MILLER DA SILVA LEHNER

ANALYSIS OF THE POPULATION STRUCTURE OF *Sclerotinia sclerotiorum* CAUSING WHITE MOLD ON COMMON BEAN IN BRAZIL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de Doctor Scientiae.

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SUMÁRIO

RESUMO

LEHNER, Miller da Silva, D.Sc., Universidade Federal de Viçosa, Fevereiro, 2015. **Uma análise detalhada da população de Sclerotinia sclerotiorum causando mofo-branco em feijoeiro comum no Brasil.** Orientador: José Eustáquio de Souza Carneiro. Co-orientadores: Eduardo Seiti Gomide Mizubuti e Trazilbo José de Paula Júnior.

O mofo-branco causado pelo fungo Sclerotinia sclerotiorum é uma das doenças mais destrutivas à cultura do feijoeiro comum no Brasil. O patógeno está amplamente disperso pelas áreas de produção e causa vultosas perdas de produção. O controle do mofo-branco é feito pelo uso intensivo de fungicidas, porque cultivares resistentes não estão disponíveis no Brasil. Para auxiliar o desenvolvimento de cultivares resistentes e prolongar a durabilidade deles é necessário conhecer a estrutura genética da população do patógeno, o que até o momento é deficiente no Brasil. Marcadores microsatellites (SSR) e grupos de compatibilidade micelial (MCGs) foram utilizados para avaliar a estrutura genética da população de S. sclerotiorum. Analisaram-se 300 isolados coletados nas principais regiões produtoras de feijão no Brasil. A população é subdividida pelos MCGs, dentro dos quais isolados podem acasalar aleatoriamente. Entretanto, quando analisada como um todo a população tem uma estrutura genética clonal. A agressividade de 20 isolados de S. sclerotiorum de distintos haplótipos SSR e de variável morfologia foi avaliada usando dois métodos de inoculação e dois cultivares de feijão. A agressividade dos isolados foi similar, exceto pela elevada agressividade do isolado Ss-217 quando inoculado em plantas do genótipo A195. Agressividade não foi correlacionada com MCGs, haplótipos SSR, pigmentação micelial, taxa de crescimento ou produção de escleródios. Um total de 282 isolados foram testados quanto à sensibilidade ao tiofanato-metílico (TM), fluazinam e procimidone, fungicidas comumente utilizados para controlar o mofo-branco do feijoeiro no Brasil. Nenhum isolado foi resistente ao fluazinam ou procimidone. Um isolado foi resistente ao TM,

por apresentar a mutação L240F no gene da β-tubulina. Esta mutação nunca havia sido descrita. A adaptabilidade do isolado resistente ao TM foi comparável a dos isolados sensíveis. Práticas de manejo de resistência devem ser implementadas para atrasar a dispersão de genótipos resistentes ao TM.

ABSTRACT

LEHNER, Miller da Silva, D.Sc., Universidade Federal de Viçosa, February, 2015. **A detailed analysis of the population of Sclerotinia sclerotiorum causing white mold on common bean in Brazil.** Adviser: José Eustáquio de Souza Carneiro. Co-advisers: Eduardo Seiti Gomide Mizubuti and Trazilbo José de Paula Júnior.

White mold caused by the fungus Sclerotinia sclerotiorum is a highly destructive disease to common beans in Brazil. The pathogen is widely dispersed in the production areas, which leads to extensive damage to common bean yields. White mold control is done by intensive use of fungicides, because resistant cultivars are not available in Brazil. To assist the development of resistant cultivars and extend the durability of any resistant materials proper understanding of the genetic structure of the pathogen population is required. Nevertheless, relatively little is known about the genetic structure of S. sclerotiorum affecting common bean in Brazil. Microsatellite (SSR) markers and mycelial compatibility groups (MCGs) were used to assess the genetic structure of the population of S. sclerotiorum. A total of 300 isolates were collected from the main producing areas of common bean in Brazil. The population is subdivided into MCGs, within which random mating takes place. However, when analyzed as a whole (all MCGs), the population has a clonal genetic structure. The aggressiveness of 20 S. sclerotiorum isolates from distinct SSR haplotypes and variable morphology was assessed using two inoculation methods and two common bean cultivars. The aggressiveness of the isolates was similar, except for a higher response of Ss-217 when inoculated on plants of the genotype A195. Aggressiveness was not correlated with MCGs, SSR haplotypes, mycelial pigmentation, growth rate or sclerotia production. A total of 282 isolates of S. sclerotiorum were screened for sensitivity to thiophanate-methyl (TM), fluazinam and procymidone, fungicides usually used for white mold control of common bean in Brazil. No isolate was resistant to fluazinam or

procymidone. One isolate was resistant to TM. The resistant isolate had a L240F mutation in the β-tubulin gene, which had never been reported. The fitness of TM-resistant isolate was comparable to sensitive isolates. Therefore, resistance management practices should be implemented to delay the spread of TM-resistant genotypes.

GENERAL INTRODUCTION

Common bean (Phaseolus vulgaris L.) is an important staple food in Brazil. After the 1980's common bean crops spread throughout Brazil, over large areas and under different systems. Many technological innovations have been incorporated into common bean crops, but the use of certified seeds of good sanitary quality is still limited. In addition, the use of inappropriate crop rotation, use of cultivars of semiprostrate or prostrate indeterminate growth habit and irrigation using center pivot during fall-winter crop season, create a favorable environment to the development of white mold epidemics, a devastating fungal disease caused by Sclerotinia sclerotiorum (Lib.) De Bary (Paula Jr. et al. 2006).

Management of white mold is difficult because S. sclerotiorum is a soilborne pathogen with a wide host range (Willetts and Wong 1980). In addition, it 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). Genetic resistance would be the best strategy to white mold control, because it is easy to implement and is environmentally friendly. However, to date, in Brazil there is no commercial cultivar with high levels of resistance to white mold and growers heavily rely on intensive fungicide treatment to control the disease. The main compounds applied are thiophanate-methyl, fluazinam and procimidone.

Breeding for white mold resistance in common bean has just begun in Brazil. However, in other countries, especially in the United States, some partially resistant breeding lines have been released (Griffiths 2009; Kelly et al. 2012; Miklas et al. 2014). In Brazil, initial studies have been conducted in order to identify sources of resistance (Carvalho et al. 2013; Souza et al. 2014; Lehner et al. 2015) or understand the genetic control of resistance (Antônio et al. 2008; Carneiro et al. 2011). On the other hand, very

few studies were conducted to assess the genetic structure of the population of S. Sclerotiorum and to evaluate pathogenic variability. The proper knowledge of the genetic structure of S. sclerotiorum population in Brazil can be helpful for optimizing control strategies, particularly the development of resistant cultivars and the maintenance of resistance durability (McDonald and Linde 2002). Thus, the aims of this study were: i. to determine the genetic structure of S. sclerotiorum affecting common bean in Brazil using SSR markers and MCGs; ii. to assess the pathogenic variation among S. sclerotiorum isolates iii. to assess the sensitivity of S. sclerotiorum isolates to the most commonly used fungicides for white mold control in Brazil.

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Chapter 1

The population of Sclerotinia sclerotiorum affecting common beans in Brazil is structured by mycelial compatibility groups that define clonal lineages within which isolates can randomly mate

Abstract

A thorough analysis of the population of Sclerotinia sclerotiorum that causes white mold in common bean in Brazil was conducted using microsatellite (SSR) loci and mycelial compatibility groups (MCGs) as markers. A total of 300 isolates were studied and 154 SSR haplotypes and 32 MCGs were identified. Two MCGs were widely distributed and accounted for 70% of the isolates. Six SSR haplotypes were associated to more than one closely related MCGs. There was no evidence of random association of alleles among loci when the population comprised by all MCGs was analyzed, suggesting that outcrossing is absent or rare. Nevertheless, there was evidence of random mating within the major MCGs. Seven genetic groups were identified, one of them comprised only by highly pigmented isolates, which have an specific allele at locus 114-4. Isolates of distinct MCGs did not differ in aggressiveness. Given that 95.6% of total genetic variation was attributed to differences among MCGs; there was strong genetic differentiation among MCGs; and the ocurrence of linkage equilibrium within MCGs, the common bean population of S. sclerotiorum is structured by MCGs. Therefore, breeders and pathologists should focus on the dynamics of MCGs in order to develop resistant cultivars and set white mold control strategies.

Keywords: Phaseolus vulgaris, microsatellite markers, genetic structure, white mold.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an ascomycete that causes white mold, a highly destructive fungal disease that affects common bean (Phaseolus vulgaris L.)

and at least a dozen other important crops. The pathogen has a necrotrophic lifestyle and secrets a wide array of cell-wall-degrading enzymes and oxalic acid to colonize its hosts. S. sclerotiorum is a homothallic fungus with a single MAT locus containing both the alpha and the high mobility group domain encoding the MAT genes. This fungus can reproduce asexually by means of sclerotia or sexually by self- or cross-fertilization (Amselem et al. 2011).

Brazil is the world's largest producer of common bean, an important staple food in this country. Currently, approximately three million hectares are estimated to be cultivated with bean in Brazil (CONAB 2014). Although this crop is susceptible to several diseases, none or a few of them have been as harmful to common bean as the white mold. This can be explained by the lack of resistant cultivars; the use of semiprostrate or prostrate indeterminate growth habit (Type III) cultivars; the use of grains instead of seeds, which contributes to pathogen dispersal; the presence of a wide range of other susceptible host crops cultivated almost year-round in the surroundings of common bean producing areas or as rotation crops in the same area; and fluctuation in common bean price, which can lead to the reduction in fungicide application and restriction in the implementation of other management strategies when the price is low.

Proper understanding of how the genetic variability of S. sclerotiorum populations is affected by evolutionary mechanisms and how it is distributed can be helpful for optimizing control strategies, particularly the development of resistant cultivars (McDonald and Linde 2002). The assessment of the variability of S. sclerotiorum has been accomplished by the use of mycelial compatibility groups (MCGs), molecular and morphological markers (Atallah et al. 2004; Sexton and Howlett, 2004; Mert-Turk et al. 2007; Attanayake et al. 2013). Except for one investigation (Malvarez et al. 2007), early studies using RFLP and/or MCGs revealed a

large prevalence of clonal populations of S. sclerotiorum based on the high frequency of a few DNA-fingerprinting genotypes or MCGs (Kohli et al. 1992; Cubeta et al. 1997; Hambleton et al. 2002). However, after the introduction of microsatellite (SSR) markers to assess genetic variation in S. sclerotiorum there seems to be a change in the scenario, with many studies reporting high variability of the pathogen (Atallah et al. 2004; Sexton and Howlett, 2004; Mert-Turk et al. 2007; Hemmati et al. 2009; Gomes et al. 2011; Attanayake et al. 2013) and only a few revealing low variability (Clarkson et al. 2013; Lehner et al. 2015). In some cases, in addition to high variability, random association of alleles was found, indicating that recombination may be occurring in the populations (Atallah et al. 2004; Sexton and Howlett, 2004; Hemmati et al. 2009; Attanayake et al. 2013; Attanayake et al. 2014). If this occurs in other areas/host plants then the control of white mold epidemics will become a considerable challenge given that S. sclerotiorum has a wide host range, can survive as sclerotia in the absence of host plants, uses an aggressive colonization mode, and will now rely on a genetically variable population.

Mycelial compatibility occurs when two isolates form a stable heterokaryon after hyphal anastomosis. Incompatibility limits genetic exchange by preventing anastomosis ultimately leading to groups of individuals (subpopulations) that differ in gene pools which may affect ecological, physiological and pathological traits (Remesal et al. 2012). When isolates of a MCG share the same set of markers, such as molecular markers, they are probably derived from a common parental strain (Leslie 1993). Consequently, isolates of a MCG are closely related and the MCG conceived as a clonal lineage (Leslie 1993). In a previous study of the genetic variability in S. sclerotiorum from common bean SSR haplotypes and MCGs were associated, but the relatively low number of isolates per MCG prevented proper analysis to test the hypothesis that each MCG is a distinct clonal lineage (Lehner et al. 2015).

The studies about variability of S. sclerotiorum affecting common bean in Brazil had conflicting results. The first study that used SSR markers reported high variability among S. sclerotiorum isolates from unspecified locations in the Cerrado region (Gomes et al. 2011). Recently, a new study was conducted using SSR markers and MCGs and low variability was found in populations of S. sclerotiorum from common bean fields in Minas Gerais state (MG) (Lehner et al. 2015). In the latter, there was no evidence of outcrossing and the population was composed of five genetic groups. Nevertheless, both studies were of limited scope in terms of sampled areas. For instance, isolates from other important common bean producing states, such as Paraná, which accounts for almost 25% of the total common bean production in Brazil (CONAB 2014) were not sampled. Therefore, a nationwide study should be conducted to investigate the genetic structure of the population of S. sclerotiorum affecting common bean. Additionally, a broader sampling would allow a better estimate of the distribution of the MCGs, its composition and to evaluate if a MCG is indeed a distinct clonal lineage.

The objectives of the present study were (i) to determine the genetic structure of S. sclerotiorum affecting common bean in Brazil using SSR markers and MCGs; (ii) to elucidate the relationship within and among S. sclerotiorum MCGs; and (iii) to assess the aggressiveness of representative S. sclerotiorum isolates from the MCGs obtained in the present study. We hypothesized that the population of S. sclerotiorum from common bean in Brazil has a clonal genetic structure. We also explored the possibility of each MCG is a clonal lineage as we suggested in a previous report (Lehner et al. 2015).

Materials and Methods

Sclerotinia sclerotiorum isolates. Three hundred S. sclerotiorum isolates were obtained from sclerotia collected from common bean plants with white mold symptoms

in fields located in the following states in Brazil: Santa Catarina (two fields, five isolates), Paraná (10 fields, 25 isolates), São Paulo (nine fields, 29 isolates), MG (21 fields, 121 isolates), Espírito Santo (11 fields, 25 isolates), Goiás (13 fields, 46 isolates), Bahia (two fields, 15 isolates) and Pernambuco (12 fields, 34 isolates) (**Fig. 1**). These states account for approximately 70% of total common bean production in Brazil (CONAB 2014). The isolates from Santa Catarina were collected in the municipalities of Curitibanos and Major Vieira; from Paraná in Araucária, Corbélia, Londrina, Mauá da Serra, Ponta Grossa, Reserva do Iguaçu, Três Barras do Paraná and Ventania; from São Paulo in Capão Bonito, Itaí, Itaporanga, Itararé and Taquarivaí, from MG in Cabeceira Grande, Paracatu, Unaí, Canaã, Coimbra, Oratórios, Porto Firme, Presidente Bernardes, Viçosa, Iraí de Minas, Patos de Minas, Ijaci, Lambari and Candeias; from Espírito Santo in Castelo, Domingos Martins, Santa Maria do Jetibá and Venda Nova do Imigrante, from Goiás in Campo Alegre de Goiás, Montividio, Rio Verde, Santo Antonio de Goiás and Silvânia, from Bahia in São Desidério; and from Pernambuco in Arcoverde, Calçado, Jucati, Jupi, Lajedo, and São João.

Most samples (85%) came from commercial fields where intensive fungicide applications were applied to control white mold. Few sampled areas (15%) are characterized as subsistence agriculture, mainly in the Zona da Mata region of MG, where fungicide application is less frequent. The incidence of white mold was low in areas with intensive fungicide application and only few and small disease foci could be found. This prevented both the usage of a structured sampling scheme and the collection of the same number of isolates in all areas. Two to 12 sclerotia were collected per field, depending on the disease incidence and field size. Nevertheless, distance among collection points within field was at least 10 m.

From each sclerotium hyphal-tip (isolates from MG) or single ascospore isolates were obtained and fungal mycelia was produced as described by Lehner et al. (2015). Hyphal-tip and single ascospore isolates were demonstrated to be equivalent in terms of inferences about the genetic variability of S. sclerotiorum as they result in similar SSR profile (Lehner et al. 2015; submitted).

DNA extraction and species-specific PCR. The genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) 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).

The primer pair SSasprF/SSasprR specific for S. sclerotiorum (Abd-Elmagid et al. 2013) was used to identify each isolate. DNA of S. sclerotiorum LMK 211 was used as a positive control and DNA of S. trifoliorum CBS 122377 was used as negative control. Additionally, 30 isolates previously identified by PCR as S. sclerotiorum were arbitrarily chosen and tested with the specific primers for S. trifoliorum (STcadF/STcadR) and S. minor (SMLcc2F/SMLcc2R). PCR reactions were performed as previously described (Lehner et al. 2015). Amplification was confirmed by using 5 µL PCR product subjected to electrophoresis in 1% agarose gel and TBE and viewed under UV light after staining with GelRed (Biotium, Hayward, CA, USA). Fragments were compared with a 100 bp DNA ladder and scored.

Mycelial compatibility group. The MCGs were determined for all S. sclerotiorum isolates as suggested by Schafer and Kohn (2006), with slight modifications (Lehner et al. 2015). The 300 isolates were paired in Petri dishes (60 x 15 mm) containing PDA supplemented with 75 µL L^{-1} of McCormick's red food coloring. Mycelial plugs (5 mm diameter) from a 2-day-old culture of the isolates were used for

pairings. The plugs were placed 18-20 mm apart from each other resulting in four pairings per dish. The dishes were kept at 23°C in the dark. Vegetative compatibility was checked visually after 3 and 6 days of incubation. The isolates were initially tested for compatibility with other isolates collected in the same geographic region. Afterwards, at least two isolates representing MCGs identified in each region were tested against each other. Each pairing was performed twice. When the results were inconsistent, two new independent pairings were done.

Microsatellite genotyping. All isolates were genotyped using the SSR loci 7-2, 8-3, 9-2, 12-2, 13-2, 36-4, 42-4, 92-4, 106-4 and 114-4 (Sirjusingh and Kohn 2001). PCR reactions were performed with Type-it Microsatellite PCR kit as suggested by the manufacturer (Qiagen, Hilden, Germany). Primers labeled with fluorescent dye (G5 dye set: 6-FAM, NED, VIC; Applied Biosystems) were used and an the allele size was determined in an automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystem) (Lehner et al. 2015). DNA of the isolate LMK 211 was used as positive control because its SSR allele sizes had been previously determined. A sample without DNA was used as negative control. Five isolates were replicated three times with independent DNA extraction to confirm the reproducibility of the results. Data were processed with GENEMARKER v.1.191 (SoftGenetics). Each allele was determined according to its amplicon size taking into account the number of repeat units in each locus.

Aggressiveness assay. The aggressiveness of representatives SSR haplotypes of all MCGs detected in the present study was assessed using a detached leaflet assay. A total of 62 haplotypes were evaluated. The number of haplotypes evaluated per MCG varied from one, for MCGs composed by one isolate, to 12, for the largest MCG composed by 146 isolates (**Fig. 2**). The common bean cultivar Pérola was used. Leaflets

of the youngest fully expanded trifoliolate leaves of 5-week-old plants were placed in filter paper moistened with 5 mL of sterilized distilled water inside of plastic boxes (11 width x 11 length x 3 cm height). One two-day-old mycelial disc (5 mm diameter) from the first subculture of each isolate was placed between the main vein and the leaflet edges. Boxes containing inoculated leaves were kept at 23°C in the 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.

Microsatellite analysis

Neutrality test

In order to check if the SSR markers were under selection, an outlier detection analysis was conducted using BayeScan software (Foll and Gaggiotti 2008). This method uses a Bayesian framework to determine if a locus is under selection. The analysis was performed three times to ensure robustness. In each run we used 100,000 iterations and the default settings of the program: 10 pilot runs with length of 5,000; sample size set of 5,000; and thinning interval of 10. Neutrality at each locus was rejected if the log10 (Bayes Factor) was higher than 1.5.

Genetic diversity and random mating. Each isolate was assigned to a haplotype using GENODIVE software (Meirmans and Van Tienderen 2004). The clonal fraction was calculated as 1 - [(number of different genotypes) / (total number of isolates)]. The gene diversity (Nei 1973) per locus averaged over all loci was calculated from clone-corrected data set using Genepop (Rousset 2008). Clone-corrected dataset was also used to estimate linkage disequilibrium across all SSR loci through the I_A and r_d indices (Agapow and Burt 2001). These indices and the evenness (E_5) were calculated

in the Poppr package (Kamvar et al. 2014) for the R program. I_A and r_d were calculated with 1,000 randomizations of the dataset.

Population structure. Clustering of genetically similar individuals were sought using the discriminant analysis of principal components (DAPC) (Jombart et al. 2010). DAPC was implemented in the Adegenet package (Jombart 2008) for the R program. The number of clusters (K) was allowed to vary from one to 30 and the optimal K was determined based on the Bayesian information criterion (BIC) (Jombart et al., 2010).

The Bruvo's distance among haplotypes was calculated using the Poppr package. This distance was used to construct a network by applying an automatic thresholding algorithm based on percolation theory using the EDENetworks software (Kivela et al. 2014). This method splits the fully connected network, so that only nodes with distance smaller than the critical threshold distance, the percolation distance (Dp), remain connected. The Dp can be entered manually or is automatically calculated by the software, based on the inner properties of the network (Arnaud-Haond et al. 2014). We set the Dp according to automatic threshold function available in the software.

Haplotype variation within and among MCGs. The number of haplotypes was determined for each MCG. Nei's distance (Nei 1978) between MCGs was calculated and a hierarchical cluster analysis according to the Ward criterion was performed. Both analyses were performed using the R program. The distribution of variation of SSR haplotypes among and within of MCGs was assessed through the hierarchical analysis of molecular variance (AMOVA) calculated using Arlequin (Excoffier and Lischer 2010). The distance method was based on the sum of squared size differences between two haplotypes for SSR data and the number of permutations was 10,100. Pairwise comparisons of MCGs were performed based on Slatkin's R_{st} index calculated with 10,000 permutations using Alerquin. To prevent unreliable conclusions, AMOVA and

pairwise comparisons were not performed for MCGs composed of less than five isolates.

To avoid biased analysis, the clonal fraction, E_5, He, I_A and r_d were calculated for MCG1 and MCG2 only, because of limitations in sample size of other MCGs.

Aggressiveness data analysis. Data from both experiments were pooled after performing Bartlett's test for homogeneity of variance. For each MCG we calculated the average lesion diameter and the standard deviation.

Results

Species-specific PCR identification. All isolates were identified as S. sclerotiorum based on the amplification of a 171 bp-fragment of the aspartyl protease gene. No amplification was detected when the primer pairs for S. trifoliorum or S. minor were used.

Mycelial compatibility group. A total of 32 MCGs were identified among the 300 S. sclerotiorum isolates. The number of isolates per MCG varied from one to 146. The second largest MCG was composed by 64 isolates and six MCGs were composed by a single isolate (**Fig. 3**). All isolates were self-compatible.

Microsatellite markers

A total of 154 haplotypes were identified among the 300 isolates analyzed. The most frequent haplotype was detected in 47 isolates and 39 haplotypes were detected in more than one isolate, resulting in an E_5 value of 0.34 and a clonal fraction of 0.49 (**Table 1**). The number of alleles at each locus varied from two (locus 42-4) to 16 (loci 114-4) with an average of eight alleles per locus. The average gene diversity over all loci was 0.60. Gene diversity varied from 0.08 (locus 42-4) to 0.76 (locus 12-2) (**Supplementary table**).

Neutrality and random mating. No outlier locus was detected, thus none were potentially under selection. The log10 (Bayes Factor) was lower than zero and not significant for all loci (data not shown). Considering the whole population (300 isolates), I_A and r_d were significantly different from zero ($P < 0.01$) (**Table 1**), thus there was no evidence of random mating.

Population structure. The most likely number of clusters was K=7. The gray group was composed by 21 isolates, 14 haplotypes and seven MCGs. The black group was composed by 17 isolates, 15 haplotypes and 4 MCGs. The red group was composed by 115 isolates, 27 haplotypes and 7 MCGs. The yellow group was composed by 64 isolates, 38 haplotypes and one MCG (MCG2). The blue group was composed by 43 isolates, 26 haplotypes and 4 MCGs. The pink group was composed by eight isolates, six haplotypes and one MCG (MCG15). The green group was composed by 32 isolates, 27 haplotypes and 12 MCGs.

The estimated percolation threshold automatically identified by EDENetworks was Dp = 0.20. At this threshold, the red and blue groups identified by the DAPC merged into a single group (**Figure 4**). There was higher heterogeneity among haplotypes of the pink, gray, black and green groups, compared with the red, blue or yellow groups.

Relationship within and among MCGs. Nine of the 11 MCGs represented by two isolates were composed by distinct haplotypes (**Fig. 3**). Three of the five MCGs represented by three isolates were composed by distinct haplotypes. Four isolates made up MCGs5 and 19, which were composed by three and four haplotypes, respectively. Five isolates made up MCGs4 and 18, which were composed by four and three haplotypes, respectively. The MCGs6 and 16 had six isolates with four and five

haplotypes, respectively. The MCGs represented by eight (MCG15) or nine (MCG3) isolates were composed by six and five haplotypes, respectively.

The MCG1 had higher clonal fraction, lower evenness and slightly lower gene diversity 0.66, 0.40 and 0.30, respectively, than MCG2 (**Table 1**). When considering the subpopulations defined by MCGs, the values of I_A and r_d did not differ from zero, thus there was evidence of random mating within MCG1 or MCG2 (**Table 1**).

The Nei's distance between MCGs varied from 0.01 for MCGs 3 and 11 to 2.30 for MCGs 13 and 14 (**Fig. 5**). Four main clusters of MCGs were identified: 1) composed by MCGs of the gray genetic group; 2) composed by MCGs of the black group; 3) composed by MCGs of the green and pink groups, plus MCG27 which has isolates in the green and red groups; and 4) composed by MCGs of the red and blue groups.

Six haplotypes were associated with more than one MCG. The haplotype coded as H26 belongs to MCGs7 and 17; H35 to MCGs1 and 25; H41 to MCGs1, 20, 30 and 31; H58 and H62 to MCGs3 and 11; and H70 to MCGs1 and 25. The Nei's distance between MCGs that shared the same haplotypes was low, the maximum value was 0.06 between MCGs1 and 25 or between MCGs7 and 17 (**Fig. 5**).

There was strong differentiation among the eight MCGs analyzed (those with at least five isolates). The R_{ST} estimates varied from 0.65 when comparing MCG2 and MCG15 to 0.99 when comparing MCG3 and MCG4 (**Table 2**). According to the AMOVA 95.6% of the total variation was attributed to genetic differences among MCGs, whereas only 4.6% was due to the variation within MCGs (**Table 3**).

Aggressiveness assay. The average lesion diameter by MCG varied from 39.3 mm ± 8.12 mm for MCG25 to 48.8 mm ± 4.37 mm for MCG28 (**Fig. 2**).

Discussion

The genetic structure of the population of S. sclerotiorum causing white mold on common bean was investigated under a different perspective of other studies by emphasizing the relationship among MCGs. The population of S. sclerotiorum as a whole has a clonal genetic structure. However, when data were analyzed as subpopulations defined by MCGs we found strong evidence of subdivision and that random mating takes place within MCGs1 or 2.

When the entire population was analyzed, its genetic structure was similar to that observed for MG subpopulation (Lehner et al. 2015), with moderate gene diversity and clonal fraction, and haplotype richness (proportion of different haplotypes) of about 50-60%. Nevertheless, the evenness estimate in the present study was about two times lower than that observed in MG. This indicates the predominance of few haplotypes in the Brazilian population. Other similar findings to the previous study (Lehner et al. 2015) were: low MCG richness (proportion of different MCGs), the predominance of only two MCGs, and linkage disequilibrium among alleles at different loci. Taken together these features support a clonal genetic structure, as expected for a homothallic fungus (Milgroom 1996; Taylor et al. 1999).

Low levels of MCG diversity are expected in populations of asexual fungi (Leslie 1993). Hambleton et al. (2002) reported a clonal structure of S. sclerotiorum in Canada, where the MCG richness was 9.8%. In other studies, in which evidence of outcrossing (linkage equilibrium) was detected, the MCG richness ranged from 49% (Atallah et al. 2004) to 90% (Attanayake et al. 2013). In the present study, MCG richness was 11%, consistent with a clonal genetic structure. However, to infer about clonality based only on MCG diversity may be misleading because the low MCG diversity may simply reflect the low number of polymorphic vic loci in the population

(Taylor et al. 1999). Thus, conclusions should be reached after assessing a set of characteristics of the population such as, high frequency of few genotypes or linkage disequilibrium among alleles at different loci, as mentioned above.

Despite the relatively low MCG richness, the haplotype richness was moderate (51%), but consistent with clonal populations of S. sclerotiorum investigated with SSR markers (Clarkson et al. 2013; Lehner et al. 20015). The high resolution of these markers has been able to reveal variation among closely related isolates (Lehner et al. 2015). Consequently, high genotypic diversity has been reported (Atallah et al. 2004; Gomes et al. 2011; Hemmati et al. 2009; Mert-Turk et al. 2007; Sexton and Howlett 2004), even when there are no evidence of outcrossing (Clarkson et al. 2013; Lehner et al. 2015). Considering the same loci, the number of alleles observed in the Brazilian population (2 to 16) was higher than that reported in the states of Washington and North Dakota in USA (Atallah et al. 2004; Attanayake et al. 2013), in China (Attanayake et al. 2013); and in Turkey (Mert-Turk et al. 2007). Compared with the UK population (Clarkson et al. 2013), the number of alleles in Brazil was higher in loci 7-2, 8-3 and 114-4, but lower in the loci 13-2 and 92-4. Regarding the Australian population (Sexton and Howllet 2004), the number of alleles in Brazil was higher in the loci 7-2, 8-3, 9-2, 12-2 and 13-2 and equal in locus 92-4 (**Supplementary table**). Nevertheless, direct comparisons must be carefully conducted, since factors such as agricultural practices, host plant genotypes, climatic conditions and methodologies used to estimate the SSR alleles may differ.

The larger sample size of the current study allowed the elucidation of important features of the population of S. sclerotiorum in Brazil. The number of isolates analyzed in the present study is approximately three times bigger than the regional studies previously conducted (Gomes et al. 2011; Lehner et al. 2015). Seven genetic groups

were found compared to five in our previous paper (Lehner et al. 2015). Previously, isolates from Ijaci in the southern region of MG putatively came from an unsampled distinct subpopulation, given that they were vegetatively incompatible with other isolates and formed a distinct genetic group. In the present study a similar situation was observed for isolates of the pink group (MCG15), which was obtained from Ventania, Paraná state. In the present study isolates from Ijaci were not compatible with isolates from other states, but were assigned in the same gray group, together with one isolate from Espírito Santo state (MCG32) and two isolates from Pernambuco state (MCG28). We suspected that the isolates from the gray genetic group have a different genetic origin (founder effect) and the high distance of the MCGs of this group compared to the other MCGs/groups (**Fig. 5**) support this claim. Probably these isolates are related by a common parent. Nevertheless, this could not be properly addressed with SSR markers. A more accurate analysis with DNA sequences, using phylogenetic and simulations of genealogies using the coalescent approach could clarify this question.

The network topology provides a clear picture of subdivision in the population of *S. sclerotiorum*. Although seven genetic groups were identified by DAPC, the haplotypes of the red and blue groups are fully linked. These results, in addition to low Nei's distance among the MCGs of these two groups, suggest a close relationship between these groups. This may be explained by the fact that 92% of the isolates that comprise the red and blue groups are from MCG1, which is widely distributed in all sampled regions (**Fig. 1**). The other genetic groups, except the pink, were also comprised by isolates from several localities. In general the groups were not associated with geographic region. However, we observed association of a specific SSR allele with the black group. Only isolates assigned to the black group have a 336 bp allele at locus

114-4. Surprisingly, mycelia of these isolates are highly pigmented and form a black colony, different from all other isolates.

The location of SSR locus and/or the number of repeats may be a key factor to determine gene expression (Li et al. 2002). Melanin biosynthesis in fungi uses the polyketide pathway (PKS) (Jacobson 2000). Locus 114-4 is positioned at chromosome 4, but using genomic analysis no putative PKS genes related to melanin production was found in this chromosome. However, it is possible that hypothetical proteins encoded by genes of unknown functions located in chromosome 4 are related to melanin production. It is also possible that locus 114-4 is located in a promoter region, which could affect the gene transcription (Li et al. 2002). In a previous study in which highly pigmented isolates were identified the allele of 336 bp at locus 114-4 was not found (Attanayake et al. 2013). Therefore, more research needs to be undertaken to investigate the possible association between mycelial pigmentation and SSR loci.

Mycelial incompatibility limits the exchange of genetic information between isolates of different MCGs. Given that outcrossing does not occur often, presumably similar to what happens in an assexual fungus, members of each MCG will form a genetically isolated subpopulation that will be subjected to the actions of evolutionary mechanisms (Leslie 1993). If each MCG is equally fit, then they will be lost by chance due to genetic drift. However, if a MCG is selectively more fit than most of the others, members of this group will tend to predominate in the population (Leslie 1993). Fitness of S. sclerotiorum isolates has been estimated by assessment of sclerotia production, mycelial growth rate and specially by pathogenicity tests (Zhou et al. 2014). Aggressiveness variation (Kull et al. 2004; Attanayake et al. 2013) as well as lack of variation (Atallah et al. 2004;) have been found. In the present study there was no variation in aggressiveness among the MCGs. The reason for this may be because the

isolates really do not differ in aggressiveness or due to high susceptibility to white mold of cultivar Pérola used in the aggressiveness assay. It seems that isolates of S. sclerotiorum exhibits variation in aggressiveness only when inoculated on common bean cultivars partially resistant to white mold (Lehner et al. unpublished data).

In the present study a large MCG was found, composed by 146 isolates and widely distributed across the sampled areas (**Fig. 1**). This MCG is likely to be older than the others, since the there was no difference in terms of aggressiveness, a surrogate used to estimate fitness. It is interesting to note that there is a North to South gradient in terms of MCG diversity. The higher the latitude, the higher the MCG diversity (**Fig. 1**). In the northeastern region, fewer fields were sampled, which may have contributed to smaller number of MCGs. Nevertheless, the MCG diversity seems to follow the gradient of common bean production in Brazil. The two largest common bean producer states, Paraná and MG, are located in the southern and southeastern regions, respectively. In the northeast, common bean areas expanded recently, especially in western Bahia state, but this area is much smaller than Paraná and Minas Gerais. In addition, for most part of the northeast environmental conditions are not favorable to white mold epidemics. In the central region, common bean competes with other crops, mainly soybean and maize, and fewer common bean crops are planted each year compared to southern and southeastern regions. Finally, MCG diversity is higher in areas where common beans are more intensively cultivated because of higher seed trade, which facilitates the introduction of new pathogen genotypes and possibly MCGs.

To the best of our knowledge no study of S. sclerotiorum considered MCG as subpopulations. Linkage disequilibrium at the population level, i.e. when the 300 isolates were analyzed, suggests that outcrossing is absent or rare. However, we found random association of alleles within MCG1 or 2, suggesting that recombination occurs

within MCGs. Based on these results, it seems that recombination does not occur between isolates belonging to distinct lineages/branches (in our case, MCGs), but frequent recombination occurs between isolates within each lineage. This pattern was suggested to be associated with bacterial populations (Maynard Simith et al. 1993, Figure 1B). Almost all studies try to identify populations of S. sclerotiorum separated by geographic region or by the host plant. In most cases these populations are usually comprised of different MCGs and tests to infer recombination are commonly carried out. However, recombination between two S. sclerotiorum isolates from distinct MCGs is likely to be a rare event. The dikaryotic phase that precedes apothecial initiation in S. sclerotiorum occurs by the duplication of nuclei directly from the vegetative hyphae (Willetts and Wong 1980). Heterokaryotic mycelia can occur through hyphal anastomosis, but just in isolates belonging to the same MCG (Leslie 1993). Thus, it does not seem appropriate to assess linkage equilibrium in populations comprised by distinct MCGs.

Contrary to what was found previously (Lehner et al. 2015), in the present study one haplotype was associated with more than one MCG. The decoupling between independent markers suggests that recombination between genetically distinct isolates may be occurring in the population (Kohli et al. 1992; Milgroom 1996; Taylor et al. 1999). Evidence for the occurrence of recombination was gathered only for isolates within a MCG. These isolates share the same vic alleles and recombination would not result in a new MCG. New MCGs could arise probably by mutation(s) at the vic loci, but the SSR loci of a mutant are likely to remain unaltered due to the unlinked nature of these markers. This is supported by the low distance observed among the MCGs that shared a haplotype (**Fig. 5**).

In the late 1990's using DNA fingerprinting Kohli and Kohn (1998) reported random association of alleles in populations of S. sclerotiorum from Canada or USA. After the introduction of SSR markers to assess genetic variation in S. sclerotiorum this result became more frequent (Atallah et al. 2004; Sexton and Howlett, 2004; Hemmati et al. 2009; Attanayake et al. 2013; Attanayake et al. 2014). However, the high mutation rate in the SSR loci could have been confused with recombination. Recently it was demonstrated that the random association of alleles is more likely be due to recombination than mutation, because the linkage disequilibrium decreases with increasing in the distances between the SSR loci (Attanayake et al. 2014). In most studies, including the present one, linkage disequilibrium or non-random association of alleles at SSR loci was detected. Linkage disequilibrium may occur due to physical linkage of genes (Milgroom 1996). After locating each SSR locus in the available sequence of S. sclerotiorum genome (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum), the 7-2 and 114-4 loci are at chromosome 4 and the 13-2 and 36-4 loci at chromosome 6. The other loci are at distinct chromosomes. The linkage disequilibrium analysis was repeated after removing loci 7-2 and 13-2, and there was no effect on the significance of the results. Thus, the linkage disequilibrium most likely is not due to physical linkage of SSR loci.

In addition to physical linkage, selection and genetic drift can affect linkage disequilibrium, but these mechanisms were ruled out. The results of the neutrality analysis do not indicate any evidence for selection in the SSR loci. Genetic drift is unlikely to have caused the observed linkage disequilibrium because populations of S. sclerotiorum have large effective sizes. The pathogen has a wide host range (Willetts and Wong 1980) and other susceptible host crops are cultivated almost year-round in the common bean producing areas, especially in tropical areas. Additionally, strong

bottlenecks in an area are unlikely given the capacity of the fungus to survive as sclerotia for many years in the soil.

Other possible cause of linkage of disequilibrium is when the analyzed samples consist of a mixture of several subpopulations. The subpopulations may be geographically or ecologically isolated, or there may be biological barriers to gene exchange (Maynard-Simith et al. 1993). The fact that linkage disequilibrium is detected in the entire population supports the existence of genetically distinct subpopulations characterized by MCGs. In addition to the results of the linkage disequilibrium tests, the R_{ST} pairwise comparisons among MCGs and the AMOVA support the hypothesis of MCGs acting as subpopulations. There was strong pairwise differentiation (R_{ST}> 0.25) of MCGs and the genetic differences among them accounted for almost all genetic variation (95.6%). Each MCG is postulated to be a clonal lineage, as suggested in a previous report (Lehner et al. 2015). They indicate that the population of S. sclerotiorum from common bean fields in Brazil is not a single, panmitic population. Instead, it is formed by several MCGs that may have distinct evolutionary trajectories.

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Figure legends

Figure 01. Sampled areas (black dots) and distribution of the seven genetic groups and the 32 mycelial compatibility groups (MCGs) detected among isolates of Sclerotinia sclerotiorum from common bean-producing regions in Brazil. The different colors refer to the genetic groups identified by discriminant analysis of principal components (DAPC). Dotted circles delimit geographically defined regions in which the analysis (pie charts) was conducted. The pie-charts represent the distribution of the genetic groups and MCGs in each geographic region.

Figure 02. Aggressiveness and number of SSR haplotypes evaluated per MCG of Sclerotinia sclerotiorum from common bean fields Brazil. Mean ± standard deviation.

Figure 03. Frequency of isolates and SSR haplotypes in each of the 32 mycelial compatibility groups of Sclerotinia sclerotiorum sampled from common bean fields in Brazil.

Figure 04. Network of Sclerotinia sclerotiorum SSR haplotypes based on Bruvo's genetic distance. Only links with genetic distance smaller than the percolation threshold (Dp = 0.20) are represented. A gradient of dark gray to light gray represents decreasing distances among haplotypes. The scatterplot of the discriminant analysis of principal components (DAPC) for the 300 isolates is shown in the lower left corner.

Figure 05. Clusters of mycelial compatibility groups (MCGs) of Sclerotinia sclerotiorum according to Nei's distance and based on the Ward criterion. The MCGs names and the dotted ellipsis are colored according to the seven genetic groups estimated by discriminant analysis of principal components (DAPC). The dotted ellipsis indicate MCGs that were assigned in more than one genetic group.

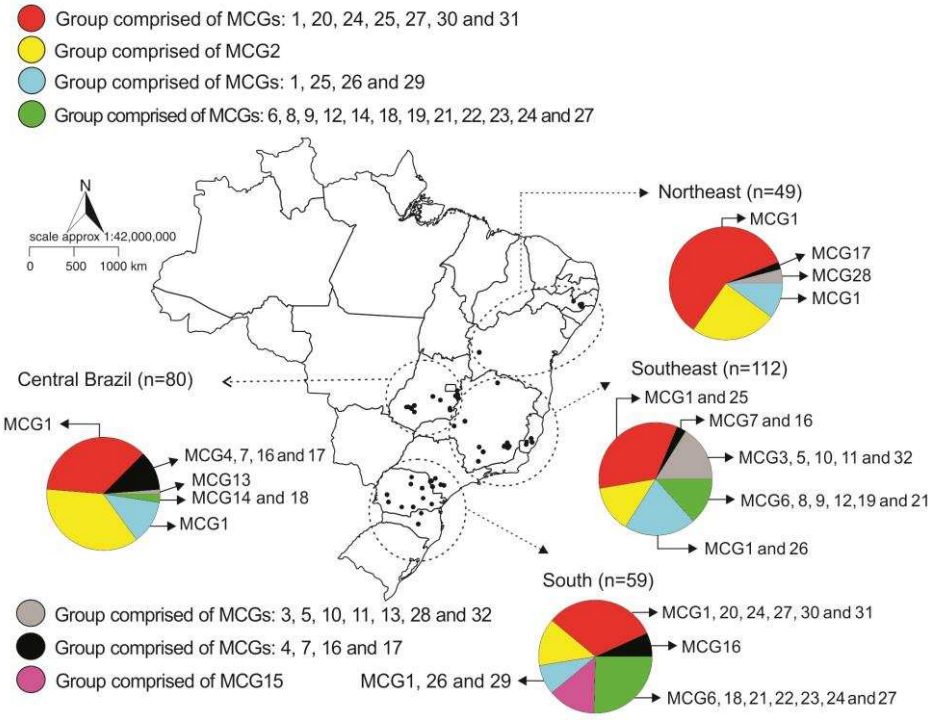


Figure 01. Lehner et al

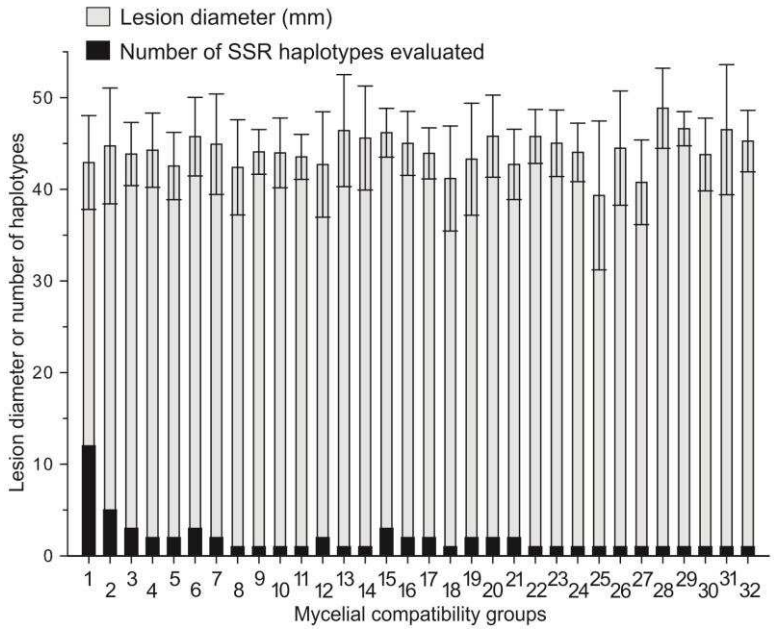
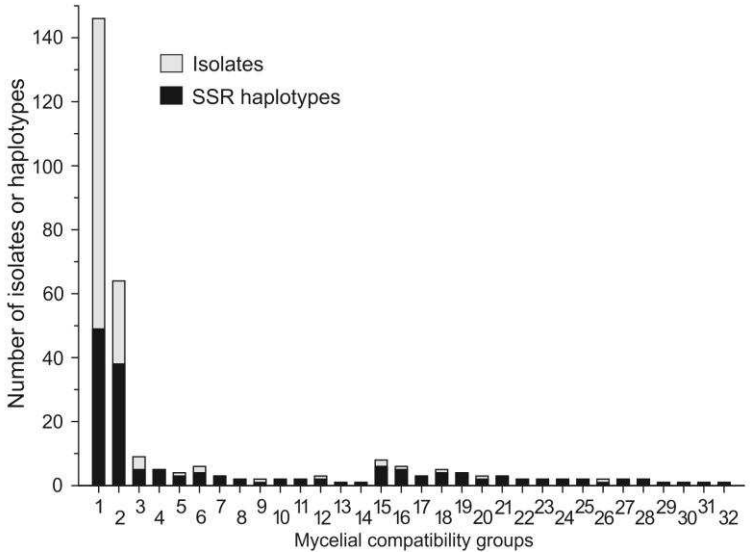


Figure 02. Lehner et al



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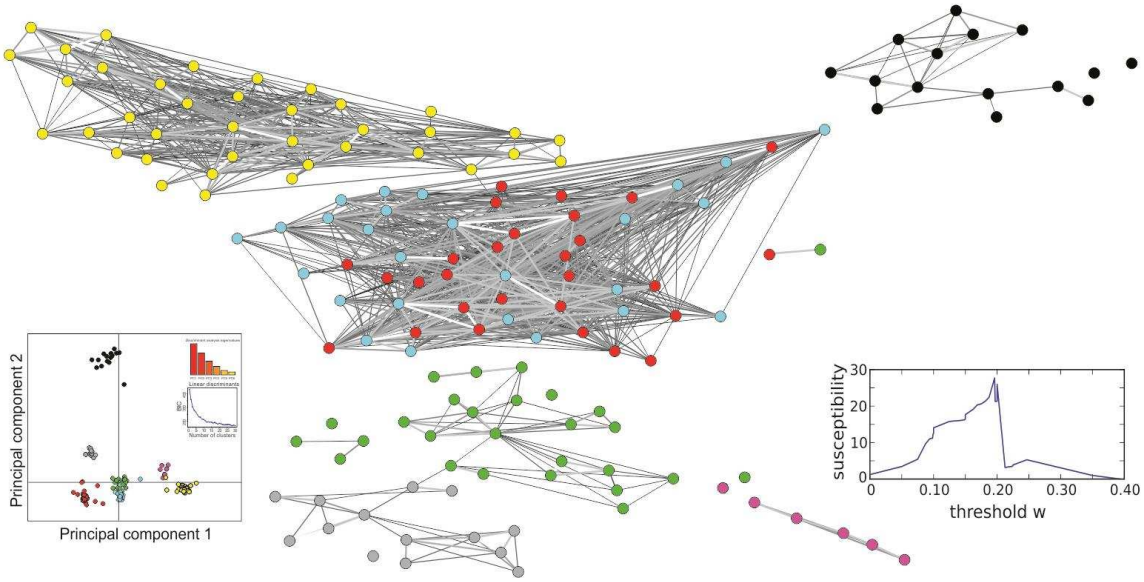

Figure 04. Lehner et al

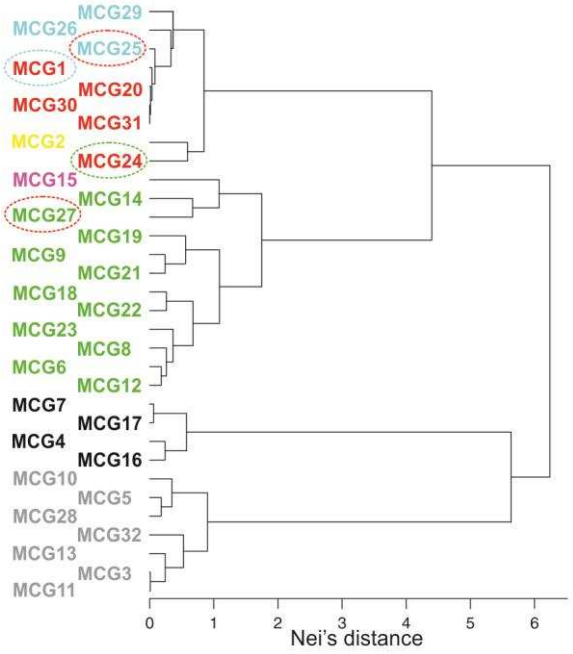

Figure 05. Lehner et al

Table 01. Basic descriptors of genetic variation in the subpopulations of Sclerotinia sclerotiorum defined based on mycelial compatibility groups (MCG). All isolates were obtained from common bean fields in Brazil.

Subpopulation	N[a]	H[b]	Clonal fraction[c]	Evenness[d]	Clone-corrected data set		
					He[e]	I_A[f]	r_d[f]
MCG 1	146	49	0.66	0.40	0.30	-0.24	-0.03
MCG 2	64	38	0.41	0.70	0.32	-0.26	-0.03
All MCGs (whole population)	300	154	0.49	0.34	0.60	0.96**	0.11**

[a] Sample size
[b] Number of haplotypes
[c] Clonal fraction calculated as 1 - [(number of different genotypes) / (total number of isolates)].
[d] Evenness (Grunwald et al. 2003)
[e] Gene diversity (Nei 1973), averaged over all loci
[f] Measures of multilocus linkage disequilibrium
**Significant at $P < 0.001$

Table 02. Genetic differentiation (R_{ST}) among mycelial compatibility groups of isolates of Sclerotinia sclerotiorum collected from common bean fields in Brazil.

Population	MCG1	MCG2	MCG3	MCG4	MCG6	MCG15	MCG16
MCG1							
MCG2	0.96						
MCG3	0.93	0.97					
MCG4	0.98	0.97	0.99				
MCG6	0.96	0.92	0.97	0.90			
MCG15	0.96	0.65	0.96	0.92	0.81		
MCG16	0.97	0.97	0.95	0.93	0.93	0.94	
MCG18	0.94	0.97	0.79	0.98	0.96	0.94	0.93

R_{ST} calculated using Arlequin (Excoffier and Lischer 2010). Number of permutations = 10,100. All indices were significant at $P < 0.001$.

Table 03. Analysis of molecular variance (AMOVA) for eight mycelial compatibility groups of isolates of Sclerotinia sclerotiorum from common bean fields in Brazil[a].

Source of variation	df	Sum of squares	Variation (%)	Fixation index	P-value
Among MCGs	7	22776.9	95.6		
Within MCGs	241	1714.1	4.4	$R_{ST} = 0.95$	< 0.0001
Total	248				

[a] Distance method based on the sum of squared size differences (R_{ST}) between two haplotypes for microsatellite data. Number of permutations = 10,100.

Supporting information

Supplementary table 01. Number of alleles found in the Brazilian population of Sclerotinia sclerotiorum from common beans compared to those found in other studies, size range and gene diversity for each microsatellite locus.

Locus	Repetitive sequence	Number of alleles	Size range (bp)	Gene diversity[a]	Number of alleles (previous reports)
7-2	$(GA)_{14}$	8	156-172	0.66	14^{b},3^{c}, 4^{d}, 2^{e}, 3^{f},6^{g}
8-3	$(CA)_{12}$	10	240-268	0.72	9^{b},7^{d},7^{g}
9-2	$(CA)_{9}(CT)_{9}$	7	354-368	0.24	8^{b},5^{g}
12-2	$(CA)_{9}$	6	211-221	0.76	9^{b},2^{c}, 1^{e}, 3^{f},5^{g},3^{h}
13-2	$(GTGGT)_{6}$	9	275-320	0.47	18^{b},2^{c},17^{d},2^{e},5^{f},7^{g}
36-4	$(CA)_{6}(CGCA)_{2}(CAT)_{2}$	3	410-414	0.22	9^{b},1^{g}
42-4	$(GA)_{9}$	2	405-407	0.08	6^{b}
92-4	$(CT)_{12}$	4	371-377	0.55	8^{b},2^{c}, 6^{d},4^{g}
106-4	$(CATA)_{25}$	13	496-584	0.66	9^{b},3^{c}, 6^{h}
114-4	$(AGAT)_{14}(AAGC)_{4}$	16	336-428	0.56	12^{b},4^{c}, 15^{d},2^{e},6^{f},9^{h}

[a] Gene diversity (Nei 1973) among individuals within populations, averaged over populations.
[b] Brazil, Gomes et al. (2011)
[c] USA (Washington), Atallah et al. (2004)
[d] UK, Clarkson et al. (2013)
[e] China, Attanayake et al. (2013)
[f] USA (North Dakota), Attanayake et al. (2013)
[g] Australia, Sexton and Howllet (2004)
[h] Turkey, Mert-Turk et al. (2007)

Chapter 2

Similar aggressiveness of phenotypically and genotypically distinct isolates of Sclerotinia sclerotiorum

Abstract

Understanding how Sclerotinia sclerotiorum aggressiveness varies among isolates may be useful for breeding programs aimed at developing common bean cultivars resistant to white mold. We used two inoculation methods and two common bean genotypes (Pérola and A195) to assess the aggressiveness of 20 S. sclerotiorum isolates collected in common bean fields from four Brazilian States. The isolates were genotyped with 10 microsatellite (SSR) loci, had their mycelial compatibility groups (MCGs) and partial sequences of the oxaloacetate acetylhydrolase (OAH) gene determined and were characterized for morphological traits. Twenty SSR and seven OAH haplotypes, 10 MCGs and high variability in colony morphology were found. Except for Ss-217, isolates showed similar aggressiveness. Ss-217 was more aggressive when inoculated on plants of the genotype A195. Aggressiveness was not correlated with MCGs, SSR or OAH haplotypes, mycelial pigmentation, growth rate or sclerotia production.

Keywords: Sclerotinia stem rot, white mold, straw test

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungus with a broad host range that causes white mold in common bean (Phaseolus vulgaris L.) (Boland and Hall 1994). In the Brazilian common bean-producing areas, S. sclerotiorum is widespread and severe white mold epidemics are frequently observed. White mold management depends almost exclusively on fungicide applications at the flowering stage (Vieira et al. 2010). However, there is increasing interest in alternative strategies,

especially genetic resistance, to control white mold, given that the use of resistant common bean cultivars is a promising option (Schwartz and Singh 2013).

The current breeding strategy for minimizing damage caused by white mold on common bean is based on the combination of physiological and avoidance resistance. Physiological resistance is conferred by plant defense mechanisms and avoidance resistance, by plant architecture-related traits (Miklas et al. 2013). Under field conditions, both types of resistance contribute to white mold control, whereas in the greenhouse or laboratory only physiological resistance can be identified (Vuong et al. 2004).

Finding new sources of resistance to white mold requires reliable disease evaluation techniques and proper understanding of pathogenic variability of S. sclerotiorum (Kull et al. 2003). Thus, common bean germplasm should be screened against appropriate S. sclerotiorum isolates, preferably genetically distinct and collected in regions where cultivars will be released (Schwartz and Singh 2013). The inoculation method can also interfere in the response of common bean genotypes to white mold (Kull et al. 2003). Two tests are often used to assess physiological resistance to white mold in common bean: inoculation of detached leaflets (Steadman et al. 1997) and the straw test (Petzoldt and Dickson 1996). Detached leaflets is a repeatable method that allows many common bean genotypes to be tested in a short space and time (Kull et al. 2003). In the straw test, the fungus is inoculated on the stem of plants and the severity assessment is done using a 1-9 scale or measuring the lesion length. Currently, the straw test is the most widely used method for detection of physiological resistance to white mold (Schwartz and Singh 2013).

In Brazil, assays aiming at screening for resistance to white mold have been carried out using the straw test method (Carneiro et al. 2010; Carvalho et al. 2013).

Usually, pathogen variation has not been considered in these studies, because little is known about the variability of S. sclerotiorum aggressiveness in the common bean-producing areas. Recently, studies were conducted to assess the genetic variability of S. sclerotiorum isolates from common bean fields in Brazil (Lehner et al. 2014; Lehner et al. 2015). Using microsatellite markers and mycelial compatibility groups (MCGs), low genetic variability was found (Lehner et al. 2015), but apparently there is high variability in morphological traits, such as mycelial pigmentation, growth rate and sclerotia production (Lehner et al. 2014). Nonetheless, variation in aggressiveness was not assessed in these studies.

Variation in aggressiveness is often assessed by measuring variables associated with the disease signs and symptoms. Studies designed to address aggressiveness of S. sclerotiorum have been done with the most economically important hosts of the pathogen: soybean (Kull et al. 2004), potato (Atallah et al. 2004), canola (Sexton and Howlett 2004; Attanayake et al. 2013), sunflower (Ekins et al. 2007; Irani et al. 2011) and common bean (Pascual et al. 2010; Otto-Hanson et al. 2011). In one study with common bean, Otto-Hanson et al. (2011) tested 156 isolates, 138 from the USA and 18 from France. In another paper, four isolates from Spain were assessed for differences in aggressiveness (Pascual et al., 2010). No studies were conducted to evaluate variation in aggressiveness among isolates of S. sclerotiorum from common bean fields in Brazil, the largest world producer of this legume. In addition, few studies have addressed the relationship between aggressiveness and molecular or morphological markers.

Our major goal in this study was to compare the aggressiveness of S. sclerotiorum isolates collected from distinct common bean fields in Brazil. We also want to determine the relationship between aggressiveness and genetic variability of the isolates. The following questions are addressed in this paper: Do different genotypes of

S. sclerotiorum from distinct geographic populations differ in aggressiveness? Are molecular or morphological markers related to pathogen aggressiveness? We addressed these questions using 20 isolates of S. sclerotiorum, two inoculation methods and two common bean genotypes. For the 20 isolates, phenotypic traits, two neutral markers (MCGs and SSR markers) and partial sequences of the oxaloacetate acetylhydrolase (OAH) gene, which is a pathogenicity determinant for S. sclerotiorum (Andrew et al. 2012), were evaluated for potential association with aggressiveness.

Materials and Methods

Fungal isolates and DNA extraction. Sclerotia of S. sclerotiorum were collected from plants with symptoms of white mold. Diseased plants were sampled in 20 common bean fields between 2010 and 2012. The fields were located in four states, which together account for about 50% of the bean production in Brazil (IBGE 2015). Sclerotia were collected in the municipalities of Oratórios, Canaã, Viçosa, Porto Firme, Paracatu (2 fields), and Unaí (3 fields), in Minas Gerais state; Itaí, Capão Bonito (2 fields), and Itararé, in São Paulo state; Santa Maria do Jetibá (2 fields) and Domingos Martins, in Espírito Santo state; and Mauá da Serra, Corbélia, Reserva do Iguaçu and Três Barras, in Paraná state. To ensure genetic uniformity, only hyphal-tip or monoascosporic isolates were used in the tests (Table 1). To obtain hyphal-tip isolates, one sclerotium from each sampled field was arbitrarily chosen, disinfested using standard procedures (Lehner et al. 2015), transferred to Petri dishes (90 mm-diameter) containing PDA medium and incubated at 23°C for six days for mycelium growth. The tip of an individualized hypha was cut with a scalpel and transferred to freshly prepared PDA dishes, which were maintained at 23°C in the dark for 10-15 days. To obtain monoascosporic isolates, the sclerotium was buried at a depth of 5 mm in autoclaved sand and incubated at 18°C/12 h photoperiod for production of apothecia. Tap water

was sprayed every day on the sand to keep the high humidity needed for carpogenic germination. From one mature apothecium of each sclerotium, one ascospore was arbitrary selected and transferred to PDA as previously described (Atallah et al. 2004). Sclerotia produced by colonies of the hyphal tip or single ascospore isolates were dried and stored at 4ºC.

All isolates were confirmed to be S. sclerotiorum as previously described (Lehner et al. 2015) using the specific primer pair SSasprF/SSasprR (Abd-Elmagid et al. 2013). To extract DNA, isolates were grown in liquid medium (10 g sucrose, 2 g l-asparagin, 2 g yeast extract, 1 g KH_2PO4, 0.1 g $MgSO_4.7H_2O$, 0.44 mg $ZnSO_4.7H_2O$, 0.48 mg $FeCl_3.6H_2O$, and 0.36 mg $MnCl_2.H_2O$) in Erlenmeyer flasks at 23°C for 7 days. 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, Madison, WI, USA) 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, USA).

Mycelial compatibility group. Mycelial compatibility was evaluated according to Schafer and Kohn (2006). The S. sclerotiorum isolates were paired in Petri dishes (60 x 15 mm) containing PDA added with 75 µL/L of McCormick's red food coloring. Disks (5-mm-diameter) from colonies grown on PDA for 2 days at 23ºC were distributed in Petri dishes with a total of four pairings per dish. The dishes were kept at 23°C in the darkness. Mycelial compatibility was checked visually at 3 and 6 days of incubation. Each pairing was replicated at least twice. Control self–self pairings were also carried out at each round of testing. When the results were inconsistent, the pairing was replicated twice. S. sclerotiorum isolates were analyzed in all-pairwise

combinations. Compatible isolates were distinguished by the absence of reaction line produced in the interaction zone between two isolates (Schafer and Kohn 2006).

Microsatellite genotyping. The isolates were genotyped as previously described (Lehner et al. 2015) using the microsatellite loci 7-2, 8-3, 9-2, 12-2, 13-2, 36-4, 42-4, 92-4, 106-4 and 114-4 (Sirjusingh and Kohn 2001). DNA of isolate LMK 211 was used as positive control since its microsatellite allele sizes had been previously determined. A sample without DNA was used as negative control. Both positive and negative controls were included in each run. A group of isolates was replicated three times with independent DNA extraction to confirm the reproducibility of the results. Data were processed with the GeneMarker Software V1.191 (Sounits Genetics). Each allele was determined according to the amplicon size of each isolate, taking into account the number of repeat units in each locus.

Analysis of DNA sequence of the oxaloacetate acetylhydrolase gene

PCR was performed in a final volume of 25 µL with 1 µL of DNA (25 ηg/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 and 12.5 µL of Dream Taq PCR Master Mix (2X) that includes dATP, dCTP, dGTP and dTTP (0.4 mM each) and 4 mM of MgCl2 (Thermo Fisher Scientific). DNA fragments were amplified using the primer pair OAHaF (5′-CGAGTTGCCCTTCAAGTT - 3′) and OAHbR-deg (5′- CCACCAGCCGYAGTAT - 3′) which flank a region of approximately 900 bp of the OAH gene (Andrew et al. 2012). 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, 72°C for 30 s, and a final extension at 72°C for 7 min. Amplification was confirmed using 5 µL of PCR product separated by electrophoresis on 1% agarose gel containing GelRed (Biotium, Hayward, CA, USA). PCR products were purified using ExoSAP-IT cleanup reagent (USB,

Cleveland, OH) and sequenced by Macrogen Services (Kumchun-ku, Seoul, Korea) using OAHaF and OAHbR-deg primers. The nucleotide sequences were edited using DNA BASER sequence assembly software (Heracle BioSoft) 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).

Aggressiveness assessment using the straw test. The straw test method (Petzoldt and Dickson 1996) was used according to Terán and Singh (2009). The cultivar Pérola and the line A195 were used in all tests. Pérola ("carioca" grain class, cream-striped) is widely used in Brazil and is susceptible to white mold (Vieira et al. 2010). The line A195 (beige colored grain with a distinctive yellow hilar ring) has partial white mold resistance (Singh et al. 2007). Experiments with each common bean genotype were carried out separately.

Plants were grown in 3 L-pots filled with the commercial substrate Tropstrato HT Hortaliças (Vida Verde, Mogi-Mirim, SP, Brazil). This substrate has pH 5.8 and is prepared with pine bark, processed peat, and vermiculite. Ten days after seedling emergence, 3 g of urea was distributed on the surface of every pot. A randomized design with four replicates was used. A pot with three plants (= experimental unit) was considered as a replicate.

Thirty days after planting, the main stem of the plants was cut 3 cm above the fourth node. A single two-day-old mycelial disc from the first subculture of each isolate was punched with a 1000 µL Eppendorf tip and capped over the cut stem of each plant. The length of the lesions was evaluated seven days after inoculation. The experiments with each common bean genotype were performed twice.

Aggressiveness assessments using the detached leaflet test. The two common bean genotypes used for the straw test were also used here. Leaflets of the youngest fully expanded trifoliate leaves of 5-week-old plants were placed on filter paper moistened with 5 mL of sterilized distilled water inside plastic boxes (11 width x 11 length x 3 cm height - gerbox). Two two-day-old mycelial discs (5 mm diameter) from the first subculture of each isolate were placed between the main vein and the leaflet edges; one disc on each side of the main vein. Boxes containing inoculated leaves were kept at 23°C in the dark. The lesion diameter was assessed 48 h after inoculation using a digital caliper. Treatments were replicated four times in a completely randomized design. The experiments were performed twice.

Mycelial growth rate and sclerotia production at three temperatures. Mycelial plugs (5 mm-diameter) from a two-day-old culture were placed in the center of Petri dishes (6 cm-diameter) containing 10 mL of PDA with 100 mg/L of chloramphenicol. The dishes were kept at 18°C, 23°C, and 28°C. Colony diameter was assessed at 24 and 29 h of incubation, and the average growth rate (mm/h) was estimated as: [(colony diameter measured with 29 h - colony diameter with 24 h)/5]. The number of sclerotia produced in each Petri dish was counted after 15 days of incubation. A completely randomized design with three replicates was used. Each replicate was considered as one colony in a plate. The experiment was performed twice.

Mycelial pigmentation. Assessment of colony pigmentation was conducted in colonies formed in the Petri dishes containing the mycelial plugs used in the previous experiment. Each colony was visually observed after 15 days of incubation and classified as non-pigmented, pigmented and highly pigmented as previously described (Lehner et al. 2014).

Data analysis

Microsatellite analyses. Each isolate was assigned to a haplotype using GENODIVE (Meirmans and Van Tienderen 2004). The GeneClass2 program (Piry et al. 2004) was used to calculate Nei's gene diversity (Nei 1987) among the isolates. To assess the genetic relationships among the haplotypes, we used the Alerquin software (Excoffier and Lischer 2010) to construct a minimum spanning network.

Aggressiveness assessment by the straw and detached leaflet tests. Data were analyzed for homogeneity of variance using Bartlett's test. Data from two experiments were pooled for statistical analysis when variances were homogeneous; experiments were analyzed separately when variances were not homogeneous. Analysis of variance (ANOVA) was performed using the R program. The overall mean and the standard deviation of the assessed variables were estimated for each cultivar. An isolate was considered less or more aggressive when the average of a given variable was either lower or greater, respectively, than the value of the standard deviation.

Mycelial growth rate and sclerotia production at three temperatures. The growing conditions for each of the three temperatures were considered as three independent experiments. Data were analyzed for homogeneity of variance using Bartlett's test. Additionally, the multivariate structure of the data was explored using a canonical discriminant analysis (CDA). Both analyses were performed in R.

Correlation analysis. The Mantel test was performed in GenAlex 6.41 with 10,000 permutations in order to estimate the correlation between the matrices of aggressiveness and genetic or phenotypic distances. The Bruvo's genetic distance (Bruvo et al. 2004) was calculated between all pairs of isolates using the Poppr package (Kamvar et al. 2014) for the R program. The Mahalanobis distance of aggressiveness or phenotypic data were also calculated using R.

Results

Genetic diversity based on mycelial compatibility groups, microsatellite markers and DNA sequences. Ten MCGs were found among the 20 S. sclerotiorum isolates (Table 1). Each isolate was a distinct SSR haplotype (Table 1; Fig. 1). The number of alleles at each locus varied from one, at locus 36-4, to seven, at locus 7-2 and 106-4 (Table 1). The mean gene diversity across all loci was 0.58.

The partial DNA sequence of AOH gene had 846 base pairs. The sequences of the Brazilian isolates shared 99.99–100% sequence similarity to S. sclerotiorum 1980 isolate (data not shown). Seven haplotypes were identified based on DNA sequence data. The isolates Ss-188, Ss-196 and Ss-79 were single haplotypes. The most frequent haplotype was detected in the isolates Ss-2, Ss-8, Ss-19, Ss-23, Ss-141, Ss-173 and Ss-203. Other haplotypes were comprised by the isolates Ss-44, Ss-51, Ss56, Ss-138 and Ss-193; isolates Ss-166, Ss-165, Ss-70 and 1980; and isolates Ss-136 and Ss-217.

Aggressiveness assessment using the straw and detached leaflet tests. The error variances for both experiments with cultivar Pérola for both straw and detached leaflet tests were similar, and the hypothesis of homoscedasticity could not be rejected. The effect of isolates on lesion length (straw test) was not significant ($F = 1.58$, $P = 0.07$), but aggressiveness varied among isolates for the lesion diameter in the detached leaflet test ($F = 1.71$, $P = 0.04$).

With the line A 195, variances between experiments were homogeneous in the detached leaflet test. Using the pooled data of the two experiments, aggressiveness was affected significantly by the isolates ($F = 4.04$, $P < 0.001$). Variances between experiments were heterogeneous in the straw test. Isolates affected aggressiveness in experiments 1 ($F = 3.27$, $P < 0.001$) and 2 ($F = 2.06$, $P = 0.018$).

In the straw test, the lesion length ranged from 5.7 (Ss-203) to 7.8 cm (Ss-136) for cv. Pérola (Figure 2A). For line A195, lesion length varied from 1.9 (Ss-2) to 4.3 cm (Ss-79) in the first experiment, and from 6.3 (Ss-166) to 10.4 cm (Ss-217) in the second experiment (Fig. 2B). In the detached leaflet test, the lesion diameter varied from 32.4 (isolate Ss-165) to 41.3 mm (Ss-51) for cv. Pérola (Fig. 2C), and from 27.3 (Ss-138) to 40.2 mm (Ss-217) for A195 (Fig. 2D). There was no difference regarding isolate aggressiveness for cv. Pérola (Fig 2A and 2B), whereas for A195 the isolate Ss-217 was more aggressive than the other isolates (Fig. 2B and 2D). Isolate Ss-136 had the second largest average values of lesion diameter and length (Experiment 2) when inoculated on plants of A195 (Fig. 2B and 2D). However, the value of lesion diameter did not differ from the overall mean.

Mycelial growth rate and sclerotia production at three temperatures. Variances between experiments were homogeneous. Incubation at 18°C ($F = 5.20$, $P < 0.001$), 23°C ($F = 9.23$, $P < 0.001$) or 28°C ($F = 4.71$, $P < 0.001$) affected the mycelial growth rate of the isolates. The average mycelial growth rate at 18°C ranged from 1.50 ± 0.43 (isolate Ss-138) to 2.20 ± 0.31 mm/h (Ss-203) (supplementary table). At 23°C growth rates varied from 1.77 ± 0.40 mm/h (Ss-79) to 2.65 ± 0.15 (Ss-203). The mycelial growth rate of isolates at 28°C was on average two times lower than that at 23°C. At 28°C, the mycelial growth rate ranged from 0.64 ± 0.12 (Ss-173) to 1.48 ± 0.08 mm/h (Ss-79).

Isolates affected the number of sclerotia produced at either 18°C ($F = 1.75$, $P = 0.04$) or at 23°C ($F = 2.91$, $P < 0.001$). The number of sclerotia per plate at 18°C varied from 19 ± 4 (isolate Ss-2) to 33 ± 8 (Ss-165) (supplementary table). The number of sclerotia per plate at 23°C varied from 11 ± 3 (Ss-188) to 30 ± 16 (Ss-23). Isolates of *S. sclerotiorum* did not produce sclerotia at 28°C.

The first two canonical variables captured 73.4% of the total variation of the morphological traits. Despite the high variability in mycelial growth rate and sclerotia production, CDA did not indicate distinct groups of isolates (Fig. 3).

Colony color. Isolates exhibited differences in mycelial pigmentation. The colonies of isolates classified as non-pigmented were white in color, the pigmented were brown and the highly pigmented were black colonies. Isolates Ss-138, Ss-193, and Ss-196 were non-pigmented. Isolates Ss-44, Ss-51, Ss-56, Ss-136, and Ss-217 were highly pigmented, whereas the other isolates were classified as pigmented (Fig.1).

Correlations. Correlations between aggressiveness and either genetic distance (based on SSR data) ($r = -0.03$, $P = 0.40$) or phenotypic distance (based on mycelial growth rate and sclerotia production) ($r = 0.17$, $P = 0.10$) were low and not significant.

Discussion

S. sclerotiorum is the most harmful soilborne pathogen of common bean in Brazil and other countries such as the USA, Canada and Argentina (Schwartz and Singh 2013). Thus, understanding the aggressiveness of the pathogen is valuable to improve the effectiveness of control practices, particularly the strategic use of cultivar resistance. We used two inoculation methods and two common bean genotypes to assess the aggressiveness of 20 S. sclerotiorum isolates from four Brazilian States. We did not estimate the incubation period due to the inherent difficulties in accurately assessing initial symptom development. The incubation period of white mold in common bean is generally short and, given the necrotrophic nature of the fungus, plant cell damage occurs within six hours of inoculation (Lumsden and Dow 1973). Latent period was not assessed, because it does not convey useful information for the purpose of estimating aggressiveness. The hyphae of S. sclerotiorum are formed on damaged cells (Lumsden

and Dow 1973), so the latent period is of limited utility to determine genotype resistance, since extensive necrosis has already occurred.

The detached leaflet and the straw test are effective methods to assess the aggressiveness of S. sclerotiorum isolates (Kull et al. 2003). The results of the present study corroborate these findings, since we observed that the aggressiveness of isolates did not change with the inoculation method. On the other hand, aggressiveness was affected by common bean cultivar. When the cv. Pérola was used, aggressiveness of the isolates was similar, regardless of the inoculation method. Using A195, however, the most aggressive isolate was Ss-217. Thus, A195 seems to be more suited to use in aggressiveness tests than cv. Pérola. One possible explanation for the lack of variation in aggressiveness when using cv. Pérola is that this cultivar is more susceptible to white mold than is A195. Pérola probably has fewer resistance genes, given that A195 is considered partially resistant to white mold (Singh et al. 2007). Aggressiveness of S. sclerotiorum has been a subject of many studies in different crops. Uniformity (Atallah et al. 2004; Sexton and Howlett 2004) as well as aggressiveness variation among isolates (Kull et al. 2004; Ekins et al. 2007; Otto-Hanson et al. 2011; Attanayake et al. 2013) have been reported. Thus, direct comparisons of experimental results must be done carefully, since isolate variability, inoculation method, host species and environmental conditions of the experiments may affect the results. Cultivar resistance also affects the results and this was clearly demonstrated in the present study, where the higher aggressiveness of the isolate Ss-217 was demonstrated to be dependent on common bean genotype.

One goal of this study was to evaluate the possible correlation between aggressiveness and genetic variability of the isolates. We found similar aggressiveness levels among 20 SSR haplotypes and no correlation between genetic distance and

aggressiveness. High genotypic diversity based on SSR markers was found among 35 S. sclerotiorum isolates from potato in the USA, but there was no variation in aggressiveness (Atallah et al. 2004). Similar results were reported for 43 S. sclerotiorum isolates from canola in Australia (Sexton and Howllet 2004). Contrary to these reports, differences in aggressiveness among SSR haplotypes of S. sclerotiorum collected in canola fields from the USA and China were detected (Attanayake et al. 2013). Isolates Ss-136 and Ss-217 were slightly more aggressive when tested on A195. They shared an identical and distinct DNA sequence of the OAH gene. It is known that oxalic acid is essential for the pathogenicity of S. sclerotiorum (Amselem et al. 2011), so it is possible that mutations in the OAH gene may interfere in the aggressiveness of isolates. Our results suggest that different SSR haplotypes of S. sclerotiorum do not necessarily have different aggressiveness levels in common bean. The pathogenic loci of S. sclerotiorum probably evolved independently of SSR loci, leading to a lack of association between aggressiveness and SSR haplotypes, as postulated for Phytophthora infestans (Goodwin et al. 1995). Therefore, further research should involve evaluations of the polymorphism in pathogenic loci and their effects on the aggressiveness of S. sclerotiorum isolates.

In some pathosystems MCG and aggressiveness were demonstrated to be associated (Douhan and Johnson 2001; Remesal et al. 2012). In S. sclerotiorum, there are studies reporting lack of variation (Atallah et al. 2004; Sexton and Howllet 2004) as well as differences in aggressiveness among MCGs (Kull et al. 2004; Irani et al. 2011; Otto-Hanson et al. 2011; Attanayake et al. 2013). In our study, isolates Ss-136 and Ss-217 belong to the same MCG and were slightly more aggressive in A195. The remaining isolates, although divided into nine other MCGs, had similar aggressiveness levels. Therefore, we have no evidence of association between MCGs and aggressiveness.

High variability in mycelial growth rate at 18°C, 23°C and 28°C and sclerotia production at 18°C and 23°C were found. Nevertheless, these characteristics were uninformative, because they did not allow separation of the isolates into distinct groups. In addition, there was no correlation between phenotypic and aggressiveness distance. When analyzing the Pearson's correlation coefficient between each pair of variables, we found significant correlation only between lesion length on A195 and mycelial growth rate at 28°C ($r = 0.51$, $P = 0.021$) and number of sclerotia at 23°C ($r = 0.56$, $P = 0.009$) and between mycelial growth rate at 28°C and lesion diameter on A195 ($r = 0.53$, $P = 0.015$) (data not shown). Due to the low magnitude and the lack of association, these correlations seem to have occurred randomly. These results are consistent with those of another study (Irani et al. 2011) and suggest that sclerotial production and mycelial growth rate have no influence on the aggressiveness of S. sclerotiorum isolates.

Isolates Ss-44, Ss-51, Ss-56, Ss-136 and Ss-217 have black mycelia and probably produce high amounts of melanin. An association of melanin and pathogenicity or aggressiveness has been reported in Colletotrichum lagenarium, Magnaporthe grisea (Howard and Ferrari, 1989) and Bipolaris sorokiniana (Chand et al. 2014). Melanin may be involved in pressure build-up in the appressorium, providing forces for mechanical penetration in the host cell (Howard and Ferrari, 1989). It may also provide rigidity in the cell wall, decreasing the pore size, meaning that a lower quantity of extracellular enzymes are released (Chand et al. 2014). In the present study all isolates were pathogenic to common bean, regardless of the site of inoculation (leaflets or stems) or melanin intensity. This result may be explained by the fact that S. sclerotiorum does not depend on pressure mediated by melanin in appressoria for penetration in the host cell. In addition to mechanical force via appressoria, the pathogen can penetrate the cuticle of the host plant using enzymes (Hegedus and

Rimmer 2005). Contrary to what is reported in the current study, melanin content was negatively correlated with extracellular enzymes and consequently aggressiveness in Bipolaris sorokiniana (Chand et al. 2014). Although isolates Ss-136 and Ss-217 had been slightly more aggressive when tested in A195, other highly-pigmented isolates showed aggressiveness levels similar to those presented by pigmented and non-pigmented isolates. Therefore, we did not find a direct relationship between mycelial pigmentation and pathogenicity or aggressiveness. Similar results were reported for eight S. sclerotiorum isolates (three darkly-pigmented) collected from rape in Australia (Garg et al. 2010).

This study has found similar aggressiveness levels among the 20 S. sclerotiorum isolates from common bean collected in four Brazilian States. Aggressiveness was not related with MCGs, SSR haplotypes or phenotypic traits, such as mycelial pigmentation, growth rate and sclerotia production. An important practical implication is that breeding programs for white mold resistance in Brazil that use artificial inoculations may use one or a few recently collected isolates, without major concerns about variation in pathogen aggressiveness.

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Figure legends

Figure 01. Minimum spanning network based on 10 SSR loci and mycelial pigmentation of Sclerotinia sclerotiorum isolates sampled from common bean fields in Brazil. Each node represents a haplotype. The number of loci one haplotype differs from another is represented between the nodes.

Figure 02. Stem lesion length measured in the straw test and lesion diameter on leaflets of common bean genotypes Pérola (A) and A195 (B). The lines indicate the overall mean of each variable. Gray bands correspond to the one standard deviation around the mean. In panel B, the dotted line indicates the mean of lesion length in experiment 1 and the dark gray band corresponds to one standard deviation around the mean of this variable.

Figure 03. Canonical discriminant analysis of the phenotypic variation observed among the 20 Sclerotinia sclerotiorum isolates. Dotted circles indicate the confidence region at 95% probability.

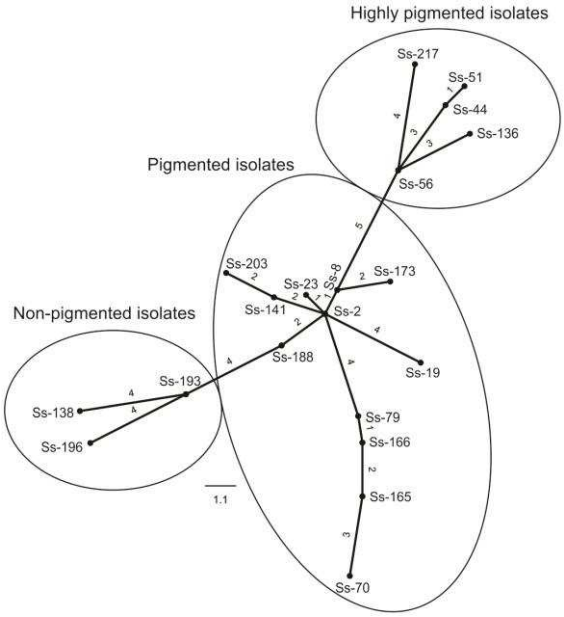

Figure 01. Lehner et al

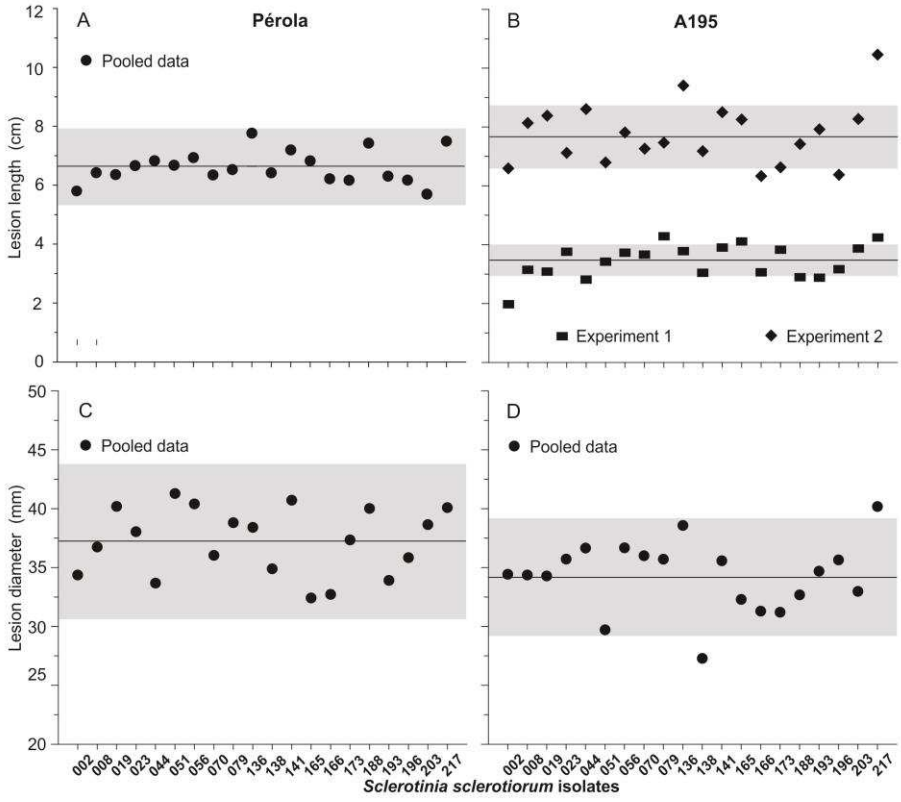

Figure 02. Lehner et al

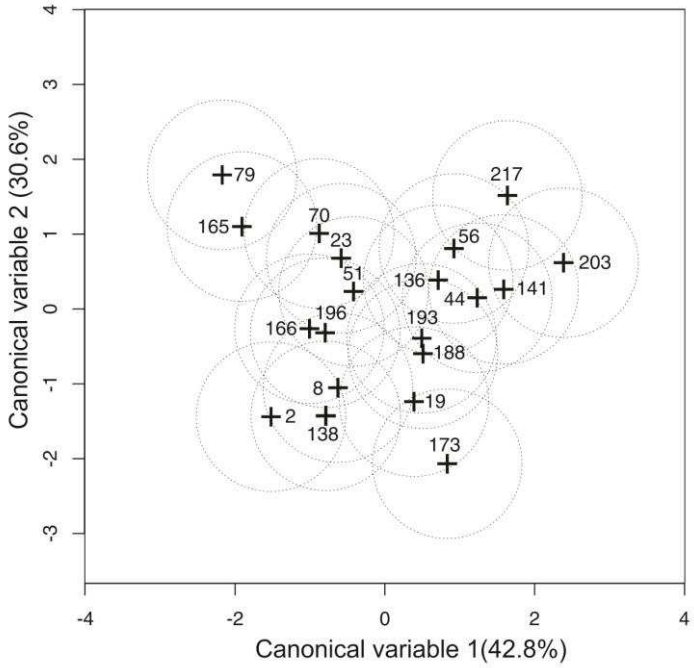

Figure 03. Lehner et al

Table 1. Microsatellite allele size in each locus and mycelial compatibility groups (MCG) of 20 Sclerotinia sclerotiorum isolates from four Brazilian states.

Isolate code[a]	Municipality-State of collection	Date of collection	Allele size (bp) at each locus										MCG
			7-2	12-2	13-2	114-4	9-2	106-4	8-3	92-4	42-4	36-4	
Ss-2	Oratórios-MG	June, 2010	160	214	305	408	356	504	248	373	405	412	1
Ss-8	Canaã-MG	June, 2010	160	216	305	408	356	504	248	373	405	412	1
Ss-19	Viçosa	June, 2010	162	218	285	408	356	504	248	373	407	412	1
Ss-23	Porto Firme-MG	July, 2010	160	212	305	408	356	504	248	373	407	412	1
Ss-44	Paracatu-MG	Aug., 2010	156	212	310	336	356	564	240	371	405	412	3
Ss-51	Paracatu-MG	August, 2010	156	212	310	336	356	568	240	371	405	412	3
Ss-56	Unaí-MG	August, 2010	160	216	310	336	356	572	240	371	405	412	3
Ss-70	Unaí-MG	August, 2010	172	222	305	412	356	568	254	375	405	410	2
Ss-79	Unaí-MG	August, 2010	170	216	305	408	356	568	248	375	405	412	2
Ss-136	Itaí-SP	Nov., 2011	160	214	310	336	356	520	242	371	405	414	4
Ss-138	Capão Bonito-SP	Dec., 2011	168	220	275	356	364	496	250	371	405	412	9
Ss-141	Capão Bonito-SP	December, 2011	160	216	305	408	356	504	250	373	405	412	1
Ss-165	Sta Maria Jetibá-ES	July, 2012	172	220	305	408	356	568	250	375	405	412	2
Ss-166	Sta Maria Jetibá-ES	July, 2012	170	220	305	408	356	568	250	375	405	412	2
Ss-173	Domingos Martins-ES	July, 2012	160	216	285	408	356	504	250	373	405	412	7
Ss-188	Mauá da Serra-PR	July, 2012	168	212	305	408	356	504	250	373	405	412	6
Ss-193	Corbélia-PR	July, 2012	168	220	290	356	356	572	250	373	405	412	8
Ss-196	Reserva do Iguaçu-PR	August, 2012	168	220	290	356	364	496	250	377	405	412	5
Ss-203	Três Barras-PR	July, 2012	160	220	305	408	356	504	250	371	405	412	10
Ss-217	Itararé-SP	August, 2012	158	214	310	336	356	528	242	371	405	412	4

[a] The isolates Ss-2, Ss-8, Ss-19, Ss-23, Ss-44, Ss-51, Ss-56, Ss-70 and Ss-79 came from hyphal tips, the others from a single ascospore.

MG = Minas Gerais state, SP = São Paulo state, ES = Espírito Santo state, PR = Paraná state

Supporting information

Supplementary table 01. Mean ± standard deviation of mycelial growth rate and sclerotia production on PDA medium of 20 Sclerotinia sclerotiorum isolates from four Brazilian states.

Isolate code	Mycelial growth rate (mm h^{-1})			Number of sclerotia	
	18°C	23°C	28°C	18°C	23°C
Ss-2	1.55 ± 0.09	1.99 ± 0.24	0.82 ± 0.13	19 ± 4	21 ± 5
Ss-8	1.63 ± 0.28	2.16 ± 0.13	0.90 ± 0.17	23 ± 5	20 ± 6
Ss-19	1.75 ± 0.13	2.35 ± 0.05	0.80 ± 0.25	28 ± 7	17 ± 5
Ss-23	1.78 ± 0.06	2.09 ± 0.07	1.09 ± 0.33	27 ± 8	30 ± 16
Ss-44	1.96 ± 0.11	2.46 ± 0.13	1.13 ± 0.31	20 ± 6	24 ± 10
Ss-51	1.71 ± 0.18	2.15 ± 0.15	1.18 ± 0.50	25 ± 5	22 ± 13
Ss-56	2.03 ± 0.17	2.37 ± 0.31	1.31 ± 0.17	27 ± 11	16 ± 9
Ss-70	1.81 ± 0.17	2.02 ± 0.08	1.24 ± 0.29	25 ± 6	26 ± 6
Ss-79	1.58 ± 0.21	1.77 ± 0.40	1.48 ± 0.08	27 ± 9	29 ± 8
Ss-136	1.77 ± 0.16	2.38 ± 0.28	1.31 ± 0.08	23 ± 8	20 ± 9
Ss-138	1.50 ± 0.43	2.15 ± 0.27	0.80 ± 0.07	26 ± 10	21 ± 6
Ss-141	1.73 ± 0.11	2.56 ± 0.13	1.14 ± 0.27	30 ± 6	25 ± 4
Ss-165	1.72 ± 0.01	1.81 ± 0.15	1.17 ± 0.38	33 ± 8	26 ± 9
Ss-166	1.60 ± 0.14	2.06 ± 0.11	1.16 ± 0.24	19 ± 8	21 ± 6
Ss-173	1.89 ± 0.15	2.44 ± 0.15	0.64 ± 0.12	23 ± 4	14 ± 5
Ss-188	1.91 ± 0.21	2.34 ± 0.20	1.14 ± 0.20	19 ± 6	11 ± 3
Ss-193	1.88 ± 0.16	2.34 ± 0.18	1.14 ± 0.20	21 ± 7	14 ± 5
Ss-200	1.62 ± 0.11	2.11 ± 0.13	1.15 ± 0.20	21 ± 6	19 ± 3
Ss-203	2.20 ± 0.31	2.65 ± 0.15	1.25 ± 0.24	22 ± 8	19 ± 4
Ss-217	1.9 ± 0.13	2.52 ± 0.19	1.39 ± 0.19	28 ± 7	28 ± 5
Average	1.75	2.24	1.11	24	21

Chapter 3

Fungicide sensitivity of Sclerotinia sclerotiorum: A thorough assessment using discriminatory dose, EC_{50}, high resolution melt analysis and description of new point mutation associated with thiophanate-methyl resistance

Abstract

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 EC_{50} values. A total of 282 isolates from the most important production areas were screened and none was resistant to fluazinam or procymidone. The EC_{50} 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 EC_{50} of the TM-resistant isolate was greater than 100 µg/mL, whereas the EC_{50} 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 HRM analysis allowed the distinction of TM-sensitive and resistant isolates by specific melting peaks and curves. The TM-resistant isolate had comparable mycelial growth, sclerotia production and pathogenicity to 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 non existent or rare. Resistance management practices should be implemented, however, to delay the spread of TM-resistant genotypes.

Key words: Phaseolus vulgaris, white mold, sclerotinia stem rot, chemical control

Introduction

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 beans. 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 (18-23°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 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 altitude.

Fungicide application is the most used strategy to control white mold in common beans. To date, there is no commercial cultivar with high levels of resistance to white

mold and growers heavily rely 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 multi-site 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, 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. But, 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 to 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 have 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 (Tm). At this temperature half of the double-stranded DNA amplicon will dissociate and become single strand DNA. The Tm 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 SNPs 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

Sclerotinia sclerotiorum isolates. Two hundred eighty two 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 (five isolates), Paraná (25), São Paulo (30), Minas Gerais (99), Espírito Santo (25), Goiás (49), Bahia (15) and Pernambuco (34) (Fig. 1). These states 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 4ºC.

Determination of discriminatory doses and EC_{50} 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), 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 of active ingredient/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 (42-50°C), but non-solidified PDA medium. The concentrations used for each fungicide were: 0 (PDA + DMSO), 0.5, 1.0, 5.0, 10 and

100 µg of TM/mL; 0, 0.0025, 0.005, 0.01, 0.05 and 0.1 µg of fluzinam/mL and 0, 0.05, 0.1, 0.25, 0.5 and 1.0 µg of procymidone/mL. Mycelial plugs (5 mm diameter) from a 2-day-old culture of the 31 isolates were placed in the center of Petri dishes (6 cm 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 (EC_{50}) using the R program. Because the variances of the two experiments were homogeneous, the mycelial growth and EC_{50} 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 diameter) from a 2-day-old culture of each isolate were placed in the center of Petri dishes (6 cm diameter) containing 10 mL of PDA amended with 5, 0.05, or 0.5 µg/mL the discriminatory dose of TM, fluazinam, and procymidone, respectively. After 24 h of incubation at 23°C in the dark, colony

diameter 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 on the fungicide-amended PDA. They were considered sensitive if they grew less than 50% compared to the control on the fungicide-amended PDA.

Analysis of DNA sequence of the β-tubulin gene from sensitive and resistant isolates 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 sucrose, 2 g l-asparagin, 2 g yeast extract, 1 g KH2PO4, 0.1 g MgSO4.7H2O, 0.44 mg ZnSO4.7H2O, 0.48 mg FeCl3.6H2O, and 0.36 mg MnCl2. H2O) 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) 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).

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 (BSA) at 50 mg/mL, 1 µL of each primer at 10 µM, 6 µL of water and 12.5 µL of Dream Taq PCR Master Mix (2X) that includes dATP, dCTP, dGTP and dTTP (0.4 mM each) and 4 mM of MgCl2 (Thermo Fisher Scientific). The primer pair used was TUB-F1 (5′-GCTTTTGATCTCCAAGATCCG-3′; nucleotide positions, 1757 to 1777) and TUB-R1 (5′-CTGGTCAAAGGAGCAAATCC3′; nucleotide positions, 2134 to 2115). 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, 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 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). 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.). PCR was performed in a final volume of 10 µL with 1 µL of DNA (25 ng/µL), 5 µL of 2 x 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, 72°C for 10 s. The HRM analysis was performed by heating the amplicon DNA gradually from 60°C up 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 thiophanate-methyl. 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-diameter) from a 2-day-old culture were placed in the center of Petri dishes (9 cm-diameter) containing 15 mL of PDA with 100 mg of chloramphenicol/L. The dishes were kept at 23°C. Colony diameter was assessed after 24 and 29 h of incubation and the average growth rate (mm/h) was estimated as: (colony diameter measured at 29 h of incubation - colony diameter 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 width x 11 length x 3 cm height - gerbox). One 2-day-old mycelial disc (5 mm-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 the 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 GENES software. Treatments (isolates) and experiments effects were considered as random factors in the analysis. The linear model fit to the data was: Yijk = m + Gi + Aj

+ GAij + Eijk . Gi is the effect of treatments, Aj is the effect of experiments, GAij is the treatment x experiment interaction term, and Eijk is the error effect.

Results

Determination of discriminatory doses. At 0.5 µg of TM/mL more than 75% of the isolates grew over 80% relative to the control (Fig. 2A). At 1.0 µg/mL, the mycelial growth varied from 45.5 % to 83.7 % and more than 75% of the isolates grew over 50% relative to the control. At 5.0 µg/mL, the mycelial growth varied from 14.5% to 31.0% and more than 75% of the isolates grew less than 25%, therefore this concentration was chosen as discriminatory for TM. At 10 and 100 µg/mL, all isolates grew between 13% and 21% relative to the control (Fig. 2A).

Compared to the control, the mycelial growth of S. sclerotiorum isolates varied from 42.8% to 76.5% at 0.0025 µg of fluazinam/mL and from 31.3% to 54.7% at 0.005 µg/mL (Fig 2B). At 0.01 µg/mL, all isolates grew between 25% and 38% relative to the control and the median value was 32%. At 0.05 µg/mL, the median was 19.2% and all isolates grew less than 25%, therefore, this concentration was chosen as discriminatory for fluazinam. At 0.1 µg/mL, the mycelial growth varied from 14% to 20.5%, relative to the control (Fig. 2B).

At 0.05 and 0.1 µg of procymidone/mL, more than 75% of the isolates grew over 80%, compared to the control (Fig. 2C). At 0.25 µg/mL, the mycelial growth ranged from 40.6% to 69.8% and the median value was 57.3%. At 0.5 µg/mL, the mycelial growth varied from 18.7% to 27.2% and 90% of the isolates grew less than 25%. Therefore, this concentration was chosen as discriminatory for procymidone. At 1.0 µg/mL, all isolates grew between 13% and 21%, in relation to the control (Fig. 2C).

The EC_{50} values for TM ranged from 0.38 µg/mL to 2.23 µg/mL (Fig. 3A) and the overall mean was 1.16 µg/mL. For fluazinam the EC_{50} values ranged from 0.003

µg/mL to 0.007 µg/mL (Fig. 3B) and the overall mean was 0.005 µg/mL. For procymidone the EC_{50} values ranged from 0.11 µg/mL to 0.72 µg/mL (Fig. 3C) and the overall mean was 0.35 µg/mL.

Sensitivity of Sclerotinia sclerotiorum isolates to different fungicides. Using the discriminatory dose of 5.0 µg/mL, one out 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 to the control, Ss-7 grew 100% at 0.5 and 1.0 µg/mL and 98%, 94% and 47% at 5.0 µg/mL, 10 µg/mL and 100 µg/mL, respectively (inset of Fig. 1A). The mycelial growth of the other 282 isolates varied from 15% to 40%, compared to 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, a 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 to the control, for fluazinam and procymidone, respectively (data not shown).

Assessment of thiophanate-methyl resistance using HRM. The TM resistant isolate showed a specific melting peak (Tm = 82.9 °C) and curve based on the HRM analysis, allowing differentiation from the sensitive isolates (Tm = 83.2°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

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 to other related species of Sclerotiniaceae, such as Botrytis 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.

A variety of 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 and/or spore germination (Russel 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 (Russel 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%. So, 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 resistant isolates 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 only one S. sclerotiorum isolate was resistant to TM. In a sample of 31 isolates, the estimated EC_{50} of sensitive isolates ranged from 0.38 µg/mL to 2.23 µg/mL, whereas the estimated EC_{50} of resistant isolate was 106 µg/mL. These results are consistent with those of other studies that found EC_{50} values of benzimidazoles resistant isolates higher than 5.0 µg/mL in S. sclerotiorum (Ma et al. 2009) and higher than 50 µg/mL in M. fructicola 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: the E198A and the 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 the 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 EC_{50} values between 0.5 µg/mL and 3.0 µg/mL (T. yallundae) or on the capacity of isolates to grow at 1 µg/mL and at 5 µg/mL, but not at 50 µg of TM/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; Matheron and Porchas 2004; Vieira et al. 2012; Mahoney et al. 2014). 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 EC_{50} value reported here supports this claim. In other studies, S. sclerotiorum isolates from canola fields in China and United States (Attanayake et al. 2013) and from one alfalfa field in USA (Attanayake et al. 2012) were assessed using the discriminatory dose of 0.005 µg of fluazinam/mL. The authors found mycelial growth ranging from 20% to 40%, and from 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. fructicola (Lim and Cha 2003) and Sclerotinia 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; Suga et al. 2011; Chatzidimopoulos et al. 2014). 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 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 to use 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 to detect any resistant isolate, without assuming the existence of a specific mutation. The TM-resistant isolate had comparable mycelial growth, sclerotia production and pathogenicity to the sensitive isolates. This suggests that it has sufficient parasitic fitness to compete with sensitive isolates in field. 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 up to date there is no evidence of loss of sensitivity.

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Figure legends

Figure 01. 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.

Figure 02. Box plots showing the percentage of mycelial growth of the 31 S. 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 of each box plot represent outliers. (A) Thiophanate-methyl. (B) Fluazinam. (C) Procymidone.

Figure 03. Density frequency of the EC_{50} values of the 31 S. sclerotiorum isolates estimated for TM, fluazinam and procymidone. The EC_{50} values (µg 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.

Figure 04. Alignment of β-tubulin amino acid sequences from S. sclerotiorum isolates. The codes Ss-7_BR1 and Ss_7_BR2 correspond to biological replicates of the TM-resistant Ss-7 isolate.

Figure 05. Differentiation of the S. sclerotiorum isolates sensitive and resistant to TM via HRM analysis. (A) Melting peaks. (B) Melting curves.

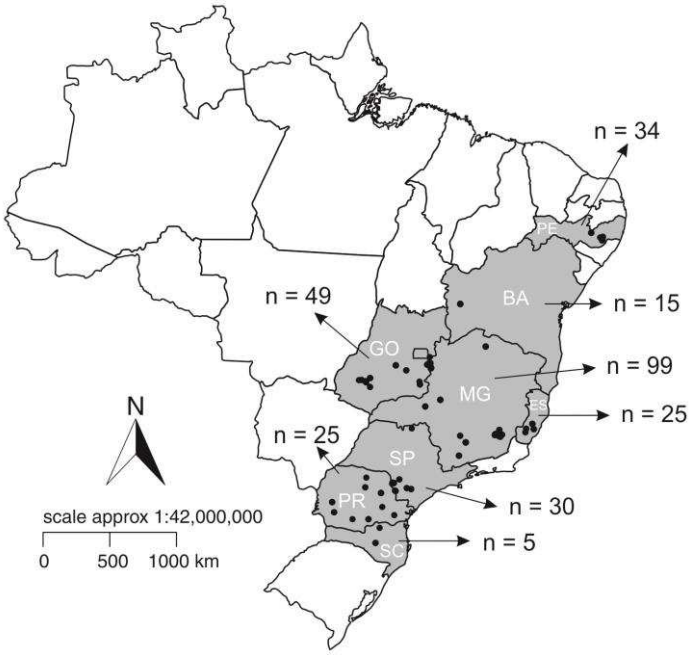

Figure 01. Lehner et al

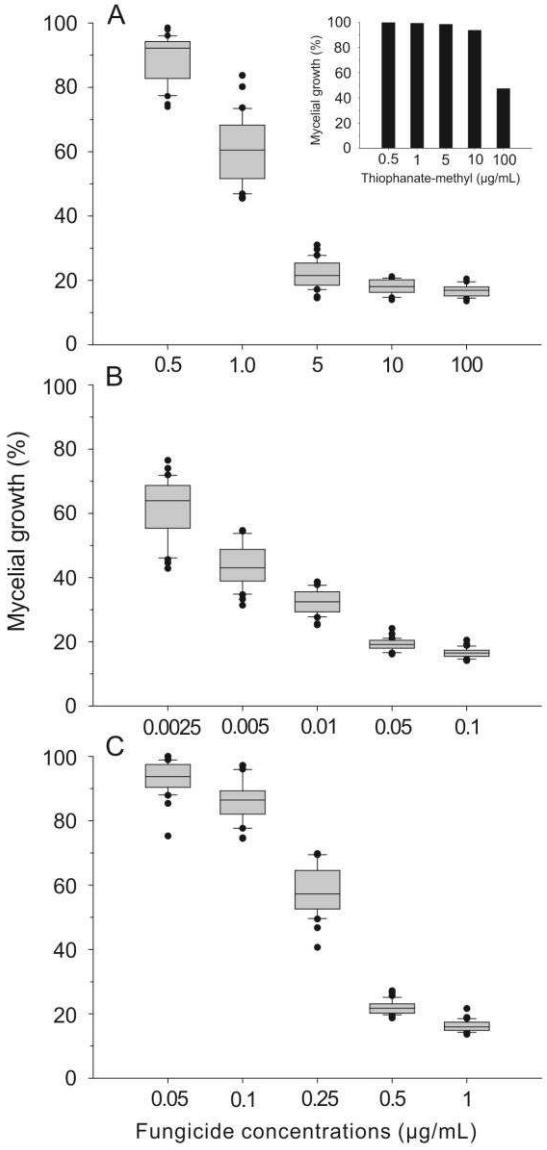

Figure 02. Lehner et al

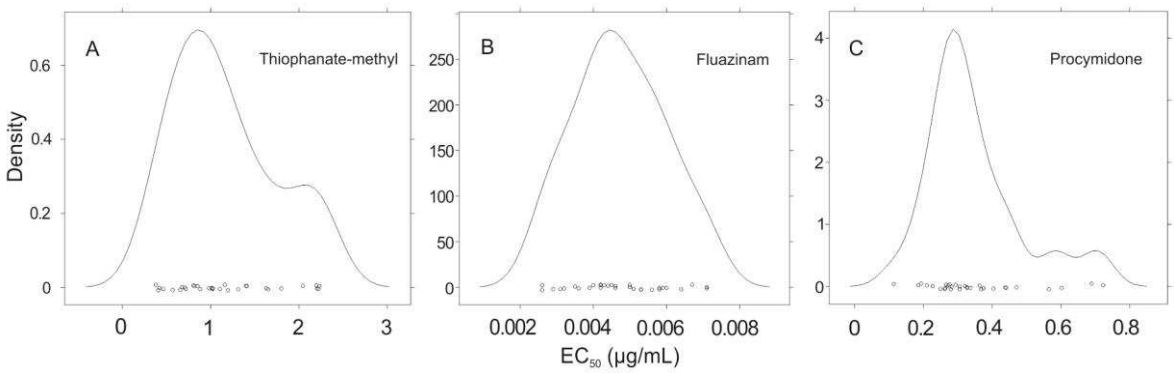

Figure 03. Lehner et al

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                                  154                                                                198                                233
                                                                                                     ↑
Sclerotinia_sclerotiorum_1980 KIREEFPDRMMATFSVVPSPKVSDTVVEPYNATLSVHQLVENSDETFCIDNEALYDICMRTLKLSHPSYGDLNHLVSAVM
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
                                            *
Sclerotinia_sclerotiorum_1980 SGVTTCLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGF
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............................
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Figure 04. Lehner et al

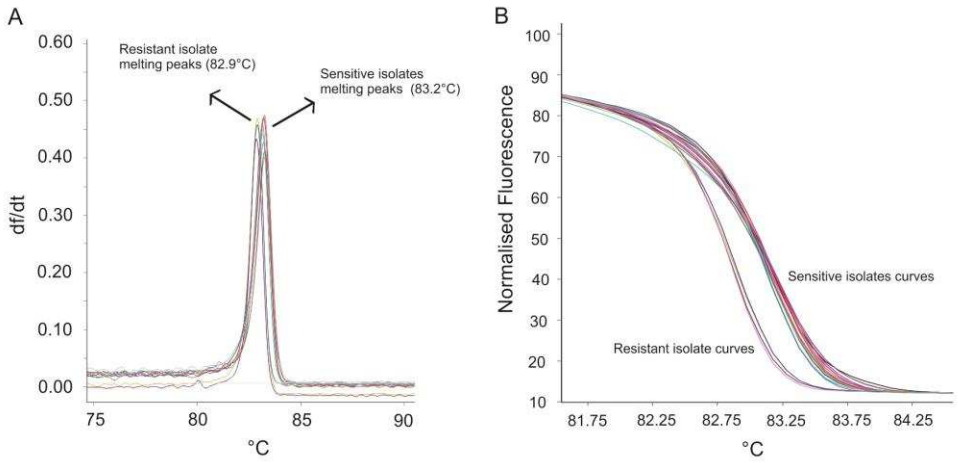

Figure 05. Lehner et al

Table 1. Geographical origin and comparison of mycelial growth, sclerotia production and pathogenicity among the TM-resistant and the sensitive isolates of S. sclerotiorum from eight Brazilian States

Isolate code	State of origin (region)	Phenotype	Mycelial growth rate (mm h^{-1}) [a]	Number of sclerotia [b]	Lesion diameter (mm) [c]
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]The mycelial radial growth was measured after 24 and 29 h of incubation on potato-dextrose-agar at 23°C in the dark. The mycelial growth rate (mm/81h) was estimated as: (colony diameter measured with 29 h - colony diameter with 24 h)/5).
[b]Sclerotia were counted after 21 days of incubation of isolates on PDA at 23°C in the dark.
[c]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.
*Mean ± standard deviation.

General conclusions

1. The population of Sclerotinia sclerotiorum affecting common bean in Brazil consist of several mycelial compatibility groups that when evaluated together provide a clonal genetic structure, without evidence of recombination.

2. The MCGs acts as isolated subpopulations within which isolates can randomly mate.

3. Different genotypes (based on microsatellite markers) and phenotypes of S. sclerotiorum have similar aggressiveness.

4. For Brazilian isolates of S. sclerotiorum resistance to fluazinam or procimidone is non existent or rare. To thiophanate-methyl, however, it exist and does not affect the mycelial growth rate, sclerotia production or pathogenicity.