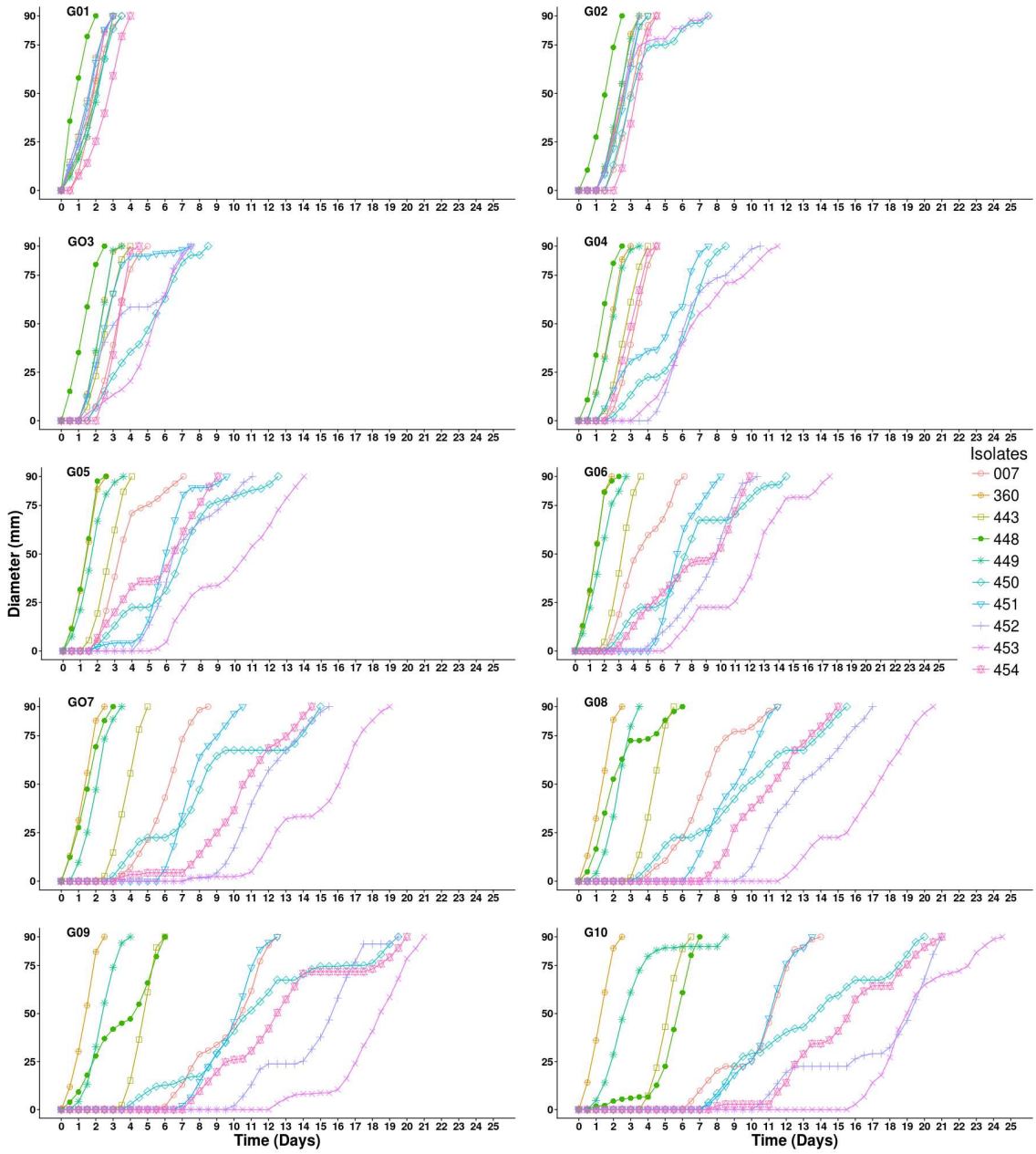

Figure 4. Mean colony diameter of TM-resistant and sensitive (UFVSs-360) isolates of *Sclerotinia sclerotiorum* as a function of time in ten generations (G01 to G10).

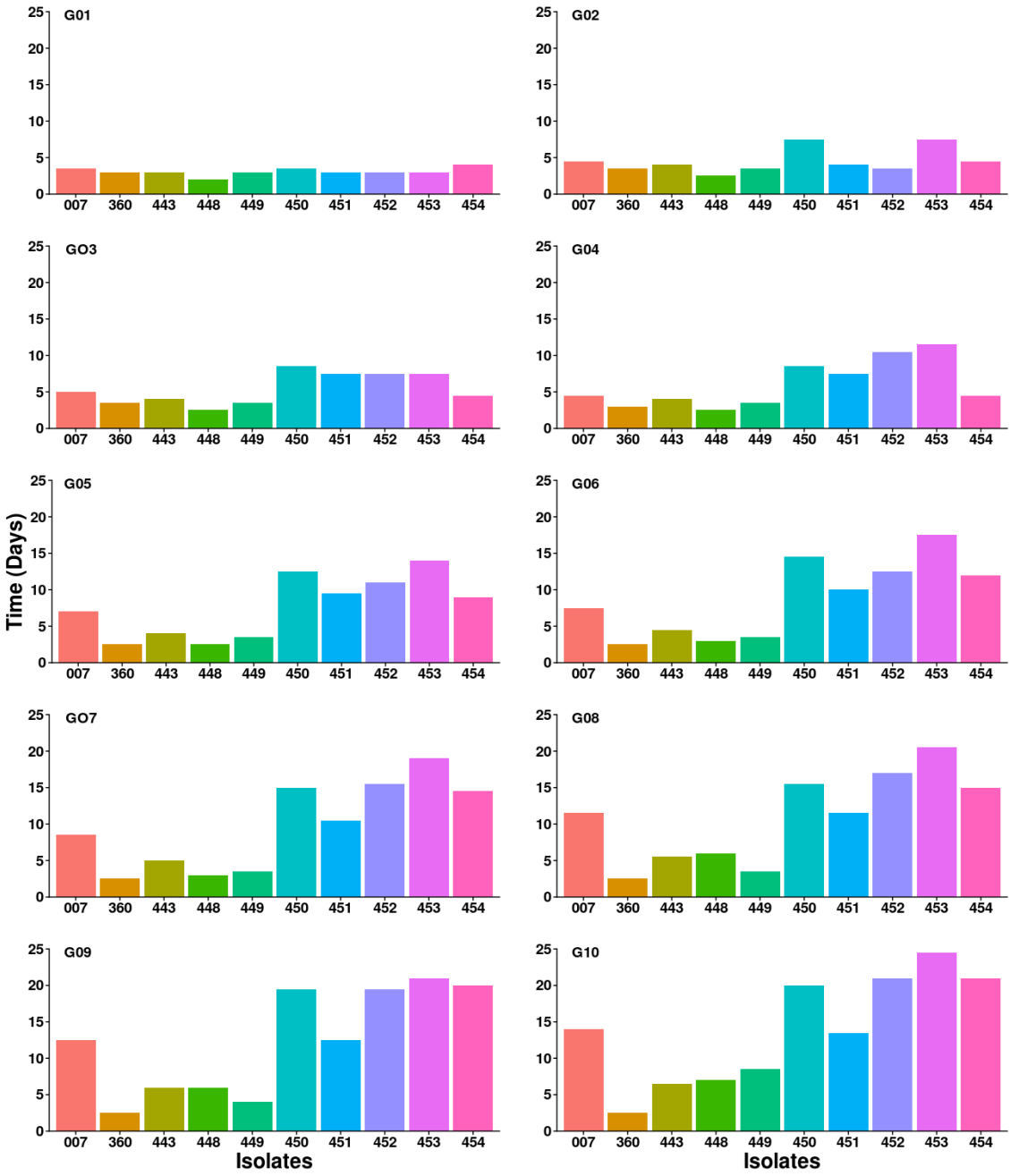

Figure 5. Time required for the colony to reach the edge of the Petri dish of TM-resistant and sensitive (UFVSs-360) isolates of *Sclerotinia sclerotiorum* in the ten generations (G01 to G10).

Partial sequence of the β-tubulin gene. Analysis of the partial sequence of the β-tubulin gene of the isolates: UFVSs-443, 448, 449, 450, 451, 452, 453, 454, 483, 484, 485, 496, 497, and isolates UFVSs-007, 451, and 452 in four different generations (G01, 03, 06, and 10) revealed the L240F mutation in the β-tubulin gene. The L240F mutation persisted throughout the successive transfers of the resistant isolates (UFVSs-007, 451, and 452). The UFVSs-360

and Ss-1980 isolates used as control had no mutation on the partial sequence of the gene.

Genotyping of isolates. The three TM-resistant (UFVSs-007, 451, and 452) isolates had the same haplotype (177/305/369/411/406/252/317/347/410/174/224/358/376). This haplotype was recovered at G01, G03, G06 and G10. Similarly, the haplotype (157/320/369/411/406/244/317/353/338/160/217/360/372) of the sensitive isolate, UFVSs-360 did not change at the sampled generations.

Discussion

Assessment of fungicide sensitivity and phenotypic stability shall be investigated regularly to predict the consequences of resistant isolates for white mold management. Thirteen isolates were resistant to TM, however, there was no evidence of resistance to fluazinam or procymidone. No resistance of *S. sclerotiorum* to fluazinam is likely due to its multisite mode of action. Studies have reported fluazinam as the most efficient fungicide to control white mold (Vieira et al. 2012; Meyer et al. 2014). Fortunately, in Brazil, the population seems to remain sensitive to this active ingredient.

In another study, the sensitivity to fluazinam, procymidone and TM was conducted with 282 *S. sclerotiorum* isolates collected from common bean fields in Brazil. There was no resistant isolates to fluazinam or procymidone. Mycelial growth of *S. sclerotiorum* obtained from lettuce in Yuma County, Washington, was highly reduced by low concentration (0.01 µg/mL) of fluazinam (Matheron and Porchas 2004). However, mycelial growth inhibition varied from 42 to 80 % and from 60 to 80 % in PDA supplemented with fluazinam (0.005 µg/mL) compared to the control for isolates from alfalfa and oilseed rape, respectively (Attanayake et al. 2012, 2013). In the present study, fluazinam was the most effective fungicide and mycelial growth inhibition varied from 64 to 84 % at 0.05 µg/mL in isolates collected from different hosts and regions. Apparently, isolates from Brazil were more insensitive to fluazinam than isolates from United States. This difference may be associated with the intensity of fungicide usage in the crops in Brazil compared to the United States. Most isolates used in this study were collected from soybean, common bean, cotton, and tomato. These are

important economic crops in Brazil, for which intensive fungicide application schedules are adopted. It is possible that the sensitivity of the population is gradually shifting in a quantitative way towards higher tolerance to fluazinam. Future collections comparing samples from different crops, under different fungicide regimes may reveal interesting facts.

Resistance to dicarboximide fungicide was not found in isolates obtained from different hosts in Brazil. However, some studies in China reported that field isolates of *S*. *sclerotiorum* resistant to a dicarboximide fungicide (dimethachlone) showed cross-resistance to iprodione and procymidone, two fungicides of the same group (Zhou et al. 2014; Firoz et al. 2015). Procymidone-resistant isolates of *Monilinia fructicola* and *Botrytis cinerea* have also been reported (Lim and Cha 2003; Liu et al. 2016). While resistance to procymidone not was found in Brazil, resistant isolates of *S*. *sclerotiorum* and species of the same family have been found. Growers must change fungicide mode of action in each season, so as to make resistance build-up more difficult.

The first *S*. *sclerotiorum* isolate (UFVSs-007) resistant to TM in Brazil was sampled in the municipality of Cabeceira Grande, in 2010. The isolate had the L240F (leucine to phenylalanine at codon 240) mutation in the β-tubulin gene (Lehner et al. 2015). After 5 years, a new sample was conducted in the region of Cabeceira Grande and Unaí, Minas Gerais state. Eight isolates, collected in common bean fields located in Cabeceira Grande and five collected in Unaí were resistant to TM. These isolates also have L240F mutation in the β-tubulin gene. Unaí and Cabeceira Grande are located in the same micro-region, the distance between the two municipalities is approximately 65 km and both are well-known for being important common bean production areas. The intense transit of agriculture machinery between farms can have contributed to the dispersal of resistant isolates of the pathogen. Alternatively, a mutant isolate, resistant to TM, could have been introduced by means of common bean seeds sold in this region.

Isolates of *S*. *sclerotiorum* resistant to TM were phenotypically unstable after successive transfers in the laboratory, which may suggest a fitness cost associated with fungicide resistance. Laboratory-induced resistant isolates of *S*. *sclerotiorum* to dimethachlone had their insensitivity reduced after 20 successive mycelial transfers on PDA (Li et al. 2015). In the present study,

isolates resistant to benzimidazole remained insensitive to TM after 10 successive generations in unamended PDA plate. Furthermore, the L240F mutation was maintained and the 13 microsatellite loci were the same after all generations. However, isolates resistant to TM probably have reduced fitness. The growth rate of the isolates UFVSs-007, 450, 451, 452, 453, and 454 was reduced after successive transfers and longer time was required for the colony to take the Petri dish area in comparison to sensitive isolate (UFVSs-360). Therefore, unstable resistant isolates have lower ability to multiply than sensitive isolates. But, unstable resistant isolates still produce sclerotia even with successive transfers and remained resistant. Consequently, they may persist in the field, but survival capacity of sclerotia needs to be assessed. Although some experiments have been conducted to understand the mechanisms that affect the instability of isolates resistant to TM this remains to be elucidated later. It is possible that activity of transposons present in the genome of *S*. *sclerotiorum* may be implicated in this process. Transposons activity in this plant pathogenic fungus is induced under stress conditions (Santana et al. 2014).

Management of resistant populations must employ fungicides with different modes of actions. This study confirmed the prevalence of TM-resistant isolates of *S*. *sclerotiorum* from common bean fields in two local populations in Brazil. Consequently, the reduced efficacy of thiophanate-methyl to control white mold, may cause yield losses. Rotation of fungicides with different modes of action must be adopted in the areas where TM-resistant isolates were already found, as well as in other regions to prevent to build-up of insensitive individuals.

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General Conclusions

The population of *Sclerotinia sclerotiorum* from different hosts and regions in Brazil has a clonal genetic structure, with a clonal fraction of 78% and two dominant large mycelial compatibility groups (MCGs) widely distributed in the country. Most MCGs are comprised by a single multilocus genotype.

The population of *S. sclerotiorum* is not structured according to host or geographic region, but rather MCGs play an important role determining genetically distinct subpopulations.

There is no evidence for the lack of sensitivity of *S. sclerotiorum* to fluazinam or procymidone fungicides. Thirteen isolates collected from common bean in the municipalities of Cabeceira Grande (8 isolates) and Unaí (5 isolates), Minas Gerais state, were resistant to thiophanate-methyl (TM). Resistant isolates to TM have been collected in this area since 2010. Resistant isolates can be maintained in the field probably due to intensive use of TM during the season.

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