RHAPHAEL ALVES SILVA

FUNGICIDE SENSITIVITY AND GENETIC STRUCTURE OF THE POPULATION OF *Alternaria grandis*

Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Eduardo Seiti Gomide Mizubuti

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

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ABSTRACT

SILVA, Rhaphael Alves, D.Sc., Universidade Federal de Viçosa, August, 2022. **Fungicide sensitivity and genetic structure of the population of *Alternaria grandis*.** Adviser: Eduardo Seiti Gomide Mizubuti.

Early blight (EB), caused by *Alternaria grandis*, *A. linariae*, or *A. solani*, is a severe disease of potato and tomato crops and fungicide application is the most used strategy to reduce yield losses. This study was conducted to: i. identify *Alternaria* spp. associated with EB of potato and tomato; ii. develop SSR markers for the dominant species causing potato EB; iii. assess the genetic variability of the population of the predominant species; and iv. assess the sensitivity of the pathogen to SDHIs, DMIs, and chlorothalonil (CT). *A. grandis* and *A. linariae* were the predominant species associated with EB of potato and tomato, respectively. Twelve SSR markers were designed for *A. grandis* and 302 isolates were genotyped to assess the genetic structure of the pathogen population. A total of 253 MLLs were identified. The population of *A. grandis* has high genetic variability, but no structure could be detected. Fungicide sensitivity was assessed using two methods: the microtiter method for SDHIs (BosCalid; FluoPyram; FluXapyroxad; PydiFlumetofen; and PenthioPyrad); and mycelial growth on fungicide-amended medium for difenoconazole (DC) and CT. Additionally, discriminatory doses (DD) were established for high-throughput screening of isolates. The EC50 values for CT and DC ranged from 109.3 to 905.4 and 0.03 to 2.27 µg/mL, respectively, and the DD for CT and DC were 1000 µg/mL and 5 µg/mL, respectively. There is no evidence of resistance to DC, but there are isolates less sensitive to CT. The EC50 values of BC, FP, FX, PF, and PP ranged from 0.3 to 0.8; 0.9 to 69.7; 0.6 to 11.3; 0.2 to 92.5; and 6.7 to 61.5 µg/mL, respectively, and the DD were 100, 25, 25, 10, and 25 µg/mL, respectively. The sensitivity to SDHIs of 113 isolates was assessed using the DD. For BC, all isolates were classified as insensitive; for PP, FX, FP, and PF, 94%, 89%, 63%, and 61% were classified as insensitive to the respective fungicides. The reduced sensitivity of *A. grandis* to BC, FX, and PP fungicides is probably associated with the widespread occurrence of the H278Y and H134R mutations in the *sdhB* and *sdhC* genes, respectively. Management of EB must employ multi-site fungicides and site-specific products with different modes of actions.

Keywords: *Alternaria* spp.. Genetic variability. Multi-site fungicides.

RESUMO

SILVA, Rhaphael Alves, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Sensibilidade a fungicidas e estrutura genética da população de *Alternaria grandis*.** Orientador: Eduardo Seiti Gomide Mizubuti.

A pinta preta (PP), causada por *Alternaria grandis*, *A. linariae*, ou *A. solani*, é uma das principais doenças da batateira e tomateiro. A estratégia de manejo mais eficiente para o controle da PP é a aplicação de fungicidas. Este estudo foi conduzido para: i. identificar as espécies de *Alternaria* associadas com a PP da batateira e do tomateiro; ii. desenvolver marcadores SSR para a espécie predominante; iii. avaliar a variabilidade genética da população da espécie predominante; e iv. avaliar a sensibilidade do patógeno a SDHIs, DMIs e clorotalonil (CT). *A. grandis* e *A. linariae* foram as espécies predominantes associadas à PP da batateira e do tomateiro, respectivamente. Doze marcadores SSR foram desenvolvidos e a estrutura genética da população de *A. grandis* foi investigada por meio de 302 isolados. Um total de 253 MLLs foram identificados. A população possui alta variabilidade genética, mas nenhuma estruturação foi detectada. A sensibilidade aos fungicidas foi avaliada com diferentes doses usando dois métodos: microtitulação para SDHIs (BosCalida; FluoPiram; FluXapiroxade; PidiFlumetofen; e PentioPirade); e crescimento micelial em meio de cultura contendo difenoconazol (DC) ou CT. Doses discriminatórias (DD) foram estabelecidas para triagem de vários isolados. Os valores de EC50 para CT e DC variaram de 109,3 a 905,4 e de 0,03 a 2,27 µg/mL, respectivamente. A DD para CT e DC foi 1000 µg/mL e 5 µg/mL, respectivamente. Não houve resistência a DC, mas existem isolados menos sensíveis a CT. Os valores de EC50 para BC, FP, FX, PF e PP variaram de 0,3 a 0,8; 0,9 a 69,7; 0,6 a 11,3; 0,2 a 92,5; e 6,7 a 61,5 µg/mL, respectivamente, e a DD foi de 100, 25, 25, 10 e 25 µg/mL, respectivamente. A sensibilidade de 113 isolados foi avaliada usando a DD para SDHIs. Para BC, todos os isolados foram classificados como insensíveis; para PP, FX, FP, e PF, 94%, 89%, 63%, e 61% foram classificados como insensíveis aos respectivos fungicidas. A sensibilidade reduzida de *A. grandis* aos fungicidas BC, FX e PP provavelmente está associada à ampla ocorrência das mutações H278Y e H134R nos genes *sdhB* e *sdhC*, respectivamente. No manejo da PP, recomenda-se o uso de fungicidas multi-sítios e sítio-específicos com diferentes modos de ação.

Palavras-chave: *Alternaria* spp.. Variabilidade genética. Fungicidas multi-sítios.

SUMMARY

GENERAL INTRODUCTION

The genus *Alternaria* was recently divided into 24 sections (Woudenberg et al. 2013) based on molecular and morphological data (Lawrence et al. 2013; Woudenberg et al. 2013). The *Alternaria* section *Porri* contains most of the large-spored species, among which the highly destructive pathogens that cause early blight (EB) on potato and tomato are classified (Woudenberg et al. 2014).

Different large-spored *Alternaria* spp. can cause EB on potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.) fields such as *A. grandis*, *A. linariae*, *A. protenta,* and *A. solani* (Rodrigues et al. 2010; Woudenberg et al. 2014; Simmons 2000). The disease caused by *Alternaria* spp. on potato is of common occurrence, can cause severe yield losses, and require intensive fungicide applications to be kept under satisfactory yield-limiting levels (Batista et al. 2006; Leiminger and Hausladen 2012). The multiplicity of species causing apparently similar diseases require further investigations on specific biological processes, mainly those associated with virulence, ecological responses and the effects of environmental conditions on development of epidemics and the evolution of fungicide resistance. Ultimately, the correct identification of *Alternaria* spp. associated with necrotic lesions is important to improve the efficacy of control measures.

To date, there are no commercial cultivars with complete resistance to EB and fungicide application is the most used strategy to manage this disease. The succinate dehydrogenase-inhibitors (SDHIs), quinone outside inhibitors (QoIs), and demethylation inhibitors (DMIs) are commonly used fungicides for EB (Agrofit 2022), but these site-specific products are considered of high and medium risk for selection of resistant individuals (FRAC code 7, 11, and 3), respectively. Multi-site fungicides such as chlorothalonil are registered to be used in potato crops in Brazil (Agrofit 2022) and have often been applied to manage EB. Chlorothalonil is a fungicide with low risk of resistance (FRAC code M05) and is frequently used as a preventive measure to control EB and reduce the risks of developing resistance to site-specific molecules.

Several studies were conducted to investigate the sensitivity of isolates of *Alternaria* spp. to fungicides and the scenario varies according to the chemical characteristics of the products. For DMIs, only one study (Zhang et al. 2020), among several conducted (Avenot et al. 2016; Fonseka and Gudmestad 2016; Landschoot et al. 2017b; He et al. 2019; Wang et al. 2016) reported resistant isolates to difenoconazole. In this single report, only one isolate had a mutation in the *CYP51* gene that led to the exchange of arginine to tryptophan (R511W) at position 511 (Zhang et al. 2020). In contrast to the scenario of resistance to the DMI, in the past years several studies reported the occurrence of resistance of *Alternaria* spp. to SDHI-fungicides in USA (Avenot et al. 2008, 2009, 2019; Fairchild et al. 2013; Gudmestad et al. 2013; Mallik et al. 2014; Miles et al. 2014; Tymon and Johnson 2014; Bauske et al. 2018; Bauske and Gudmestad 2018; Yang et al. 2015), Belgium (Landschoot et al. 2017b, 2017a), China (Shi et al. 2015), Germany (Metz et al. 2019; Nottensteiner et al. 2019) and Greece (Malandrakis et al. 2015). The mutations associated with *A. solani* SDHI-resistant isolates had been found in the subunits of the *AsSdh* gene: *SdhB*, *SdhC*, and *SdhD* (Mallik et al. 2014). However, until now there are no SDHI-resistant individuals publicly reported in Brazil.

Intensive use of SDHI-fungicides can select resistant *Alternaria* spp. individuals relatively fast because of the site-specific mode of action and the biological characteristics of the pathogens. *Alternaria* spp. can produce copious numbers of asexual spores in one season leading to large population sizes. The larger the population, the higher the chances of a large number of mutants resistant to the compounds. In addition to the implications of high genetic variability to resistance to fungicides, another important management component that can be seriously compromised is the durability of cultivar resistance. Thus, quantifying the amount of genetic variation and its distribution, i.e. the genetic structure of the population of *Alternaria* spp., is key to establish better EB management strategies.

The genetic structure of the population of a pathogen is determined by the extant amount and distribution of the genetic variation (McDonald and Linde 2002). There is very limited information about the population of *Alternaria* spp. affecting potato in Brazil. Only two studies were conducted to assess the genetic variability of large-spored *A. solani*. The two studies were based on individuals collected from potato and tomato in Brazil. The authors used RAPD and AFLP markers and also partial sequences of ITS, *Alt A1*, and *gpdh* to assess the genetic structure and to investigate the evolutionary processes that shape the genetic variation of the pathogen population (Lourenço et al. 2009, 2011). They reported that the population of *A. solani* in Brazil was clonal and structured by hosts. However, most likely, the structuring according to host was later found to be due to the occurrence of different species associated with early blight in potato and in tomato (Rodrigues et al. 2010). There is no information about the most prevalent species causing EB in Brazil. Moreover, no study was conducted to assess the genetic variability of the populations of these pathogens. Therefore, there is a need to determine the most frequent species and to quantify the amount and distribution of the genetic variation of the populations of different *Alternaria* spp. using highly polymorphic neutral markers such as simple-sequence repeats (SSRs or microsatellites). The knowledge generated by such studies can support the implementation of better control strategies; the development of breeding programs targeting varieties with durable resistance and to increase the usage time of fungicide molecules.

CHAPTER 1: *Alternaria grandis* is the predominant species associated with potato early blight in Brazil

ABSTRACT

Early blight (EB) is a severe disease of potato and tomato crops worldwide and can be caused more oftenly by three species of *Alternaria*: *A. grandis*, *A. solani*, and *A. linariae*. Previous studies using a smaller number of isolates sampled from a reduced number of regions suggested that *A. grandis* was the predominant EB pathogen associated with potato in Brazil. The main objective of the current study was to identify the causal agent of EB using 314 isolates of *Alternaria* from the most important potato and tomato growing regions in Brazil. The identification of 300 isolates collected from potato in seven different regions of Brazil and 14 isolates obtained from tomato in Minas Gerais state during 2019, 2020, and 2021 growing seasons was based on two molecular approaches: High resolution melting (HRM) and phylogenetic analyses. Isolates were submitted to HRM analysis based on polymorphisms in the calmodulin gene. All *Alternaria* spp. isolates from potato were identified as *A. grandis* and all isolates from tomato were identified as *A. linariae*. Thirteen *A. grandis* isolates collected from different potato-producing regions, one *A. solani* from Guarapuava, Paraná state, three *A. linariae* collected from tomato in Minas Gerais state, a representative isolate of *A. solani* (CBS 109157), and ex-type isolates of *A. grandis* (CBS 109158) and *A. linariae* (CBS 109156) were selected for phylogenetic analyses based on the partial sequences of the internal transcribed spacer regions 1 and 4, *Alternaria* major allergen, glyceraldehyde-3-phosphate dehydrogenase, and RNA polymerase II beta subunit. Two monophyletic groups were identified, one was composed of all *A. linariae* with its ex-type. The other clusters were composed of *A. grandis* and *A. solani* isolates, but all *A. grandis* isolates grouped closer together. The results support the previous conclusions that *A. grandis* and *A. linariae* are the predominant species associated with EB in potato and tomato, respectively, in Brazil.

INTRODUCTION

The genus *Alternaria* includes saprophytic, endophytic and pathogenic species distributed worldwide. These pathogenic species can cause diseases in plants and animals (Thomma 2003). *Alternaria* plant-pathogenic species with large conidia, or large-spored species, cause several diseases on cultivated and uncultivated plants, including potato (*Solanum tuberosum* L.) and tomato (S. *lycopersicum* L.) (Peixoto et al. 2021). The genus *Alternaria* belong to the phylum Ascomycota, class Dothideomycetes, order Pleosporales, and to the Pleosporaceae family.

New species of large-spored *Alternaria* are supported by both morphological and molecular markers. A robust taxonomic study recognized many *Alternaria* species after summarizing all the results based on morphological characteristics (Simmons 1992; 2000; 2007). Later, molecular tools used to investigate *Alternaria* taxonomy supported some of the morphospecies (Pryor and Michailides 2002; Hong et al. 2005; Andrew et al. 2009; Lawrence et al. 2012). Recently, eight taxonomic sections were defined in the species-group *Alternaria* based on morphological and molecular data. The sections were: *Brassicicola*, *Panax*, *Gypsophilae*, *Radicina*, *Sonchi*, *Alternantherae*, *Alternaria* and *Porri* (Lawrence et al. 2013). Another phylogenetic study was conducted and divided the genera *Alternaria* into 24 sections (Woudenberg et al. 2013). The section *Porri* have species that can cause early blight (EB) in potato and tomato crops.

The section *Porri* is composed of asexual species that produce large, non concatenated conidia with long beaks (Woudenberg et al. 2014). For many years, *A. solani* Sorauer was considered as the only causal agent of EB on Solanaceae (Rotem 1994). However, a study reviewed the species associated with Solanaceae and *A. grandis* and *A. tomatophila* (syn. *A. linariae*) were also included as pathogenic to potato and tomato, respectively (Simmons 2000). *A. solani*, *A. grandis*, and *A. linariae* can cause disease in both crops, but *A. solani* and *A. grandis* are most commonly found associated with EB on potato, while *A. linariae* cause disease preferently on tomato. In Brazil, *A. grandis* and *A. linariae* seem to be the most prevalent species causing EB on potato and tomato, respectively (Rodrigues et al. 2010; Peixoto et al. 2021).

A. grandis was first reported causing EB on potatoes in the United States, by Dr. Barbara J. Christ in 1996 (Simmons 2000). *A. grandis* and *A. solani* belong to the Sect. *Porri*, but conidia of *A. grandis* are larger (conidia body: 141-192 length x 26-38 µm width; and beak length: 160-200 µm) than those of *A. solani* (conidia body: 109-115 length x 18-26 µm width and beak length: 80-118) (Simmons, 2000). Based on molecular characters, *A. grandis* differs by one nucleotide at position 99 (T to C) on GPDH (Woudenberg et al. 2014) and eight nucleotides in the calmodulin gene (Gannibal et al. 2014); therefore, *A. grandis* was not synonymized under *A. solani* (Woudenberg et al. 2014).

The correct identification of the pathogen species associated with a disease is very important to improve its management. Therefore, the objective of this study was to identify the *Alternaria* spp. associated with EB of potato and tomato.

MATERIAL AND METHODS

Sampling of *Alternaria* species

Three hundred monosporic *Alternaria* isolates were obtained from potato leaves with EB symptoms sampled in the main producing regions of the following Brazilian states: Bahia (N = 34 isolates), Goiás (6), Minas Gerais (100), Paraná (82), Rio Grande do Sul (31), Santa Catarina (15), and São Paulo (32) during 2019 (N = 34 isolates), 2020 (142), and 2021 (124) growing seasons. Also, 14 monosporic *Alternaria* isolates were obtained from tomato leaves with EB symptoms sampled in Minas Gerais states (Fig. 1). Leaves with typical lesions of EB were collected at different points in the field. At least three individuals (isolates) were obtained per site.

The isolates of *Alternaria* species were obtained after transferring conidia formed on necrotic lesions present in the leaves to potato-dextrose-agar medium (PDA). A stereomicroscope was used to aid the direct isolation procedures. Petri plates containing PDA and conidia were kept at 25°C in darkness. After 5 days, a mycelium disc was transferred to solid V8 medium amended with $CaCO_3$ (170 mL of V8 juice, 15 g of agar, and 3 g of $CaCO_3$ per liter of distilled water) on Petri plates and incubated at 25°C for 7 days when aerial mycelium was removed after flooding the colony with 10 mL of distilled water and scraping with a sterile scalpel. The water in Petri plates was discarded and plates kept at 25°C, under near-UV light (black light 40W) for 12 h and

12 h in the dark to induce sporulation. Thereafter, a single conidium was transferred to PDA on Petri plates, incubating at 25°C for 5 days. After incubation, mycelium discs were transferred to 2-mL microtubes with (Castellani preservation method, Castellani, 1939) and without water. Microtubes were stored at 8°C.

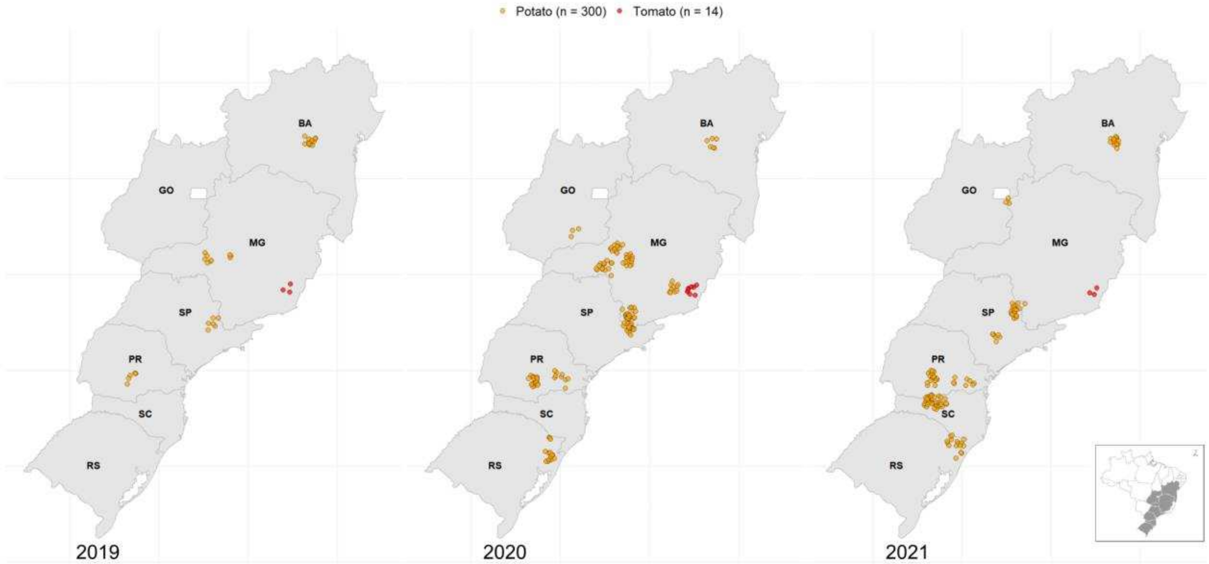

Figure 1. Location of origin of *Alternaria* spp. isolates from potato (orange) and tomato (red) fields in different states of Brazil, during the 2019, 2020, and 2021 growing seasons. Map of Brazil is depicted in the inset and shaded states in the map are those from which samples were obtained. States of Brazil: RS = Rio Grande do Sul; SC = Santa Catarina; PR = Paraná; SP = São Paulo; MG = Minas Gerais; GO = Goiás; and BA = Bahia.

DNA extraction

The isolates were cultivated in liquid medium (10 g sucrose, 2 g L-asparagine, 2 g yeast extract, 1 g KH_2PO_4, 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 1 L dH_2O) in Erlenmeyer flasks at 24 °C in the dark to allow for mycelium growth□. After 7 days, the mycelium was carefully washed with distilled water, transferred to filter paper to dry for 24 h, and macerated in TissueLyser II (QIAGEN, Germany). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. DNA integrity was analyzed by electrophoresis on a 1% agarose gel and quality was assessed using spectrophotometry. DNA was diluted to 25 ng/µl and stored at - 20 °C.

Identification of the predominant *Alternaria* species

Species identification using high resolution melting (HRM) analysis

The identification of all individuals of large-spored species, *A. solani*, *A. grandis* and *A. linariae*, was performed by high resolution melting (HRM) analysis with primers designed based on polymorphisms in the calmodulin gene (CaM) (Álvarez et al. unpublished data). This reaction was conducted on an HRM-equipped real-time rotary analyser (Rotor-Gene Q) using the Type-it HRM PCR Kit (Qiagen) following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed in a final volume of 10 µL with 5 µL of 2 x HRM Master mix, 0.35 µl of each primer (CaMF/CaMR) at 10 µM, 1 µL of DNA (25 ng/µl), and 3.3 µL of RNase-free water (Type-it HRM PCR Handbook; Qiagen). The thermal profile consisted of a denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 54 °C for 42 s, and 72 °C for 30 s. The PCR products were subjected to a melt program:denaturation of double-stranded DNA at 95 °C for 10 s. Melting curves were plotted and analyzed using the Rotor-Gene Q Detection software v. 2.02. For each isolate, two PCR reactions were conducted independently. A confidence threshold of 90% was applied to define the species. The DNA of a representative isolate of *A. solani* (CBS 109157) and *A. grandis* (UFVAg-352), and ex-type isolates of *A. grandis* (CBS 109158) and *A. linariae* (CBS 109156) were used as controls in all HRM analysis.

Species identification using phylogenetic analysis

Fungal isolates

Fourteen monosporic *Alternaria* isolates collected from potato in different regions: Bahia (N = 1 isolates), Goiás (1), Minas Gerais (3), Paraná (4), Rio Grande do Sul (2), Santa Catarina (1), and São Paulo states (2) and three isolates collected from tomato plants in the municipalities of Cajuri, Coimbra and Ervália, in Minas Gerais states, were selected for phylogenetic analysis.

PCR assay

The genomic regions: internal transcribed spacer regions 1 and 4 (ITS), *Alternaria* major allergen (*Alt a1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and RNA polymerase II beta subunit (RPB2) regions were used for phylogenetic analyses of large-spored *Alternaria* species (Gannibal et al. 2014; Woudenberg et al. 2014; Lawrence et al. 2013). The PCR were performed using GoTaq DNA Polymerase (Promega Corp., Madison, WI). Amplification was performed with primers ITS1 and ITS4 and RPB2-5f2 (Sung et al. 2007), Alt-for and Alt-rev (Hong et al. 2005), gpd1 and gpd2 (Berbee et al. 1999) and fRPB2-7cR (Liu et al. 1999) for ITS, *Alt a1*, *gapdh*, and RPB2 partial genes, respectively.

The PCR condition was: an initial step of 95 °C for 2 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 50, 55, 58 and 60 °C for ITS, *Alt a1*, *gpdh*, and RPB2, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Reaction was confirmed by electrophoresis on a 1.5% agarose gel. PCR products were purified using ExoSAP-IT cleanup reagent (USB, Cleveland, OH), lyophilized and sequenced using Sanger technology.

Sequences of these genomic regions were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/) for the isolates of *A. alternata* (CBS 916.16), *A. grandis* (CBS 109158), *A. solani* (CBS 109157), and *A. linariae* (CBS 109156).

Phylogenetic analysis

The quality of the sequences and contig assembly were performed using SeqAssem software (Hepperle, 2004). Alignments were conducted using MUSCLE with MEGA11 software (Tamura et al. 2021). The alignments were concatenated using Sequence matrix v1.8.

The concatenated datasets of large-spored *Alternaria* species (ITS, *Alt a1*, *gapdh*, and RPB2) were used in maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference analyses. The MP analysis was performed with MEGA11 software (Tamura et al. 2021) using 1000 bootstrap and tree bisection-reconnection. ML and Bayesian analysis were performed in the CIPRES Science Gateway Platform (Miller et al. 2010). The ML analysis was conducted with RaxML-HPC using the GTR+CAT substitution model and 1000 bootstraps (Stamatakis 2006). Bayesian inference analysis was performed with MrBayes (Huelsenbeck and Ronquist 2001). The jModelTest2 (Darriba et al. 2012) was used to determine the best nucleotide substitution model for each partition. The best model was HKY, GTR, JC, and GTR for *Alt a1*, *gapdh*, ITS, and RPB2, respectively, based on corrected Akaike information criterion or Bayesian information criterion. The Markov Chain Monte Carlo (MCMC) analysis was conducted with four chains starting with a random tree topology. The resulting trees were printed using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Inkscape was used to edit the tree (Inkscape Project 2020). In all analyses, *A. alternata* (small-spored; CBS 916.16) was used as the outgroup.

RESULTS

Species identification

HRM analysis

Normalized HRM curves were created to genotype isolates based on the profile of *A. solani* (CBS 109157) and *A. grandis* (UFVAg-352) and ex-type isolates of *A. grandis* and *A. linariae*. All isolates were grouped with a confidence threshold greater than 90% (Fig S1). Two hundred ninety-nine isolates collected from potato were identified as *A. grandis*. Only one isolate from potato collected in Guarapuava, Paraná state, was *A. solani*. No *A. linariae* was found associated with potato in Brazil. Fourteen isolates from tomato were identified as *A. linariae*.

Phylogenetic analyses

The *Alt a1, gpdh,* ITS, and RPB2 sequence alignments had 471, 576, 555, and 932 characters with 6, 5, 1, and 20 parsimony-informative sites, respectively. The concatenated sequences had a total length of 2534 characters. *A. linariae*, *A. grandis*, and *A. solani* grouped into two clades. One clade was composed of three isolates collected from tomato plants in the municipalities of Cajuri, Coimbra and Ervália together with the ex-type of *A. linariae* with posterior probability of 1.0 and bootstrap support of 100% and 98% for MP and ML, respectively. The bootstrap values of MP and ML were used in the Bayesian inference analysis tree because all methods; MP, Fig. S2; ML, Fig. S3; and Bayesian inference analyses generated trees with the same topology (Fig. 3).

Another clade was composed of 14 isolates identified as *A. grandis* in HRM analysis and one isolate identified as *A. solani* in HRM analysis. Isolates of *A. grandis* grouped more closely with the ex-type of *A. grandis* with posterior probability of 0.9 and bootstrap support of 65% and 93% for MP and ML, respectively (Fig. 3).

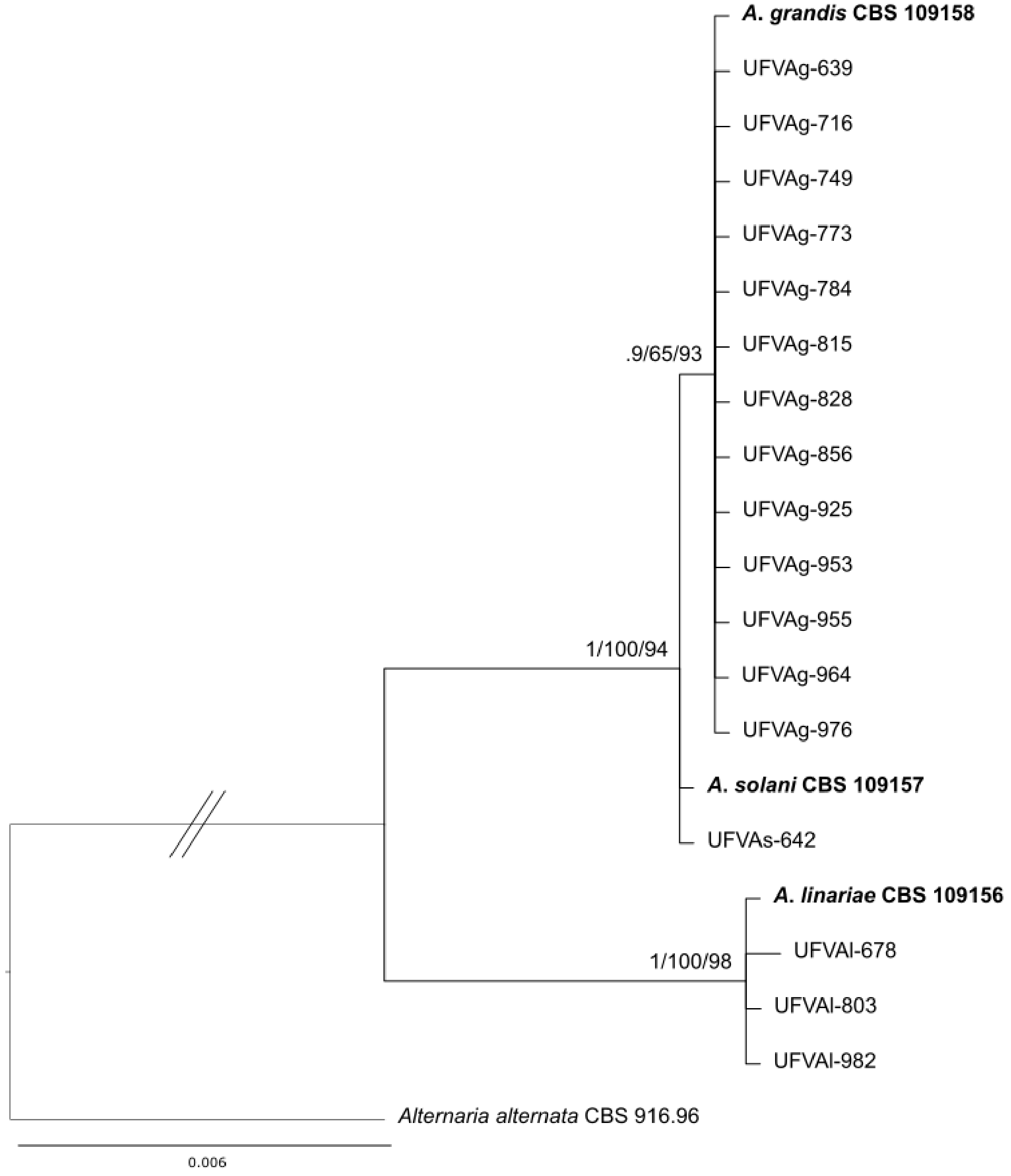

Figure 3. Bayesian phylogenetic tree based on the *Alt a1, gpdh,* ITS, and RPB2 sequences of representative *Alternaria* isolates collected from potato and tomato plants in Brazil. Posterior probability and bootstrap support values for MP and ML, respectively, are indicated near the node. The representative isolate of *A. solani* (CBS 109157) and ex-types isolates of *A. grandis* (CBS 109158) and *A. linariae* (CBS 109156) are in bold. *A. alternata* (small-spored) isolate CBS 916.96 was used as an outgroup. The black line bar indicates the number of nucleotide substitutions.

DISCUSSION

The correct identification of the species of a pathogen associated with a disease is important to improve the efficacy of control measures. In the current study, we classified 314 isolates of *Alternaria* spp. collected in the main potato-producing regions in Brazil and in one tomato producing region in Minas Gerais state, using HRM analysis. Additionally, phylogenetic analyses with partial sequences of the *Alta1*, *gapdh*, ITS, and RPB2 genes were also conducted. In Brazil, the large-spored *A. grandis* and *A. linariae* are the predominant species associated with EB of potato and tomato, respectively.

In the current study, the majority of isolates obtained from potato were identified as *A. grandis*. Only one isolate was classified as *A. solani*. This isolate was collected from potato plants grown in an experimental field. One of the plots in this field was inoculated with spores of *A. solani*. Thus, it is highly likely that this *A. solani* isolate originated from lesions caused by the inoculated one. Since 2010, not a single isolate *A. solani* was obtained from infected plants sampled from different potato growing regions.

Molecular studies conducted to identify the large-spored *Alternaria* spp. showed that the calmodulin gene is highly polymorphic and is useful to resolve species associated with potato and tomato (Gannibal et al. 2014; Peixoto et al. 2021). *A. grandis* differs by eight and ten nucleotides on the calmodulin gene from *A. solani* and *A. linariae*, respectively (Gannibal et al. 2014). Based on this, species-specific primers were developed to be used in HRM analysis designed to sort large-spored *Alternaria* spp. associated with potato and tomato (Álvarez et al., unpublished results). All isolates were differentiated with a confidence level greater than 90%.

The *Alternaria* species-groups formed by the HRM analyses were confirmed using a multilocus phylogenetic approach based on partial sequences of the *Alta1*, *gapdh*, and RPB2 genes and a portion of the ITS genomic region. Similar to what has been reported in other studies that used molecular data and morphological characteristics, the predominant species in potato and tomato are *A. grandis* and *A. linariae*, respectively (Lawrence et al. 2013; Gannibal et al. 2014; Rodrigues et al. 2010; Peixoto et al. 2021). Rodrigues et al. (2010) were the first to study the diversity of *Alternaria* species in Brazil. Nine isolates collected from tomato and 19 from potato plants sampled from five regions between 2005 and 2008 were analyzed. All isolates from potato and tomato were identified as *A. grandis* and *A. linariae*, respectively. Recently, a study was conducted to characterize 120 *Alternaria* isolates obtained from different Solanaceae species and regions in Brazil (Peixoto et al. 2021). In this last study, 36 large-spored isolates were collected from potato since 2003 and all were identified as *A. grandis*. Therefore, *A. grandis* seem to be the main species causing potato EB in Brazil since 2003.

A. grandis was first reported causing EB on potato in the US and the species was described using morphological characters (Simmons 2000). Subsequently, molecular tools confirmed the species classification (Lawrence et al. 2013). Nevertheless, *A. solani* is frequently referred to as the causing agent of EB in potato crops in many studies. The overlap in conidia dimensions, an important morphological character for taxonomic purposes, may introduce some difficulty to the correct identification of the pathogen. DNA sequences of informative genomic regions are crucial for the correct species identification by non-taxonomists. The misidentification of the morphospecies may explain why *A. solani* was listed as the main species associated with EB on potatoes worldwide. In Brazil, isolates collected from potato fields since 2003 were identified as *A. grandis* (Rodrigues et al. 2010; Peixoto et al. 2021). Therefore, the lack of data of DNA sequence may have led to the incorrect usage of *A. solani* as the species causing EB on potato in Brazil by other authors (Lourenço et al. 2009, 2011). The results reported to date confirmed that *A. grandis* is of widespread occurrence and the predominant species associated with potato in Brazil.

SUPPORTING INFORMATION

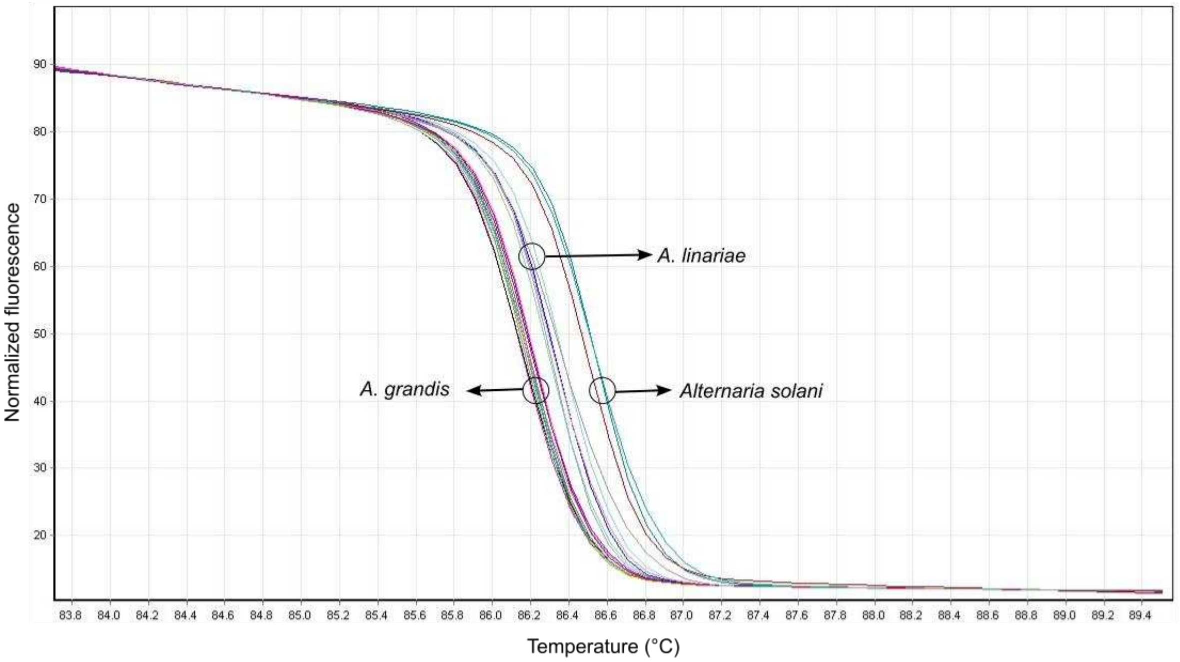

Figure S1. Normalized high resolution melting (HRM) analysis curves showing different melting profiles of isolates of *Alternaria grandis*, *A. linariae*, and *A. solani*.

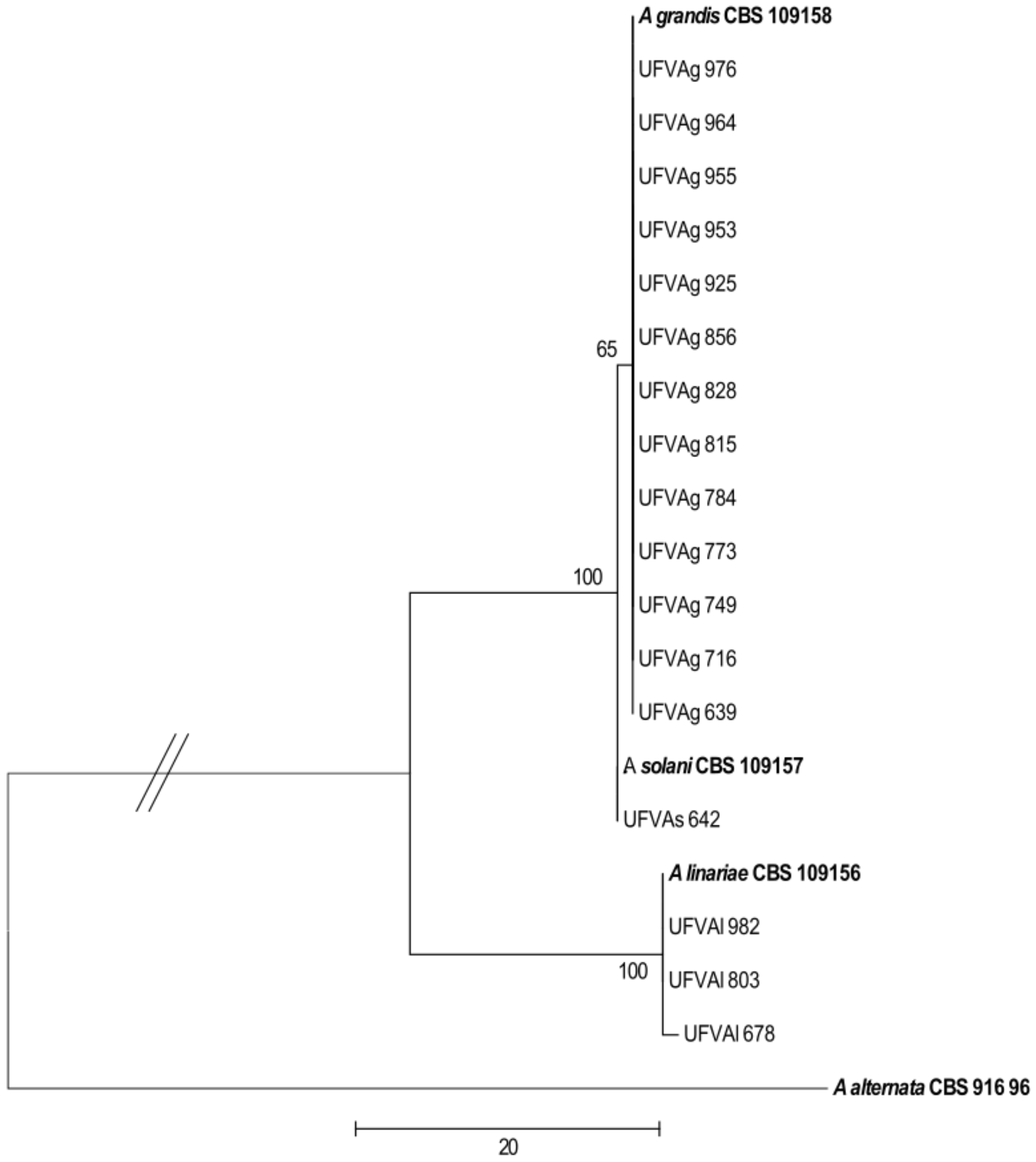

Figure S2. Maximum parsimony (MP) phylogenetic tree based on the *Alt a1, gpdh,* ITS, and RPB2 sequences of representative *Alternaria* isolates collected from potato and tomato plants in Brazil. Bootstrap support values are indicated near the node. The representative isolate of *A. solani* (CBS 109157) and ex-types isolates of *A. grandis* (CBS 109158) and *A. linariae* (CBS 109156) are in bold. *A. alternata* (small-spored) isolate CBS 916.96 was used as outgroup. The black line bar indicates the number of changes over the entire sequence.

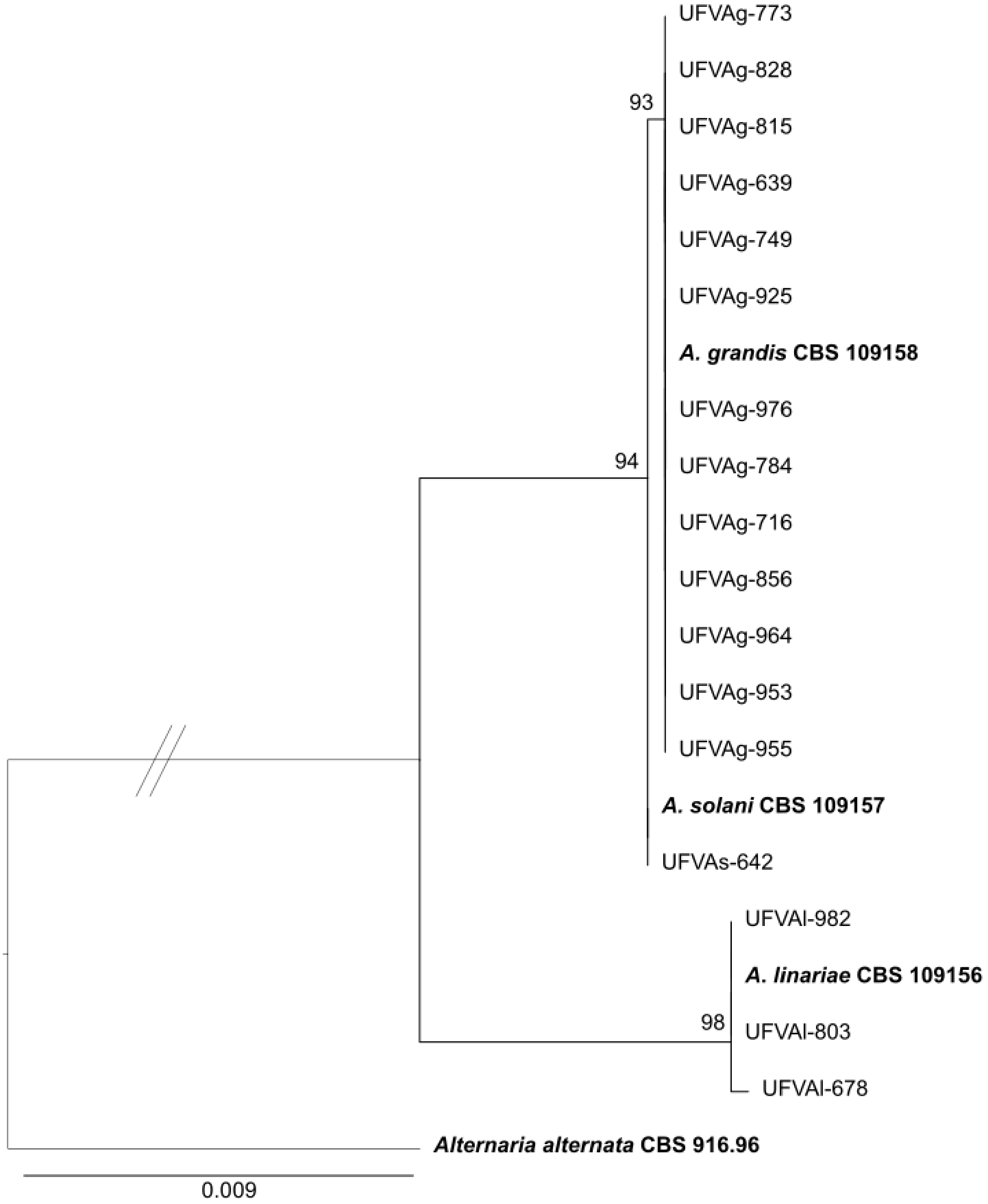

Figure S3. Maximum likelihood phylogenetic tree based on the *Alt a1, gpdh,* ITS, and RPB2 sequences of representative *Alternaria* isolates collected from potato and tomato plants in Brazil. Bootstrap support values are indicated near the node. The representative isolate of *A. solani* (CBS 109157) and ex-types isolates of *A. grandis* (CBS 109158) and *A. linariae* (CBS 109156) are in bold. *A. alternata* (small-spored) isolate CBS 916.96 was used as outgroup. The black line bar indicates the number of nucleotide substitutions per site.

CHAPTER 2: Development of SSR markers for *Alternaria grandis* and the genetic structure of the pathogen population

ABSTRACT

Early blight (EB) is a severe disease of potato worldwide. More than one species of *Alternaria* can cause EB. In Brazil, *Alternaria grandis* is the causal agent of the disease in potato, but the complete lack of information regarding the genetic aspects of the pathogen population prevents the proper understanding of evolutionary processes and their epidemiological consequences. Thus, the objective of this study was to develop SSR markers for *A. grandis* and to assess the genetic variability of the pathogen population. Twelve SSR markers were designed for *A. grandis*. A total of 302 isolates of *A. grandis* were genotyped and the genetic structure of the pathogen population was investigated. All loci were polymorphic and the number of alleles ranged from 2 to 23 with an average of seven alleles per locus. Gene diversity ranged from 0.01 to 0.85 and the allelic evenness per locus ranged from 0.32 to 0.99. A total of 253 MLLs were identified. There is high genetic variability, but no structure was detected. More than 97% of the total genetic variation was estimated to be due to the variation found within regions or years. Therefore, the population of *A. grandis* was not structured by regions or years. Linkage disequilibrium was detected in the total population, therefore, there was no evidence of random mating. The evolutionary mechanisms that most influence the variability of the population are mutation and gene flow. The high amount of genetic variability and the intense movement of pathogen genotypes among regions must be considered when considering deployment of resistant cultivars or fungicide usage.

INTRODUCTION

Before the study by Rodrigues et al. (2010) all publications about potato and tomato early blight (EB) in Brazil assumed that *Alternaria solani* Sorauer was the only species associated with this disease countrywide. The identity of 19 isolates of *Alternaria* spp. from potato and nine from tomato was determined and *A. grandis* Simmons was the only species found associated with EB on potato and *A. linariae* (Woudenberg et al. 2014) (syn. *A. tomatophila*) was the most common species recovered from tomato samples (Rodrigues et al., 2010). The isolates were collected from seven different regions in Brazil between 2005 to 2008. A recent report analyzed 299 isolates of large-spored *Alternaria* spp. causing EB on potato and all were classified as *A. grandis* (Silva et al., unpublished; Chapter 1).

A. grandis was first reported causing EB on potatoes in Pennsylvania, the United States, by Dr. Barbara. J. Christ in 1996 (Simmons 2000). Both *A. grandis* and *A. solani* belong to the *Alternaria* section *Porri*, that is comprised of asexual species that produce large, long-beaked, and non concatenated spores (Woudenberg et al. 2014). Conidia of *A. grandis* (body: 141-192 length x 26-38 µm width and beak length: 160-200 µm) are larger than those of *A. solani* (109-115 length x 18-26 µm width and beak length: 80-118 x 5-8 µm) (Simmons, 2020), but there is considerable overlapping of the dimensions. Based on molecular characters, *A. grandis* differs by one nucleotide at position 99 (T to C) in the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (Woudenberg et al. 2014) and eight nucleotides in the calmodulin gene (Gannibal et al. 2014). Thus, molecular tools are crucial to sort the species of large-spored *Alternaria* associated with EB on Solanaceae.

Early blight caused by *A. grandis* occurs anywhere potatoes are grown in Brazil, but, to date, there is no reliable estimate of yield losses yet. The main foliar symptoms of EB caused by *A. grandis* are identical to those reported for EB caused by *A. solani*; i.e. necrotic lesions with concentric rings in aerial tissues (Rotem 1994). In some regions in Brazil, potatoes are grown year-round and epidemics of EB can occur in all seasons, but the disease is more severe in the summer due the high temperature and higher precipitation (Batista et al. 2006). Epidemics of EB can have progress rates as high as those known for another quick and destructive foliar disease of potatoes, late blight, caused by *Phytophthora infestans*. To prevent yield losses, potato growers adopt frequent applications of multisite and site-specific fungicides such as demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs). Up to 8 applications of fungicides per season are commonly made for the management of EB alone (Batista et al. 2006). The intensive use of chemical fungicides has led to the selection of resistant individuals to different active ingredients (Silva et al. in preparation, Chapter 3).

Despite the widespread occurrence of EB epidemics there is no information regarding the genetic variability of *A. grandis*. Possible reasons for this paradox are: i. The relatively recent description of *A. grandis* as a species associated with potato EB (Simmons 2000); ii. The recent report of the occurrence of *A. grandis* in potato production areas worldwide (Bessadat et al. 2017; Landschoot et al. 2017c; Peixoto et al. 2021; Ivanović et al. 2022; Ayad et al. 2019); and iii. The lack of high resolution genetic markers such as microsatellites (SSR) for studying the genetic variability in the population of *A. grandis*.

Different molecular markers have been used to assess the genetic variability of the population of the sister species, *A. solani*. All studies reported high genetic variability of the population and correlation between genetic markers and geographic origin of isolates has not been reported so far (Weir et al. 1998; van der Waals et al. 2004; Pérez Martínez et al. 2004; Lourenço et al. 2011; Meng et al. 2015). One of the first studies used RAPD analysis to investigate genetic variation of 35 isolates collected from potato and tomato plants in the USA (Weir et al. 1998). Genetic differences were detected between groups of isolates formed according to the hosts to which they were associated. In South Africa, random amplified microsatellite (RAMS) analysis was used to assess the variability of 46 isolates from potato (van der Waals et al. 2004). There was high genetic diversity and high degree of gene flow among regions in South Africa. Therefore, the population was not structured by region. In a broader study, the genetic variability of *A. solani* was assessed using the AFLP profile of 122 isolates from potato and tomato from different countries. The population was structured by host. Moreover, genetic differences were detected among isolates of the EUA, Brazil, Turkey, and Cuba, but isolates from Cuba were not grouped by region (Pérez Martínez et al. 2004). The first study conducted in Brazil to assess the variability of the population of *A. solani* used both AFLP and RAPD markers and a collection of 150 isolates obtained from tomato and potato (Lourenço et al. 2011). The population of *A. solani* was structured by host. The first use of SSR markers in genetic studies of populations of *A. solani* was conducted in China with 268 isolates collected from four potato growing regions (Meng et al. 2015). Gene flow was reported to occur among regions and there was evidence of random mating.

Proper understanding of the genetic structure and the evolutionary processes affecting the population of *A. grandis* associated with potato can be useful to breeding programs, and for the deployment of resistant cultivars (when available), and even for establishing strategies to mitigate problems associated with fungicide resistance. The genetic structure of a pathogen population is determined by the extant amount and distribution of the genetic variation (McDonald and Linde 2002). However, it is important to use markers that are distributed throughout the genome, spanning different chromosomes and capable of revealing polymorphisms in a supposedly asexual pathogen such as *A. grandis*. The objectives of this study were to develop a set of SSR markers for *A. grandis* and to quantify the amount of genetic variation and its pattern of distribution in the populations of *A. grandis* in Brazil.

MATERIAL AND METHODS

Sampling and isolation of *A. grandis*

Isolates were obtained from potato leaves with typical EB symptoms sampled in the main regions of potato production in the following states in Brazil: Bahia (N = 34 isolates), Goiás (6), Minas Gerais (103), Paraná (84), Rio Grande do Sul (31), Santa Catarina (15), and São Paulo (32) during 2019 (33), 2020 (142), 2021 (124), and 2022 (3) growing seasons. Samples were collected at different points in the fields. Colonies were obtained after directly transferring conidia from lesions examined under a stereomicroscope and using a fine needle. Conidia were transferred to potato-dextrose-agar medium (PDA) on Petri plates and kept at 25 °C in darkness. After 5 days, a 5 mm-diameter mycelium disc was transferred to solid V8 medium amended with $CaCO_3$ (170 mL of V8 juice, 15 g of agar, and 3 g of $CaCO_3$ per liter of distilled water) on Petri plates and incubated at 25 °C for 7 days when aerial mycelium was removed using 10 mL of distilled water and a sterile scalpel. The water in Petri plates was discarded and plates kept at 25°C, under near-UV light (black light 40W) for 10 h and 14 h in the dark to induce sporulation. Thereafter, a single conidium was transferred to PDA on Petri plates that were kept at 25°C for 5 days. After incubation, mycelium discs were transferred to 2-mL microtubes with (Castellani preservation method, Castellani, 1939) and without water. Microtubes were stored at 8°C.

DNA extraction

DNA was extracted from all isolates using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) following the manufacturer's instructions. The isolates were cultivated in liquid medium (10 g sucrose, 2 g L-asparagine, 2 g yeast extract, 1 g KH_2PO_4, 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 1 L dH_2O) in Erlenmeyer flasks kept at 150 rpm and 25 °C in the dark to allow for mycelium to grow□. After 7 days, the mycelium was carefully washed with distilled water and transferred to filter paper to dry for 24 h. After drying, the mycelium was macerated in TissueLyser II (QIAGEN, Germany) and genomic DNA was extracted. DNA integrity was analyzed by electrophoresis on a 1% agarose gel and quality was assessed by spectrophotometry. DNA was diluted to 25 ng μl^{-1} and stored at -20 °C.

Development of SSR markers

SSR markers for *A. grandis* were initially screened *in silico* using the genome of *A. solani* available from NCBI (ASM295215v1). SSR motifs in the *A. solani* genome were identified using Krait ver 1.2.2 (Du et al. 2018). As a selection criterion, the minimum number of repeat units of di-, tri-, tetra, penta and hexa nucleotides was set as nine.

A subset of 230 SSR loci having at least 9 repeats with motifs of 2, 3, 4, 5, and 6 nucleotides was found in the genome of *A. solani* (CBS 143772). These loci were validated by inspecting the scaffold NCBI - ASM283723v1 from another isolate of *A. solani* to assess polymorphisms. Two SSR loci per each chromosome (N = 10 chromosomes) were selected for further analysis. Primer3 (Untergasser et al. 2012) was used to design primers using the following parameters: product size ranging from 100 to 500 bp, with optimal primer length of 20 bp, optimum melting temperature of 58°C, and anchoring position at least 5 bp away from the SSR locus.

Assessment of the polymorphism of the candidate SSR markers

The polymorphism at the 20 candidate SSR loci was accessed with 10 isolates of *A. grandis* haplotypes 1, 4, and 5 that were previously defined based on the *Alt a1* gene (Lourenço et al. 2009). These data were originated from different isolates collected from potato crops, in different locations. The candidate markers were also tested with ex-types isolates of *A. grandis* (EGS 44-108) and *A. linariae* (EGS 42-156), and one representative isolate of *A. alternata* and *A. solani* (EGS 44-098). PCR reactions were conducted with GoTaq DNA Polymerase (Promega Corp., Madison, WI). Amplification was confirmed by electrophoresis on a 2 % agarose gel. From the 20 candidate loci, 13 SSR primers that amplified all isolates of *A. grandis* were selected to be sequenced in order to confirm the motifs. PCR products were purified using ExoSAP-IT cleanup reagent (USB, Cleveland, OH) and sequenced. The contigs were obtained with SeqAssem Software (Hepperle, 2004) and aligned using MEGA 11.0 (Tamura et al., 2013).

SSR genotyping

Twelve polymorphic SSR loci were selected to assess the genetic structure of the population of *A. grandis*. The forward primers were labeled with 6-FAM, VIC, NED, or PET fluorescent dyes. These 12 SSR primers were used to check the allele size of 10 isolates of *A. grandis*, one isolate of *A. solani* and ex-types isolates of *A. grandis* and *A. linariae* mentioned before. The 12 SSR markers designed for *A. grandis* and two additional markers, PAS4 and Ad8, previously described for *A. solani* (Meng et al. 2015; Benichou et al. 2009) were used to genotype 302 isolates of *A. grandis* (Table S1). PCR reactions were performed using the Multiplex PCR 5X Master Mix kit following the recommendation of the manufacturer (New England Biolabs, Inc.). Amplification was confirmed by electrophoresis on a 1.5% agarose gel. Three sets of PCR multiplex reactions each with four primer-pairs and one multiplex reaction with two primer-pairs were prepared to genotype the isolates (Table S2). The allele size of all isolates was accessed using multiplex PCR. Fragment analyses were conducted on ABI 3730xl DNA Analyzer (Applied Biosystems) using a LIZ 500 size standard. Fragment size was scored using the software GeneMarker v.1.191 (SoftGenetics).

Genetic diversity

The estimates of gene diversity and allelic evenness per locus were calculated with the package poppr in the R program (Kamvar et al. 2014). Genotype accumulation curves were generated using 1,000 resamplings to assess the SSR loci power (Arnaud- Haond et al. 2007). Multilocus genotypes (MLGs) were obtained for the total population, but different MLGs could have originated from the same multilocus lineage (MLL) due to somatic mutations or scoring errors (Bailleul et al. 2016). Therefore, to avoid miscalling of MLLs the possible fortuitous MLGs generated due to low frequency somatic mutations or errors was assessed by superimposing histograms of the genetic distance spectra (GDS) values of the observed population and two simulated sexual populations, one outcrossing and another with selfing and outcrossing. If there was a gap between the narrow peaks at the beginning and at larger distances of the distributions, a threshold can be defined (Arnaud-Haond et al. 2007). After that, groups of MLGs belonging to the same MLLs were selected. The GDS values were calculated based on the divergence of SSR motifs (Rozenfeld et al. 2007) for each pair of unique MLGs. The relationship between MLLs was investigated with a minimum spanning network (MSN) constructed with the Bruvo genetic distance in the Poppr package.

Population structure

Initially, a model-free method, the discriminant analysis of principal components (DAPC), was used to inspect for clusters of individuals and their relation with geographic (region) or temporal (year) features (Jombart et al. 2010). The optimal number of principal components to be retained was determined with cross-validation (Jombart 2008). The number of clusters (K) was found with find.cluster function based on the Bayesian information criterion (BIC). DAPC was conducted with clone-correct data to select one isolate from each MLL using the adegenet package (Jombart 2008).

The structuring of the population according to region or year was tested using the analysis of molecular variance (AMOVA). AMOVA was conducted to estimate the distribution of genetic variation among and within region or year using Arlequin v. 3.5 (Excoffier et al. 2007). AMOVA analyses were performed only for the states of BA, MG, PR, RS, SC, and SP due to sample size restrictions.

Random mating

The standardized version (*r_d*) of the index of association was used to infer the occurrence of random mating in the total population. This index was calculated with clone-correct data and 999 permutations (Agapow and Burt 2001).

RESULTS

Assessment of the polymorphisms of the candidate SSR markers

The polymorphism at the 20 candidate SSR loci was accessed with 10 isolates of *A. grandis* haplotypes 1, 4, and 5 based on the *Alt a1* gene that were previously characterized (Lourenço et al. 2009)**.** From the 20 candidate SSR loci, five had null alleles, and two primer-pair for two loci did not amplify the target fragment. Among the 13 SSR loci analyzed, 12 were polymorphic and one was monomorphic. The number of alleles varied from one to six (Table 1). Therefore, 12 SSR markers were selected for further analysis.

Table 1. General characteristics of the simple sequence repeat (SSR) loci identified for the analysis of 10 isolates of *Alternaria grandis* haplotypes 1, 4, and 5 based on the *Alt a1* gene that were previously characterized (Lourenço et al. 2009).

Locus	Repeat Motif	Size range	Chromosome[a]	No. of alleles
ssr-115	(CAA)20	205 - 217	1	4
ssr-343	(CTA)56	285	1	*
ssr-195	(CA)19	130 - 142	2	3
ssr-222	(TC)17	166	2	*
ssr-164	(CT)29	131 - 135	3	2
ssr-169	(CA)30	141 - 149	3	3
ssr-016	(CAA)11	178	4	-
ssr-062	(AC)9	169 - 185	4	5
ssr-047	(AC)26	181 - 193	5	2
ssr-172	(GAA)14	159	5	*
ssr-090	(AAC)32	232	6	-
ssr-097	(TG)24	204	6	*
ssr-032	(CA)19	142 - 186	7	5
ssr-056	(TGC)9	167 - 173	7	3
ssr-020	(CA)31	122 - 152	8	3
ssr-106	(ACA)12	189 - 327	8	6
ssr-043	(CT)15	151	9	*
ssr-105	(TCT)20	181 - 241	9	4
ssr-073	(CAG)11	169 - 172	10	2
ssr-083	(TAC)25	191	10	1

[a] Chromosome of *A. solani* (ASM295215v1) used as reference

* Null alleles

\- No amplification of the target fragment

Genetic diversity

Twelve (this study) and two (Meng et al. 2015; Benichou et al. 2009) SSR markers were polymorphic and used for the genetic analyses of the population. This set of 14 markers was able to capture most of the genotypic variation in the population (Fig. S1). A total of 253 multilocus genotypes (MLGs) were identified among the 302 isolates. These 253 unique MLGs were defined as 253 MLLs, because no gap was observed in the GDS histogram (Fig. S2).

The number of alleles ranged from two (loci 105; 073; 164; and PAS4) to 23 (locus Ad8) with an average of seven alleles per locus of *A. grandis* (Table 2). Gene diversity ranged from 0.01 to 0.85 (average, 0.40) and the allelic evenness per locus ranged from 0.32 to 0.99 (average, 0.63) (Table 2).

Table 2. Description of microsatellite loci used to characterize the population of *Alternaria grandis*.

Locus (Dye)	**Motif**	**N° of allele**	**Size range**	**Gene diversity**	**Evenness**
SSR-106 (6-FAM)	(ACA)12	17	201-357	0.57	0.41
SSR-032 (VIC)	(CA)19	10	177-205	0.61	0.54
SSR-115 (NED)	(CAA)20	3	211-217	0.12	0.47
SSR-195 (PET)	(CA)19	4	136-142	0.04	0.32
SSR-062 (6-FAM)	(AC)9	4	169-181	0.49	0.91
SSR-169 (VIC)	(CA)30	4	143-149	0.52	0.89
SSR-056 (NED)	(TGC)9	3	167-173	0.42	0.83
SSR-105 (PET)	(TCT)20	2	183-186	0.03	0.37
SSR-047 (6-FAM)	(AC)26	12	172-236	0.77	0.74
SSR-020 (VIC)	(CA)31	5	130-154	0.45	0.64
SSR-073 (NED)	(CAG)11	2	167-170	0.01	0.33
SSR-164 (PET)	(CT)29	2	134-136	0.28	0.67
PAS4 (VIC)	(GTC)5	2	204-207	0.50	0.99
Ad8 (PET)	(GAA)15	23	173-326	0.85	0.65

In the minimum spanning network, the Bruvo's genetic distance varied from 0.03 to 0.30 (Fig. 1).

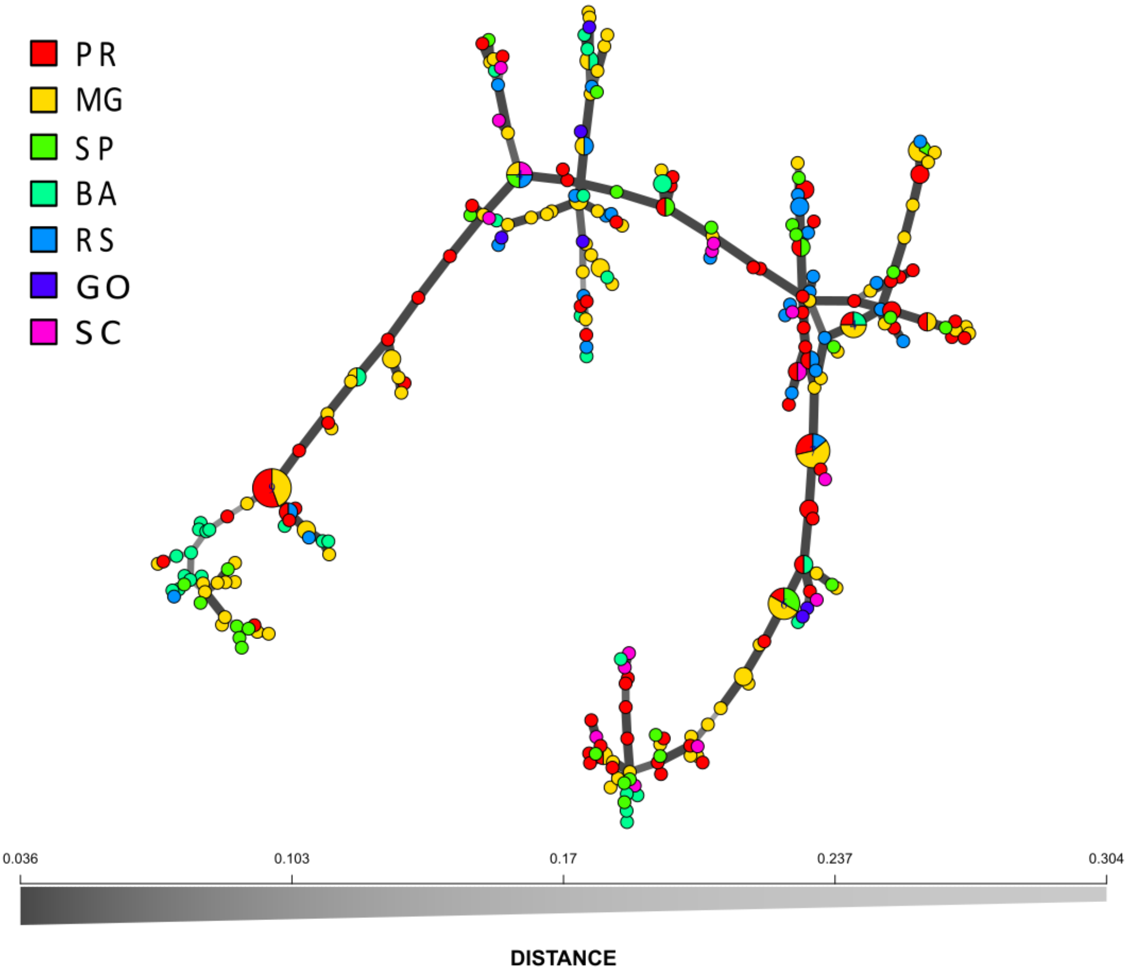

Figure 1. Minimum spanning network using Bruvo's genetic distance showing the relationship between MLL of 302 isolates of *Alternaria grandis*. Each node represents a unique MLL, node size represents the number of individuals from a given MLL. Node colors represent the origin of each isolate. The edge widths and shading of the lines connecting the nodes are proportional to Bruvo genetic distance. Edge length is arbitrary.

Population structure

The optimal number of principal components retained for the DAPC analyses was 70 based on the lowest root mean squared error. The most likely number of clusters was three (k = 3) based on BIC. Each cluster was composed of isolates from different regions in Brazil. Isolates from BA, MG, PR, RS, and SP were present in all three groups (Fig. 2). Isolates from GO and SC were present in two of the three groups. No isolates from GO and SC were clustered in group 3 (orange) and 2 (blue), respectively. Group 1 (black) was composed of 101 isolates of BA, GO, MG, PR, RS, SC, and SP states. Group 2 (blue) was composed of 34 isolates of BA, GO, MG, PR, RS, and SP states. Group 3 (orange) composed of 118 isolates of BA, MG, PR, RS, SC, and SP states (Fig. 2). In relation to temporal features, each cluster was composed of isolates collected in different years. Isolates collected in 2019, 2020, and 2021 were present in all three groups (Fig. 2). Three isolates collected in 2022 were present in group 1 (two isolates) and 3 (one isolate). Group 1 (black) was composed of 101 isolates of 2019, 2020, 2021, and 2022. Group 2 (blue) was composed of 34 isolates of 2019, 2020, and 2021. Group 3 (orange) composed of 118 isolates of 2019, 2020, 2021, and 2022 (Fig. 2).

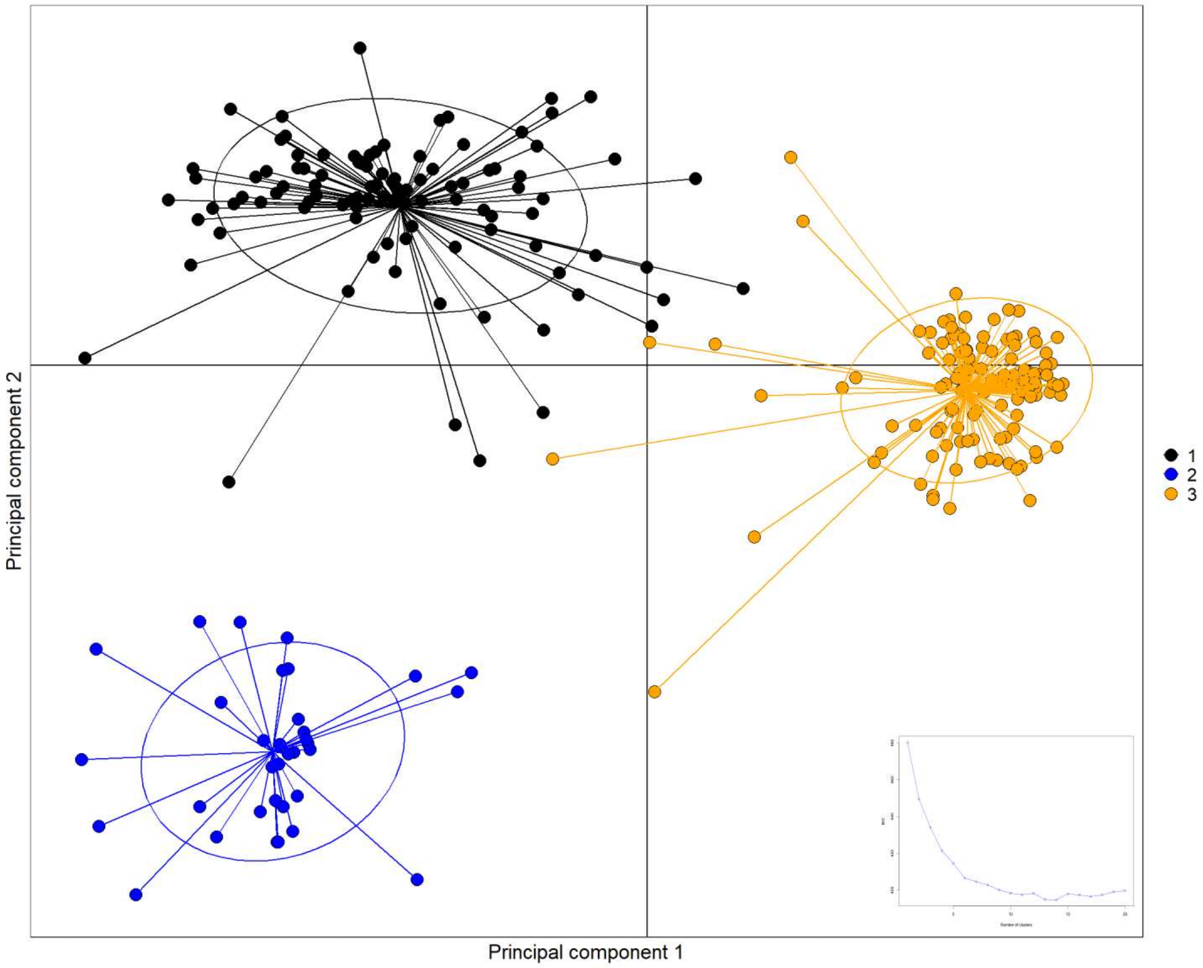

Figure 2. Scatterplots of discriminant analysis of principal components (DAPC) of isolates of *Alternaria grandis* from seven different regions in Brazil. Number of clusters was three (K = 3) based on the Bayesian information criterion (Inset bottom right). Clusters are indicated by different colors and inertia ellipses. Dots represent individuals.

AMOVA was conducted to assess the genetic variation among regions and years. A total of 98.1% and 99.3% of the genetic variation was found within regions and years, respectively (Table S3). Only 1.9% and 0.7% was due to variation among regions and years, respectively.

Random mating

There is no evidence of random mating in the population of *A. grandis*. Linkage disequilibrium was detected among all pairs of loci. The population had significant r_d values (P < 0.01).

DISCUSSION

This study was the first conducted to develop a set of highly polymorphic SSR markers and to understand the genetic variability of the population of *A. grandis* associated with potato EB in Brazil. Fourteen polymorphic SSR markers were selected for use in population genetics studies of large-spored *Alternaria* species. The Brazilian population of *A. grandis* has high genetic variability but linkage disequilibrium was detected in the total population. Thus, there was no evidence of random mating and recombination may be rare. Furthermore, the population was not structured by region or year.

There is no evidence of recombination between genetically distinct individuals in the population of *A. grandis*, although there was high genotypic diversity. The same results were found in the population of *A. solani* in Brazil (Lourenço et al. 2011). Large-spored *Alternaria* spp. reproduce asexually. No sexual structures were found in *A. solani* or *A. grandis* so far and no "unconventional" recombination mechanisms have been reported in the population of these pathogens. Therefore, the genetic variation is probably more influenced by mutation. Results reported in this study were similar to population genetics studies conducted in other countries with different molecular markers used to assess the genetic variability of the population of the sister species, *A. solani* (Weir et al. 1998; van der Waals et al. 2004; Pérez Martínez et al. 2004).

All studies reported high genetic variability of the population and the lack of correlation between genetic markers and geographic origin of isolates (Weir et al. 1998; van der Waals et al. 2004; Pérez Martínez et al. 2004; Meng et al. 2015). The first study that used SSR markers to assess the genetic variability of *A. solani* was conducted in China with 268 isolates collected from four potato growing regions (Meng et al. 2015). There was high genotypic diversity, evidence of gene flow among regions and random mating in the populations of three regions. However, in the region with the highest genetic variation, there was no evidence of random mating. The authors suggest that the high genetic variability may be influenced by the intensive movement of potato seeds that are often imported and favor the introduction of new genotypes.

There is very limited information about the population of *Alternaria* species affecting potato in Brazil. Only two studies were conducted to assess the genetic variability of *A. solani* and there is no study with *A. grandis*. The two studies were based on individuals collected from potato and tomato in Brazil. The authors used genealogical approach, RAPD and AFLP markers to assess the genetic structure and to investigate the evolutionary processes that shape the genetic variation of the pathogen population (Lourenço et al. 2009, 2011). There was an association between host and genetic distance, but there was no correlation among genetic distance and geographic origin of the isolates. Additionally, there was no evidence of recombination in the population of *A. solani*, but there was high genetic variability and structuring by host. In the current study, the results for *A. grandis* were similar to the studies with the putative *A. solani* population in Brazil. The population had high genetic diversity, but there was no evidence of random mating. Also, there was no association between genetic markers and geographic origin of the isolates.

Gene flow between regions seems to be an important evolutionary mechanism that affects the variability of the population of *A. grandis* in Brazil. The population was not structured by region. This suggests widespread dispersal of the isolates among different regions. Indeed, isolates from MG, PR, RS and SP were clustered in all seven genetic groups and isolates from BA were found in six of the groups. *A. grandis* are probably dispersed in infected potato tubers moved by humans.

SUPPORTING INFORMATION

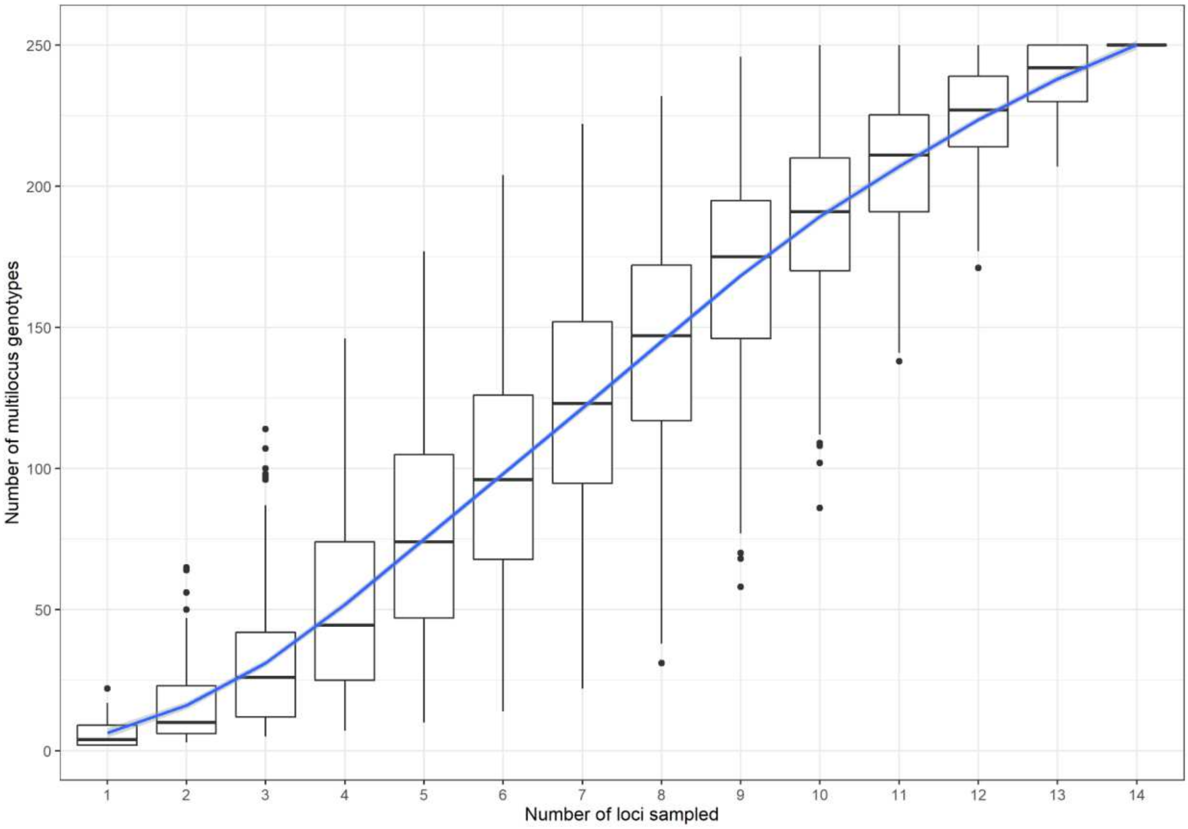

Figure S1. Genotype accumulation curve with 14 SSR loci for the population of *Alternaria grandis*.

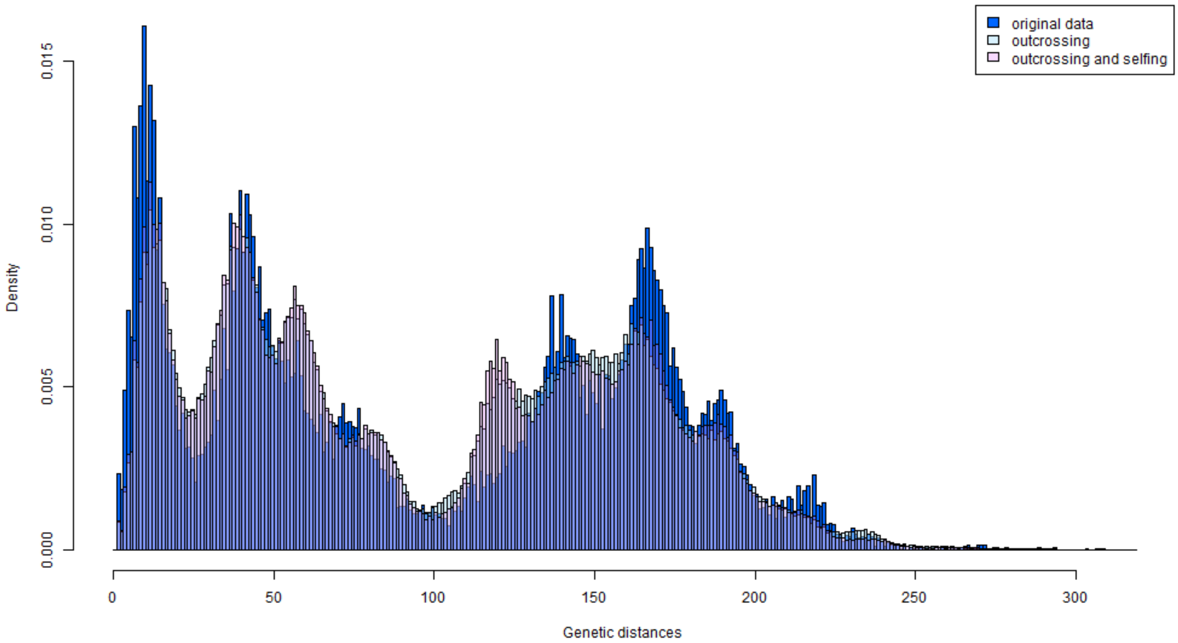

Figure S2. The genetic distance spectrum (GDS) histogram of the genotype population of *Alternaria grandis* collected from different regions in Brazil. The genetic distance was calculated based on the difference in length between alleles (Rozenfeld et al. 2007). Therefore, the x-axis represents the number of allele differences.

Table S1. Geographic origin and allele size at each microsatellite locus of 302 isolates of *Alternaria grandis* collected from main regions of potato production in Brazil.

Isolate code (UFVAg-)	**Municipality**	**State**	**Microsatellite allele size**													
			Locus													
			ssr-106	**ssr-032**	**ssr-115**	**ssr-195**	**ssr-062**	**ssr-169**	**ssr-056**	**ssr-105**	**ssr-047**	**ssr-020**	**ssr-073**	**ssr-164**	**PAS4**	**Ad8**
637	Guarapuava	PR	204	181	214	136	179	145	170	183	220	152	170	134	204	251
638	Guarapuava	PR	204	181	214	142	177	147	170	183	220	152	170	136	204	224
639	Guarapuava	PR	204	181	214	136	179	145	173	183	218	152	170	134	204	254
640	Guarapuava	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	254
641	Guarapuava	PR	204	181	214	142	177	147	170	183	220	152	170	136	207	224
643	Patrocínio	MG	204	181	214	136	169	145	173	183	220	152	170	136	207	257
644	Patrocínio	MG	204	179	214	136	177	147	170	183	190	152	170	134	204	248
645	Patrocínio	MG	204	183	217	136	179	147	173	183	220	152	170	136	204	254
646	Patrocínio	MG	204	181	214	136	179	145	173	183	218	152	170	136	204	254
647	Patrocínio	MG	204	179	214	136	177	147	170	183	192	152	170	136	204	248
648	Patrocínio	MG	204	179	214	136	177	147	170	183	190	152	170	136	204	248
649	Rio Paranaíba	MG	204	181	214	136	179	145	173	183	220	152	170	136	207	254
650	Rio Paranaíba	MG	207	181	214	136	179	147	173	183	220	150	170	136	204	254
651	Rio Paranaíba	MG	204	181	214	136	179	145	173	183	218	152	170	136	204	254
652	Estiva-Gerbi	SP	303	201	214	136	177	145	173	183	222	152	170	136	204	206
653	Estiva-Gerbi	SP	303	201	214	136	177	147	173	183	220	150	170	136	204	206
656	Holambra	SP	204	181	214	136	179	145	173	183	218	152	170	134	207	254
657	Holambra	SP	204	181	214	136	179	147	173	183	218	150	170	136	204	254
658	Holambra	SP	201	183	217	136	179	147	173	183	220	152	170	136	204	254
687	Holambra	SP	204	181	214	136	179	145	173	183	220	152	170	136	207	254
691	Mucugê	BA	204	179	214	136	177	145	170	183	192	152	170	136	207	248
692	Mucugê	BA	204	183	214	136	179	147	173	183	218	152	170	134	204	254

693	Mucugê	BA	324	201	214	136	177	145	173	183	220	152	170	136	204	254
694	Mucugê	BA	309	203	214	136	179	145	173	183	222	150	170	136	207	254
695	Mucugê	BA	204	181	214	136	179	145	173	183	218	152	170	136	204	254
696	Mucugê	BA	306	201	217	136	179	147	173	183	218	152	170	136	204	206
697	Mucugê	BA	306	201	214	136	179	145	173	183	220	152	170	136	204	206
698	Mucugê	BA	306	201	214	136	179	147	173	183	222	152	170	136	204	257
699	Mucugê	BA	333	183	217	136	179	147	173	183	190	152	170	136	207	206
700	Mucugê	BA	333	181	214	136	179	145	173	183	190	150	170	136	204	206
701	Mucugê	BA	204	181	214	136	179	147	173	183	220	150	170	136	204	257
702	Mucugê	BA	204	181	214	136	179	147	173	183	220	150	170	136	207	257
703	Mucugê	BA	315	201	217	136	179	147	173	183	220	152	170	136	204	257
704	Rio Paranaíba	MG	204	179	214	136	177	145	170	183	192	152	170	136	207	248
705	Rio Paranaíba	MG	204	179	214	136	177	145	170	183	190	154	170	136	207	248
706	Rio Paranaíba	MG	204	183	214	136	179	147	173	183	218	152	170	136	207	254
710	Mucugê	BA	204	181	214	136	177	149	173	183	222	150	170	136	207	227
711	Mucugê	BA	204	201	217	136	179	145	173	183	222	152	170	136	204	254
712	Mucugê	BA	204	181	214	136	177	147	173	183	218	152	170	136	204	254
713	Mucugê	BA	309	201	214	136	179	145	173	183	222	152	170	136	204	254
714	Mucugê	BA	309	201	214	136	179	143	173	183	220	152	170	136	204	257
715	Mucugê	BA	204	201	217	136	179	147	173	183	222	150	170	136	204	173
716	Ponta Grossa	PR	204	181	214	136	179	145	173	183	220	152	170	136	204	254
717	Ponta Grossa	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	254
718	Ponta Grossa	PR	204	183	217	136	179	147	173	183	220	150	170	136	204	254
719	Ponta Grossa	PR	204	181	214	136	179	145	173	183	216	150	170	136	204	254
720	Ponta Grossa	PR	204	181	214	136	179	147	170	183	220	152	170	136	204	257
721	Ponta Grossa	PR	204	181	214	136	179	147	170	183	218	148	170	136	207	251
722	Patrocínio	MG	312	181	214	136	177	145	173	183	222	152	170	136	204	257
723	Patrocínio	MG	204	181	214	136	179	147	173	183	220	152	170	136	204	254
724	Patrocínio	MG	204	181	214	136	179	147	173	183	218	152	170	136	207	254

725	Patrocínio	MG	318	181	214	136	177	145	173	183	222	152	170	136	204	206
726	Patrocínio	MG	204	181	214	136	179	147	173	183	220	152	170	136	207	251
727	Patrocínio	MG	204	181	214	136	179	147	173	183	220	152	170	134	207	254
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730	Patrocínio	MG	204	181	214	136	179	147	173	183	220	150	170	136	207	254
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732	Patrocínio	MG	204	195	214	136	179	147	173	183	220	152	170	136	207	263
733	Patrocínio	MG	204	181	214	136	179	147	173	183	218	152	170	134	207	254
734	Perdizes	MG	312	181	214	136	177	145	173	183	224	152	170	136	204	218
735	Perdizes	MG	204	181	214	136	179	145	173	183	218	150	170	134	204	254
736	Uberaba	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	263
737	Uberaba	MG	204	181	214	136	179	147	173	183	220	152	170	136	204	254
738	Uberaba	MG	204	181	214	136	179	147	173	183	220	152	170	136	204	254
739	Uberaba	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	254
740	Uberaba	MG	204	181	214	136	179	147	173	183	220	150	170	136	204	251
741	Uberaba	MG	204	181	214	136	179	147	173	183	220	152	170	136	207	257
742	Uberaba	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	257
743	Uberaba	MG	204	181	214	136	179	147	173	183	220	152	170	136	204	254
744	Uberaba	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	254
745	Sacramento	MG	309	203	214	136	177	145	173	186	224	152	170	136	204	206
746	Sacramento	MG	204	181	214	136	179	147	170	183	220	150	170	136	204	251
747	Perdizes	MG	204	181	214	136	179	145	173	183	220	150	170	136	207	239
748	Perdizes	MG	204	181	214	136	179	147	173	183	218	152	170	136	204	254
749	São Gotardo	MG	204	181	214	136	179	145	173	183	220	152	170	136	207	254
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751	São Gotardo	MG	312	181	214	136	177	147	173	183	222	152	170	136	207	206
752	São Gotardo	MG	204	181	214	136	179	145	173	183	220	152	170	136	207	254
753	São Gotardo	MG	204	181	214	136	179	147	173	183	220	152	170	136	207	257

754	São Gotardo	MG	204	181	214	136	179	147	170	183	218	152	170	136	204	227
755	São Gotardo	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	254
756	São Gotardo	MG	204	181	214	136	179	147	173	183	218	152	170	134	207	260
757	São Gotardo	MG	312	181	214	136	177	147	173	183	174	152	170	134	207	206
758	Rio Paranaíba	MG	204	181	214	136	179	145	173	183	218	152	170	134	207	254
759	Rio Paranaíba	MG	204	181	214	136	179	147	173	183	172	152	170	134	207	254
760	Rio Paranaíba	MG	312	181	214	136	177	147	173	183	220	152	170	134	207	206
761	Guarapuava	PR	204	181	214	136	179	145	170	183	218	150	170	134	207	251
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763	Guarapuava	PR	312	183	214	136	177	147	173	183	222	152	170	136	207	206
764	Guarapuava	PR	204	181	214	136	179	145	170	183	218	150	170	134	207	251
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766	Guarapuava	PR	315	181	214	136	177	145	173	183	222	152	170	134	207	206
767	Guarapuava	PR	204	181	214	136	179	147	173	183	218	152	170	136	204	257
768	Guarapuava	PR	204	181	214	136	179	147	170	183	216	152	170	134	204	260
769	Guarapuava	PR	204	181	214	136	179	147	173	183	220	152	170	136	207	254
770	Guarapuava	PR	204	181	214	136	179	145	170	183	218	152	170	136	207	251
771	Guarapuava	PR	204	181	214	136	179	145	170	183	218	152	170	134	204	206
772	Guarapuava	PR	204	181	214	136	179	147	170	183	218	152	170	136	204	224
773	São Francisco de Paula	RS	336	179	214	136	177	145	173	183	190	152	170	136	204	206
774	São Francisco de Paula	RS	204	181	214	136	179	145	170	183	216	150	170	136	204	254
775	São Francisco de Paula	RS	204	201	217	136	179	147	173	183	192	152	170	136	207	254
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777	São Francisco de Paula	RS	204	181	214	136	181	145	173	183	218	150	170	136	204	254
778	São Francisco de Paula	RS	204	179	214	136	177	147	170	183	192	152	170	136	204	248
779	São Francisco de Paula	RS	204	181	214	136	179	147	173	183	220	152	170	136	204	257
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781	São Francisco de Paula	RS	204	181	214	136	179	145	170	183	220	150	170	136	204	221
782	Contenda	PR	204	181	214	136	179	145	173	183	218	150	170	136	207	254

783	Contenda	PR	312	181	214	136	177	147	173	183	222	152	170	136	204	206
784	Contenda	PR	204	181	214	136	179	147	173	183	220	154	170	136	207	254
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787	São Francisco de Paula	RS	204	181	214	136	179	145	170	183	220	150	170	136	207	221
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792	Andradas	MG	204	177	214	136	177	147	170	183	192	152	170	136	204	248
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794	Guarapuava	PR	204	181	214	136	179	145	173	183	220	150	170	136	207	254
795	Guarapuava	PR	204	181	214	136	179	145	170	183	220	152	170	136	207	221
796	Guarapuava	PR	303	181	214	136	177	147	173	183	220	148	170	136	207	233
797	Guarapuava	PR	204	181	214	136	179	145	170	183	220	152	170	136	204	224
798	Guarapuava	PR	204	181	214	136	179	147	170	183	220	154	170	136	207	251
799	Guarapuava	PR	204	181	214	136	179	145	170	183	220	152	170	136	204	251
800	Morrinhos	GO	204	179	214	136	177	145	170	183	190	152	170	136	204	248
801	Morrinhos	GO	204	179	214	136	177	147	170	183	192	152	170	136	204	251
802	Morrinhos	GO	204	179	214	136	177	145	173	183	218	150	170	136	207	254
812	Conselheiro Lafaiete	MG	204	201	214	136	177	145	173	183	218	152	170	136	207	206
813	Conselheiro Lafaiete	MG	204	201	214	136	177	145	173	183	218	152	170	134	207	206
814	Conselheiro Lafaiete	MG	204	181	214	136	179	145	173	183	222	152	170	136	204	257
815	Ouro Branco	MG	312	181	214	138	177	145	173	183	220	150	167	134	207	251
816	Ouro Branco	MG	303	193	214	136	177	147	170	183	220	152	170	136	204	248
817	Ouro Branco	MG	309	179	214	140	179	145	173	183	228	152	170	134	204	230
818	Ouro Branco	MG	306	177	214	136	177	145	173	183	224	148	167	136	207	269
819	Itaverava	MG	204	181	214	136	179	145	173	183	218	150	170	134	204	257
820	Itaverava	MG	315	201	214	136	177	145	173	183	222	152	170	136	204	251

821	Itaverava	MG	336	179	211	140	179	145	170	183	224	154	170	136	207	239
822	Bom Jesus	RS	204	183	214	136	179	145	170	183	218	152	170	136	207	251
823	Bom Jesus	RS	204	183	214	136	179	147	173	183	218	152	170	136	204	254
824	Bom Jesus	RS	204	193	214	136	177	147	170	183	192	150	170	136	207	248
825	Bom Jesus	RS	312	177	214	136	179	145	173	183	218	150	170	134	207	254
826	Bom Jesus	RS	309	193	214	136	177	147	170	183	190	148	170	136	204	251
827	Bom Jesus	RS	351	183	214	136	177	147	173	183	220	154	170	136	207	254
828	São José dos Ausentes	RS	204	181	214	136	179	145	173	183	220	152	170	136	204	254
829	São José dos Ausentes	RS	201	193	214	136	177	147	173	183	190	152	170	136	204	248
830	São José dos Ausentes	RS	204	181	214	136	179	147	170	183	218	152	170	134	207	221
831	São Francisco de Paula	RS	204	181	214	136	179	145	170	183	218	152	170	134	204	251
832	São Francisco de Paula	RS	204	181	214	136	179	145	173	183	218	152	170	136	204	251
833	São Francisco de Paula	RS	204	183	214	136	177	147	173	183	190	152	170	134	207	224
834	Bom Jesus	RS	204	181	214	136	179	145	170	183	218	150	170	136	207	251
835	Senador Amaral	MG	339	179	214	136	177	145	173	183	190	152	170	136	207	206
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839	Camanducaia	MG	309	201	214	136	177	145	173	183	222	152	170	136	204	248
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843	Camanducaia	MG	204	181	214	136	179	147	173	183	218	152	170	134	204	248
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845	Camanducaia	MG	204	177	214	136	177	145	170	183	192	152	170	136	204	326
846	Camanducaia	MG	204	177	214	136	177	147	170	183	190	152	170	136	204	239
847	Camanducaia	MG	297	179	214	136	177	145	170	183	190	150	170	134	207	248
848	Camanducaia	MG	312	181	214	136	177	147	173	183	222	152	170	136	204	206
849	Camanducaia	MG	330	179	214	136	177	147	173	183	188	152	170	134	204	248

850	Bueno Brandão	MG	204	179	214	136	177	145	170	183	190	152	170	136	204	206
851	Bueno Brandão	MG	204	179	214	136	177	147	170	183	190	152	170	136	204	248
852	Bueno Brandão	MG	204	181	214	136	179	145	173	183	218	152	170	134	204	248
853	Bom Repouso	MG	204	179	214	136	177	145	170	183	192	152	170	136	204	254
854	Bom Repouso	MG	204	179	214	136	177	145	170	183	192	152	170	136	204	245
855	Bom Repouso	MG	204	181	214	136	177	147	170	183	190	152	170	136	204	245
856	Ipuiúna	MG	318	201	214	136	179	145	173	183	222	150	170	136	204	245
857	Ipuiúna	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	257
858	Ipuiúna	MG	204	179	214	136	177	147	170	183	190	152	170	136	204	254
859	Ipuiúna	MG	204	181	214	136	179	147	173	183	220	154	170	136	204	251
860	Ipuiúna	MG	204	181	214	136	179	145	167	183	218	150	170	136	204	248
861	Ipuiúna	MG	309	201	214	136	177	145	173	183	222	152	170	136	204	245
862	Ipuiúna	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	254
863	Ipuiúna	MG	309	201	214	136	177	147	173	183	218	152	170	136	207	248
864	Ipuiúna	MG	309	201	214	136	177	147	173	183	222	152	170	136	204	248
865	Espírito Santo do Dourado	MG	204	181	214	136	179	145	173	183	220	152	170	136	204	251
866	Espírito Santo do Dourado	MG	204	181	214	136	179	147	173	183	218	152	170	136	204	260
867	Espírito Santo do Dourado	MG	309	179	214	136	179	147	173	183	220	152	170	136	204	254
868	Espírito Santo do Dourado	MG	312	181	217	136	177	145	173	183	222	152	170	136	204	227
869	Espírito Santo do Dourado	MG	204	201	214	136	177	147	173	183	220	152	170	134	204	254
870	Espírito Santo do Dourado	MG	204	179	214	136	179	145	173	183	220	152	170	134	204	206
871	Irati	PR	315	181	214	136	177	145	173	183	224	152	170	136	204	254
872	Irati	PR	204	181	214	136	179	145	173	183	220	152	170	136	207	257

873	Irati	PR	204	181	214	136	179	147	173	183	218	152	170	136	207	251
874	Contenda	PR	309	181	214	136	179	145	173	183	190	152	170	136	204	206
875	Contenda	PR	351	179	214	136	177	147	173	183	190	152	170	136	207	206
876	São José dos Ausentes	RS	204	181	214	136	179	145	170	183	220	152	170	136	204	206
877	São José dos Ausentes	RS	324	181	214	136	177	147	173	183	220	152	170	136	207	257
878	São José dos Ausentes	RS	204	183	214	136	179	147	170	183	220	152	170	136	204	221
879	Divinolândia	SP	309	181	214	136	179	145	173	183	218	152	170	136	204	251
880	Divinolândia	SP	204	181	214	136	179	147	173	183	220	152	170	136	207	254
881	Guarapuava	PR	204	181	214	136	179	145	173	183	220	150	170	136	204	254
882	Guarapuava	PR	204	181	214	136	179	147	173	183	220	150	170	136	204	209
883	Guarapuava	PR	204	181	214	136	179	147	173	183	218	154	170	136	204	254
884	Guarapuava	PR	204	181	214	136	179	145	173	183	220	152	170	136	204	254
885	Guarapuava	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	254
886	Guarapuava	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	257
887	Guarapuava	PR	204	181	214	136	179	147	170	183	220	150	170	136	204	251
888	Guarapuava	PR	204	181	214	136	179	147	170	183	218	152	170	136	207	254
889	Guarapuava	PR	309	201	214	136	179	147	173	183	220	152	170	136	204	251
890	Guarapuava	PR	204	181	214	136	179	145	173	183	220	150	170	136	204	254
891	Guarapuava	PR	204	181	214	136	179	147	173	183	220	152	170	134	204	254
892	Guarapuava	PR	204	181	214	136	179	145	173	183	218	152	170	134	204	254
893	Guarapuava	PR	357	179	214	136	177	145	173	183	190	152	170	136	204	251
894	Guarapuava	PR	204	181	214	136	179	145	170	183	220	152	170	136	204	254
895	Guarapuava	PR	204	181	214	136	179	147	170	183	220	152	170	136	204	251
896	Guarapuava	PR	306	201	214	136	179	145	173	183	222	148	170	136	204	206
897	Guarapuava	PR	204	181	214	136	179	145	173	183	220	130	170	136	204	254
898	Palmas	PR	204	181	214	136	179	145	173	183	218	152	170	136	204	254
899	Palmas	PR	306	193	214	136	177	147	170	183	192	152	170	136	204	245
900	Palmas	PR	306	203	214	136	177	145	173	186	222	152	170	136	204	203
901	Palmas	PR	312	181	214	136	177	147	173	183	224	152	170	136	207	206

902	Palmas	PR	204	181	214	136	179	147	170	183	220	152	170	136	204	251
903	Palmas	PR	204	183	214	136	179	147	170	183	218	152	170	136	207	251
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905	Palmas	PR	312	181	214	136	177	147	173	183	222	152	170	134	204	206
906	Palmas	PR	204	181	214	136	179	147	173	183	218	152	170	134	207	257
907	Palmas	PR	312	181	214	136	177	145	173	183	222	152	170	136	204	206
908	Palmas	PR	315	181	214	136	177	147	173	183	222	152	170	136	204	206
909	Palmas	PR	312	181	214	136	177	147	173	183	220	150	170	134	207	206
910	Palmas	PR	204	181	214	136	179	147	173	183	218	152	170	134	204	257
911	Palmas	PR	204	201	214	136	177	147	173	183	222	152	170	136	204	206
912	Palmas	PR	315	181	214	136	177	147	173	183	220	152	170	136	207	206
913	Palmas	PR	204	181	214	136	179	147	170	183	218	152	170	136	207	218
914	Palmas	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	254
915	Palmas	PR	312	181	214	136	177	145	173	183	222	152	170	136	207	206
916	Palmas	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	269
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918	Palmas	PR	204	181	214	136	179	147	170	183	218	152	170	136	204	251
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920	Palmas	PR	204	181	214	136	179	147	173	183	220	152	170	136	204	254
921	Palmas	PR	204	181	214	136	179	143	170	183	220	152	170	136	207	251
922	Água Doce	SC	204	181	214	136	179	145	170	183	218	154	170	136	204	254
923	Água Doce	SC	312	181	214	136	177	147	173	183	222	150	170	136	207	218
924	Água Doce	SC	204	183	214	136	179	147	173	183	218	152	170	136	207	257
925	Água Doce	SC	315	181	214	136	177	145	173	183	220	150	170	136	207	206
926	Água Doce	SC	204	181	214	136	179	147	170	183	220	152	170	136	207	221
927	Água Doce	SC	204	181	214	136	179	147	173	183	218	152	170	136	207	254
928	Calmon	SC	204	181	214	136	179	145	170	183	220	152	170	134	204	251
929	Calmon	SC	204	183	214	136	177	147	173	183	220	152	170	136	207	254
930	Calmon	SC	204	181	214	136	177	147	173	183	220	152	170	136	207	251

931	Calmon	SC	315	181	214	136	177	145	173	183	220	150	170	136	204	206
932	Calmon	SC	312	181	217	136	177	145	173	183	220	152	170	136	204	206
933	Calmon	SC	312	183	214	136	177	147	173	183	222	152	170	136	204	206
934	Calmon	SC	204	181	214	136	179	145	173	183	218	150	170	136	207	218
935	Calmon	SC	204	183	214	136	179	147	173	183	218	150	170	136	204	251
936	Calmon	SC	204	181	214	136	177	147	173	183	220	152	170	136	204	254
937	Contenda	PR	312	179	214	136	179	147	173	183	218	152	170	136	207	251
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939	Contenda	PR	309	181	217	136	177	147	173	183	222	152	170	136	204	206
940	Mucugê	BA	204	179	214	136	177	145	173	183	192	152	170	136	207	218
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942	Mucugê	BA	309	201	214	136	179	147	173	183	220	152	170	136	207	257
943	Mucugê	BA	204	177	214	136	177	145	170	183	192	150	170	136	204	254
944	Mucugê	BA	306	181	214	136	179	147	173	183	222	152	170	136	207	206
945	Mucugê	BA	312	179	214	136	177	147	170	183	190	152	170	136	204	248
946	Mucugê	BA	204	181	214	136	179	145	173	183	220	150	170	136	207	254
947	Mucugê	BA	204	181	214	136	179	147	173	183	222	152	170	136	207	254
948	Mucugê	BA	309	201	214	136	179	147	173	183	220	152	170	134	207	263
949	Mucugê	BA	312	181	214	136	179	145	173	183	220	150	170	136	207	206
950	Mucugê	BA	204	181	214	136	179	147	173	183	222	152	170	136	207	254
951	Mucugê	BA	204	181	214	136	179	147	173	183	218	152	170	134	207	254
952	Mucugê	BA	201	181	214	136	177	145	173	183	190	150	170	136	207	257
953	Mucugê	BA	306	181	217	136	179	147	173	183	220	152	170	136	204	206
954	Mucugê	BA	201	183	214	136	179	147	173	183	222	150	170	136	207	275
955	Cristalina	GO	204	179	214	136	177	145	170	183	192	150	170	136	207	248
956	Cristalina	GO	204	181	214	136	177	147	173	183	190	152	170	136	207	254
957	Cristalina	GO	204	181	214	136	177	145	173	183	190	150	170	136	204	254
958	Aguaí	SP	204	181	214	136	179	145	173	183	220	152	170	136	207	254
959	Aguaí	SP	312	181	214	136	177	147	173	183	220	154	170	136	204	206

960	Aguaí	SP	312	181	217	136	177	147	173	183	222	154	170	136	207	206
961	Aguaí	SP	201	181	214	136	179	145	173	183	218	150	170	136	204	254
962	Aguaí	SP	204	181	214	136	179	145	170	183	218	152	170	136	207	251
963	Aguaí	SP	204	181	214	136	179	147	173	183	218	152	170	136	207	254
964	Vargem Grande do Sul	SP	204	181	214	136	179	145	173	183	218	150	170	136	204	257
965	Vargem Grande do Sul	SP	204	181	214	136	179	145	173	183	220	152	170	136	207	239
966	Vargem Grande do Sul	SP	336	177	214	136	179	147	173	183	220	152	170	136	207	251
967	Casa Branca	SP	204	201	214	136	177	145	173	183	222	150	170	136	204	206
968	Casa Branca	SP	318	181	214	136	177	147	173	183	222	152	170	136	207	206
969	Casa Branca	SP	309	201	214	136	177	147	173	183	222	152	170	136	207	248
970	Casa Branca	SP	309	205	214	136	177	147	173	186	224	152	170	136	204	206
971	Casa Branca	SP	309	201	214	136	179	145	173	183	222	152	170	136	207	272
972	Casa Branca	SP	309	205	214	136	177	143	173	186	222	152	170	136	207	206
973	Vargem Grande do Sul	SP	204	181	214	136	179	145	170	183	216	150	170	136	207	224
974	Vargem Grande do Sul	SP	204	181	214	136	179	143	170	183	218	152	170	136	207	224
975	Vargem Grande do Sul	SP	204	181	214	136	179	147	170	183	220	150	170	136	207	251
976	Tatuí	SP	204	181	214	136	179	145	170	183	218	150	170	136	204	251
977	Tatuí	SP	309	201	217	136	177	145	173	183	222	152	170	136	207	248
978	Tatuí	SP	204	181	214	136	177	147	170	183	192	150	170	136	207	239
979	Tatuí	SP	309	203	214	136	179	147	173	183	218	154	170	136	207	206
980	Tatuí	SP	204	179	214	136	179	147	170	183	220	152	170	136	207	254
981	Tatuí	SP	306	201	214	136	177	147	173	183	220	150	170	136	204	260
985	Guarapuava	PR	312	199	214	136	177	147	173	183	222	152	170	136	204	257
986	Guarapuava	PR	204	181	214	136	179	147	170	183	220	150	170	136	204	254
987	Guarapuava	PR	318	201	214	136	177	147	173	183	222	152	170	136	204	266

[a] BA = Bahia, GO = Goiás, MG = Minas Gerais, PR = Paraná, RS = Rio Grande do Sul, SP = São Paulo, and SC = Santa Catarina state

Table S2. Four sets of PCR multiplex reactions used to genotype the population of *Alternaria grandis*.

PCR multiplex	Locus	Fluorescent dye	Motif
1	SSR-106	6-FAM	(ACA)12
	SSR-032	VIC	(CA)19
	SSR-115	NED	(CAA)20
	SSR-195	PET	(CA)19
2	SSR-062	6-FAM	(AC)9
	SSR-169	VIC	(CA)30
	SSR-056	NED	(TGC)9
	SSR-105	PET	(TCT)20
3	SSR-047	6-FAM	(AC)26
	SSR-020	VIC	(CA)31
	SSR-073	NED	(CAG)11
	SSR-164	PET	(CT)29
4	PAS4	VIC	(GTC)5
	Ad8	PET	(GAA)15

Table S3. Two analyses of molecular variance (AMOVA) conducted to test grouping according to six geographic regions and year in Brazil.

Source of variation	**df**	**Sum of squares**	**Variation (%)**
Geographic regions			
Among region	5	29461.0	1.9
Within region	287	913805.0	98.1
Total	292	943266.0	
Year			
Among year	2	10171.9	0.7
Within year	296	943485.6	99.3
Total	298	953657.5	

CHAPTER 3: Sensitivity of *Alternaria grandis,* the causal agent of potato early blight, to DMI, SDHIs, and multi-site fungicides

ABSTRACT

Early blight is a severe and widespread disease of potatoes in Brazil and is caused by the ascomycete *Alternaria grandis*. Site-specific and multi-site fungicides are intensively used to reduce yield losses due to early blight. This study was conducted to assess the sensitivity of *A. grandis* to site-specific (SDHIs and DMIs), and multi-site (chlorothalonil - CT) fungicides and to identify mutations in the subunits of the *Sdh* gene. Fungicide sensitivity was assessed with different doses using two methods: the microtiter method for SDHIs (BosCalid; FluoPyram; FluXapyroxad; PydiFlumetofen; and PenthioPyrad); and mycelial growth for DifenoConazole (DC) and CT. Discriminatory doses (DD) were established for high-throughput screening of isolates. The EC50 values ranged from 109.3 to 905.4 and from 0.03 to 2.27 µg/mL for CT and DC, respectively, and the DD for CT and DC were 1000 µg/mL and 5 µg/mL, respectively. There is no evidence of resistance to DC, but there are isolates less sensitive to CT *in vitro*. The EC50 values of BC, FP, FX, PF and PP for 32 isolates of *A. grandis* ranged from 0.3 to 0.8; 0.9 to 69.7; 0.6 to 11.3; 0.2 to 92.5; and 6.7 to 61.5 µg/mL, respectively, and the DD were 100, 25, 25, 10, and 25 µg/mL, respectively. The sensitivity to SDHIs of 113 isolates was assessed using the DD. For BC, all isolates (100%) were classified as insensitive; for PP, FX, FP, and PF, 94%, 89%, 63%, and 61% were classified as insensitive to the respective fungicides. The reduced sensitivity of *A. grandis* to BC, FX, and PP fungicides is probably associated with the widespread occurrence of the H278Y and H134R mutations in the *sdhB* and *sdhC* genes, respectively. No mutation was detected in the *sdhD* gene. Management of early blight must employ multi-site fungicides and site-specific products with different modes of actions.

INTRODUCTION

Early blight, caused by *Alternaria grandis*, is one of the most severe diseases on potato (*Solanum tuberosum* L.) worldwide and can cause considerable yield losses in high temperature and humidity conditions (Batista et al. 2006). Intensive fungicide application is the most commonly used strategy to manage this disease because there are no commercial cultivars with complete resistance to early blight. Consequently, the intensive use of site-specific fungicides can select resistant *A. grandis* individuals relatively fast. The reasons for the rapid change in the profile of the pathogen population regarding fungicide sensitivity may be due to the mode of action of the fungicides and the biological characteristics of the pathogens: the abundant production of assexual spores in one season allows a large effective population size which in turn increases the chances for the occurrence of many mutants resistant to chemical compounds.

Site-specific fungicides as demethylation inhibitors (DMIs) and modern succinate dehydrogenase inhibitors (SDHIs Frac target code C2) (https://www.frac.info/frac-teams/working-groups/sdhi-fungicides/information) such as boscalid, fluopyram and fluxapyroxad, and multi-site as chlorothalonil are registered to be used in potato crops (Agrofit 2022), and they have often been applied to early blight control. The DMIs are broad-spectrum fungicides that affect the C14-demethylation step during ergosterol biosynthesis and inhibits fungal growth mostly due to perturbation of the cell membrane (Siegel 1981). The first DMIs were developed in the 1960's and triadimefon and triadimenol are examples of early products that became widely used in many crops. Several generations of DMIs succeeded the first products and in the past 30 years, the most recently developed DMIs were difenoconazole, metconazole, and prothioconazole (Ishii 2015).

Specifically for the management of leaf spots caused by Ascomycetes, difenoconazole has been widely recommended in cereals, fruits and vegetable crops; including potato early blight (Agrofit 2022). Difenoconazole is a translaminar DMI fungicide that presents medium risk of resistance development (FRAC code 3). Three main resistance mechanisms known for DMI fungicides have been postulated: target-site modification; *cyp51* gene overexpression; and increased fungicide efflux. Resistance to DMIs in *Alternaria* spp. that affect different crops has been reported

(Avenot et al. 2016; Fonseka and Gudmestad 2016; Wang et al. 2016; Zhang et al. 2020), but there is only one study (Zhang et al. 2020) that reported resistant isolates of small-spored *Alternaria* species to difenoconazole. Only one isolate collected from potato crop grown in China had a mutation in the *cyp51* gene that led to the exchange of arginine to tryptophan (R511W) at position 511 and difenoconazole induce overexpression of the *cyp51* gene in DMI-resistant isolates (Zhang et al. 2020). Currently, there is no information about the sensitivity of the *A. grandis* populations to difenoconazole in Brazil.

The SDHI fungicides target the succinate dehydrogenase (SDH) in complex II and block respiration (Avenot and Michailides 2010). These fungicides present medium to high risk of resistance development (FRAC code 7). The SDH complex has four subunits; a flavoprotein (SDHA), an iron-sulfur (SDHB), and two membrane-bound (SDHC and SDHD) subunits. Among the four subunits, SDHI fungicides are known to have higher affinity with the SDHB, SDHC, and SDHD subunits. These three subunits comprise the ubiquinone binding pocket of respiration complex II (Sierotzki and Scalliet 2013). Mutations in any of the subunits can result in reduced sensitivity to SDHI products. Mutations associated with *A. solani* SDHI-resistant isolates had been found in the three *sdh* genes: *sdhB*, *sdhC*, and *sdhD* (Mallik et al. 2014): Two nucleotide substitutions that lead to the replacement in the SDHB subunit of the histidine to arginine (H278R) or to tyrosine (H278Y) residue at position 278; one in the SDHC that exchange H to R (H134R) or H to glutamine (H134Q) at position 134; and two in the SDHD that replace a aspartic acid to glutamic acid (D123E) at codon 123 or H133R. In the past years several studies reported the occurrence of resistance of *A. solani* to SDHI fungicides in the USA (Wharton et al. 2012; Fairchild et al. 2013; Gudmestad et al. 2013; Mallik et al. 2014; Miles et al. 2014; Tymon and Johnson 2014; Bauske et al. 2018; Budde-Rodriguez et al. 2021), Belgium (Landschoot et al. 2017a), China (Shi et al. 2015), Germany (Metz et al. 2019; Nottensteiner et al. 2019), and Sweden (Mostafanezhad et al. 2022). Isolates of *A. solani* were sorted into three groups of sensitivity according to the EC50 values for boscalid: sensitive (<5 µg/mL), moderately resistant (5 to 20 µg/mL), and highly resistant (>20 µg/mL) (Gudmestad et al. 2013). However, until now there was no information available on the sensitivity of *A. grandis* to SDHIs.

Chlorothalonil is a fungicide with low risk of resistance (FRAC code M05). This fungicide is frequently used in the preventive control program of early blight with twofold purposes: to act as a protectant fungicide preventing new infections and to reduce the risk of developing resistance to site-specific fungicides. There was little information regarding the sensitivity of *Alternaria* species to multisite fungicides. One study was conducted to assess the sensitivity of *A. solani* collected from potato in seven fields at different times of the season to multisite fungicides (Holm et al. 2003). Isolates from five fields collected at the end of season were less sensitive to chlorothalonil than isolates collected at the beginning. In another study, the sensitivity of 60 isolates of *A. solani* collected from tomato was assessed against mancozeb and chlorothalonil (Abu-El Samen et al. 2016). Most isolates had reduced sensitivity to both fungicides.

The objectives of this study were to (i.) assess the sensitivity of the *A. grandis* populations from Brazil to boscalid (BC); fluopyram (FP); fluxapyroxad (FX); penthiopyrad (PP); pydiflumetofen (PF); difenoconazole (DC); and chlorothalonil (CT); and (ii) identify mutations in the *sdh* genes in resistant isolates to SDHI fungicides.

MATERIAL AND METHODS

Sampling and isolation of *Alternaria grandis*

A total of 297 isolates of *A. grandis* were obtained from potato plants with early blight symptoms. The samples were collected in the main regions of potato production in Brazil: Bahia (N = 33 isolates), Goiás (6), Minas Gerais (100), Paraná (80), Rio Grande do Sul (31), Santa Catarina (15), and São Paulo (32) during 2019 (32), 2020 (141), and 2021 (124) (Fig. 1). These states account for 99.7% of the potato production in the country (IBGE 2021).

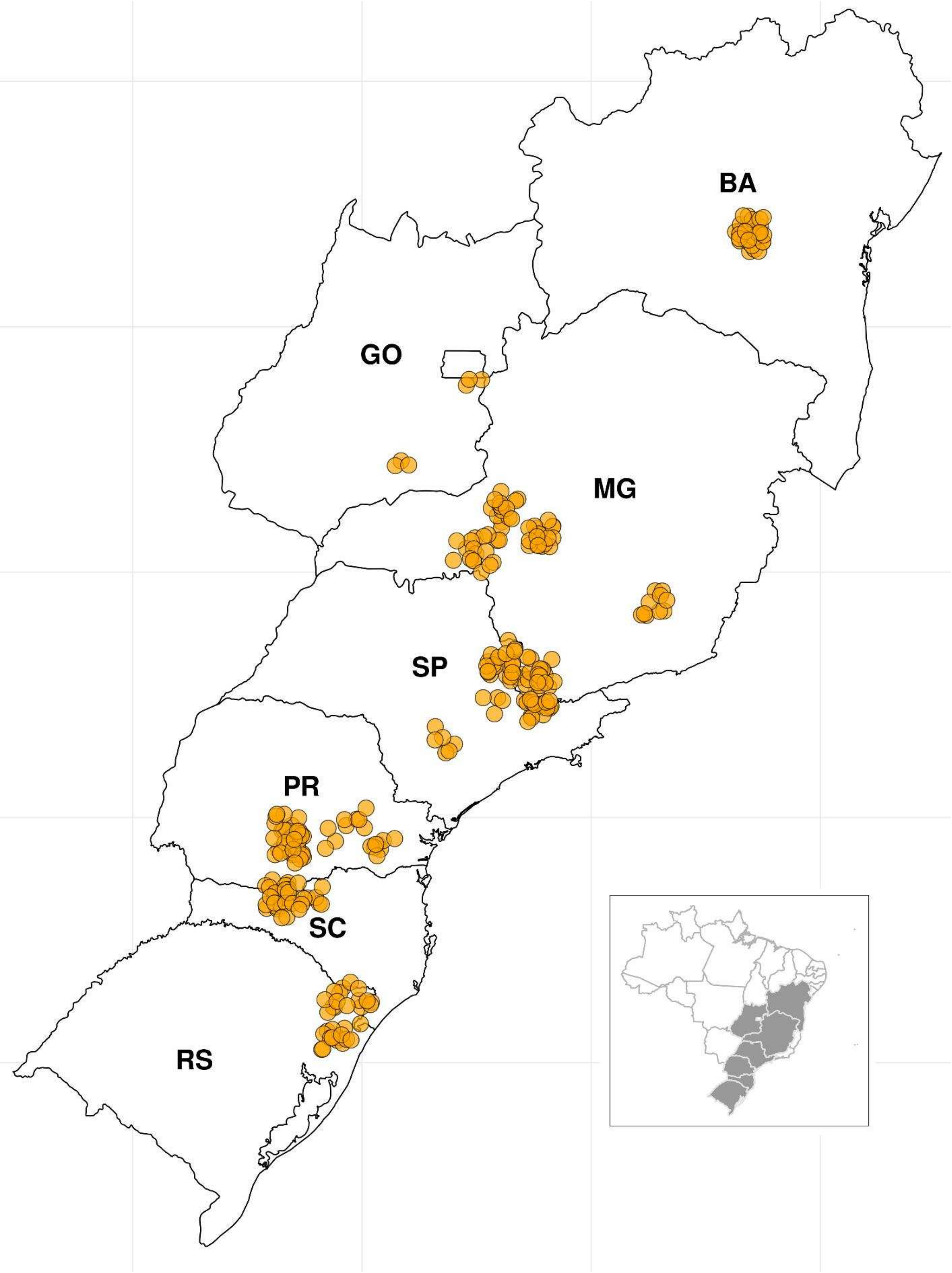


Figure 1. Location of origin of *Alternaria grandis* isolates from potato fields in different states of Brazil, during the 2019, 2020, and 2021 growing seasons. Map of Brazil is depicted in the inset and shaded states in the map are those from which samples were obtained. States of Brazil: RS = Rio Grande do Sul; SC = Santa Catarina; PR = Paraná; SP = São Paulo; MG = Minas Gerais; GO = Goiás; and BA = Bahia.

Symptomatic potato leaves were examined under a stereomicroscope searching for typical lesions of early blight. Conidia were transferred to potato dextrose agar (PDA) medium on Petri plates (70 mm - diameter) and incubated at 25 °C for 5 days. Mycelium discs (5 mm - diameter) from the colonies were transferred to the center of Petri plates (90 mm - diameter) with solid V8 medium amended with $CaCO_3$ (170 mL of V8 juice, 15 g of agar, and 3 g of $CaCO_3$ per liter of distilled water) and incubated at 25 °C for 7 days when aerial mycelium was removed using 10 mL of distilled water and a sterile scalpel. After that, the water in Petri plates was discarded and plates kept at 25 °C, under near-UV light (black light 40W) for 12 h and 12 h in the dark to induce sporulation.

Only monoconidial isolates were used in the assays. A single conidium was transferred to PDA on Petri plates and kept at 25°C for 5 days. After incubation, 5 mm-diameter mycelium discs were transferred to 2 mL microtubes with and without water (Castellani, 1939). Microtubes were stored at 8 °C.

Sensitivity to DMI and chlorothalonil

The sensitivity of mycelial growth of 54 isolates to chlorothalonil (CT; Bravonil Ultrex, Syngenta; 825 g/kg of a.i.) and difenoconazole (DC; Syngenta; 250 g/L of a.i.) was screened to define discriminatory doses. These fungicides were dissolved in DMSO (0.1%) to obtain a stock solution of 100 mg of active ingredient per mL. Mycelial plugs (5 mm - diameter) from a 5-day-old culture were placed in the center of Petri dishes (60 mm - diameter) with PDA medium supplemented with the fungicide at final concentrations of 0 (PDA + DMSO); 25; 50; 100; 500; and 1000 µg/mL for CT and 0 (PDA + DMSO); 0.05; 0.1; 0.5; 1; 5; and 10 µg/mL for DC. Plates were maintained at 25 °C in the dark for 6 days. Colony diameters of each isolate were measured in two perpendicular directions using a digital caliper, then mycelial plug diameter (5 mm) was subtracted. The assay was set on a complete randomized design with four replicates (one plate = one replicate) per concentration of fungicide. The experiment was performed twice.

The effective dose at which mycelial growth was inhibited by 50% (EC50) was calculated with the *drc* package (Ritz et al. 2015) in the R program using a log-logistic model with three parameters (LL.3). The fungicide concentration that reduced the mycelial growth of 75% of the isolates by less than 25% of the growth recorded for the control treatment was selected to be used as discriminatory doses for CT and DC.

Assessment of fungicide sensitivity using discriminatory doses

The sensitivity of 297 isolates of *A. grandis* was assessed using discriminatory doses for CT and DC defined above. Mycelial plugs (5 mm - diameter) from a 5-day-old culture were placed in the center of Petri plates (60 mm - diameter) with PDA supplemented with CT and DC at 1000 µg/mL and 5 µg/mL, respectively. Plates were maintained at 25 °C in the dark for 6 days. Colony diameters were measured in two perpendicular directions using a digital caliper. The assay was set on a complete randomized design with four replicates (one plate = one replicate) per concentration of fungicide. The experiment was performed twice. Resistant isolates were considered as those with colony diameter greater than 75% in the discriminatory dose compared to positive control.

Sensitivity to SDHI fungicides

The sensitivity of isolates of *A. grandis* was assessed using the microtiter assay (Stammler and Speakman 2006) to the following fungicides: boscalid (BC; Sigma-aldrich, 98% purity); fluopyram (FP; Sigma-aldrich, 98% purity); fluxapyroxad (FX; Sigma-aldrich, 98% purity); penthiopyrad (PP; Sigma-aldrich, 98% purity); and pydiflumetofen (PF; Adepidyn^{TM}; Syngenta 18.35%). These fungicides were dissolved in 0.1% of DMSO to obtain 100 mg of BC/mL, 100 mg of FP/mL, 25 mg of FX/mL, 25 mg of PP/mL, and 100 mg of PF/mL for the stock solutions. The serial dilutions of each fungicide were prepared from stock solutions. Fungicide was added to liquid Czapek-Dox broth medium (60 g of sucrose, 4 g of $NaNO_3$, 2 g of K_2HPO_4, 1 g of $MgSO_4$. $7H_2O$, 1 g of KCl and 0.02 g of $FeSO_4$/liter). The final concentration for each fungicide was: 0 (CD + DMSO); 0.01; 0.1; 1.0; 5; 10; 50; 100 µg/mL.

To prepare conidia suspension, *A. grandis* isolates were grown on oatmeal agar medium for 7 days and the following steps were as mentioned above to obtain monoconidial isolates. The spores were harvested with distilled water and the suspension was adjusted to 2 x 10^5 conidia per mL using a hemocytometer. Fifty microlitre conidia suspension was added in each well of the microtiter plates containing 50 µL of Czapek-Dox broth amended with fungicides at each of the concentrations previously mentioned. Chloramphenicol (100 µg/mL) and streptomycin (200 µg/mL) were added to prevent bacteria contamination. The negative control was Czapek-Dox broth amended with fungicides, but no conidia. The conidia suspension and the fungicide were mixed for 15s and absorbance was read at 405 nm (absorbance at time zero) using a Multiskan GO microplate spectrophotometer (Thermo Scientific). The microtiter plates were kept at 25 ºC in the darkness for seven days. After that, the absorbance was read at 405 nm. The values were corrected by comparison with the absorbance at time zero. The assay was done with three replicates. One well in a plate was considered as one replicate per isolate and fungicide concentration. The experiment was performed twice.

The EC50 values for each fungicide was calculated with the *drc* package (Ritz et al. 2015) in R program using a log-logistic model with four parameters (LL.4). Discriminatory doses for each SDHIs were defined based on EC50 values and isolates were grouped in classes of sensitivity previously defined (Gudmestad et al. 2013).

Assessment of SDHIs sensitivity using discriminatory doses

The sensitivity of 113 isolates of *A. grandis* was assessed to BC, FP, FX, PP, and PF using the discriminatory doses defined above. Fifty microlitre conidia suspension was added in each well of the microtiter plates containing 50 µL of Czapek-Dox broth amended with 100 µg/mL for BC; 25 µg/mL for FP, FX and PP; and 10 µg/mL for PF. Chloramphenicol (100 µg/mL) and streptomycin (200 µg/mL) were added to prevent bacteria contamination. The negative control was Czapek-Dox broth amended with fungicides, but no conidia. The conidia suspension and the fungicide were mixed for 15s and absorbance was read at 405 nm (absorbance at time zero) using a Multiskan GO microplate spectrophotometer (Thermo Scientific). The microtiter plates were kept at 25 ºC in the darkness for seven days. After that, the absorbance was read at 405 nm. The values were corrected by comparison with the absorbance at time zero. The assay was done with four replicates. One well in a plate was considered as one replicate per isolate and fungicide concentration. Resistant isolates were considered as those with germination greater than 75% in the discriminatory dose compared to positive control.

DNA extraction

The isolates were cultivated in liquid medium (10 g sucrose, 2 g L-asparagine, 2 g yeast extract, 1 g KH_2PO_4, 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 1 L dH_2O) in Erlenmeyer flasks kept at 150 rpm and 25°C in the dark to allow for mycelium growth□. After 7 days, the mycelium was washed with distilled water, transferred to filter paper to dry for 24 h, and macerated in TissueLyser II (QIAGEN, Germany). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) following the manufacturer's instructions. DNA integrity was analyzed by electrophoresis on a 1% agarose gel and quality was assessed by spectrophotometry. DNA was diluted to 25 ng/µl and stored at - 20 °C.

Sequencing of *sdh* genes

Thirty BC-resistant isolates and two sensitive ones were selected to screen for mutations in the *sdh* genes (B, C, and D subunits) known to be associated with SDHI resistance. The PCRs were performed using GoTaq DNA Polymerase (Promega Corp., Madison, WI) with primers developed to amplify the *sdh* genes: SDHB-F and SDHB-R; SdhC-F2 and SdhC-R3; and SDHD-2F and SDHD-1R for subunit B (Mallik et al. 2014), C (Yang et al. 2015), and D (Miles et al. 2014), respectively. The PCR condition was an initial step of 95 °C for 2 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 30s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Reaction was confirmed by electrophoresis on a 1.5% agarose gel. PCR products were purified using ExoSAP-IT cleanup reagent (USB, Cleveland, OH), lyophilized and sequenced.

Molecular detection of mutations in the *Sdh* genes

PCR assays were used to detect mutations in *sdh* genes of *A. grandis* isolates (Mallik et al. 2014). First, multiplex PCR was performed to detect H134R and H133R mutations in the *sdhC* and *sdhD* genes, respectively. Multiplex PCRs were performed in a final volume of 12.5 µL with 1 µL of DNA (25 ng/µL); 2.5 µL of Colorless GoTaq Reaction Buffer; 0.1 µL of SdhBsen, 0.07 µL of SdhC, and 0.1 µL of SdhD forward and reverse primers at 10 mM; and 1 U of GoTaq DNA Polymerase (Promega Corp., Madison, WI). PCR conditions were: an initial step of 95 °C for 2 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Two amplifications of 235 and 475 bp or 235 and 72 bp occurred when a mutation was detected in *sdhC* and *sdhD* genes, respectively. A 235 bp product was amplified for the wild type (no mutation). If no product was amplified, additional PCR assay was conducted using the mismatch amplification analysis (MAMA-PCR) (Mallik et al. 2014). As control, in all PCR assays were conducted with DNA of isolates with mutation in the *sdhB* and *sdhC* and without mutation.

The MAMA-PCRs were conducted to identify the H278R (MAMAB1-F and MAMABM-R primers) or H278Y (MAMAB1-F and MAMABR-R primers) mutations in *sdhB* gene. PCR program was the same as the multiplex PCR previously described, but annealing temperature was 60°C. The MAMA-PCRs were performed in a final volume of 12.5 µL with 1 µL of DNA (25 ng/µL); 2.5 µL of Colorless GoTaq Reaction Buffer; 0.1 µL of each primer at 10 mM; and 1 U of GoTaq DNA Polymerase (Promega Corp., Madison, WI).

RESULTS

Sensitivity to DMI and chlorothalonil

The EC50 values for DC after analyzing 54 isolates ranged from 0.03 to 2.27 µg/mL and the overall mean was 0.50 µg/mL (Fig. 2). There was a trend of increase of the median of mycelial growth at each dose when assessing isolates collected in later years (Fig. 3A). Fifty-three and 46 isolates grew more than 50% in relation to the control when in medium amended with the fungicide at 0.05 and 0.1 µg/mL, respectively (Fig. 3A). At 0.5, 1, and 5 µg/mL, the percent of mycelial growth ranged from: 20.4 to 75.0%; 15.2 to 65.3%; and 8.2 to 36.7% compared to the control, respectively. At 10 µg/mL, all isolates grew less than 23% in relation to the control. Therefore, the dose of at 5 µg/mL was selected as discriminatory for DC because more than 75% of isolates grew less than 25% compared to the control.

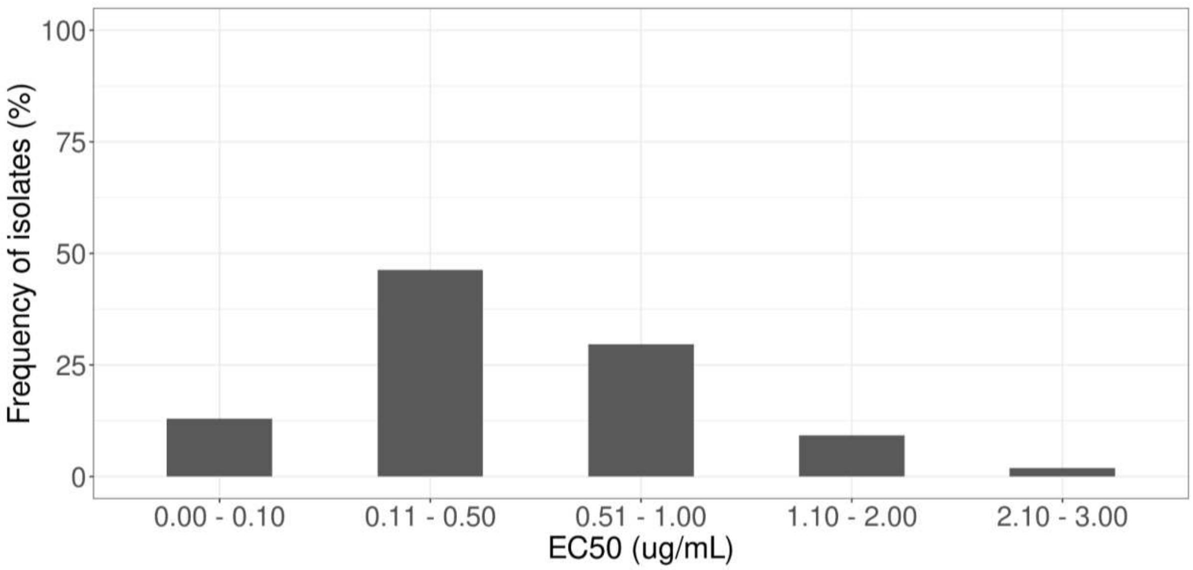


Figure 2. Frequency of isolates of *Alternaria grandis* (N=54) in different classes of effective concentration of difenoconazole that inhibits 50% of the mycelial growth (EC50).

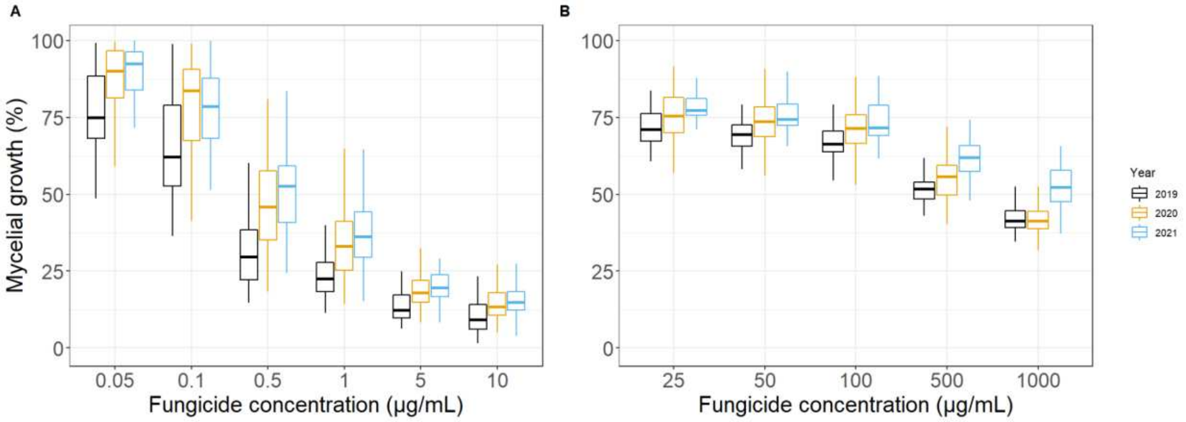


Figure 3. Boxplot showing the percent of mycelial growth compared to the control (BDA + DMSO) of the 49 isolates of *Alternaria grandis* collected in 2019 (N = 12 isolates), 2020 (N = 17), and 2021 (N = 20). A. difenoconazole, and B. chlorothalonil.

The EC50 values for CT ranged from 109.3 to 905.4 µg/mL and the overall mean was 569.8 µg/mL (Fig. 4). There was a trend of increase of the median of mycelial growth at each dose when assessing isolates collected in later years (Fig. 3B). Moreover, for 18 isolates the EC50 for CT was greater than 1000 µg/mL. The isolates grew over 50% compared to the control at 25, 50, and 100 µg/mL of CT (Fig. 3B). At 500 and 1000 µg/mL, the percent of mycelial growth ranged from 40.6 to 70.5% and 31.7 to 61.3% compared to the control, respectively. No isolate, in any dose, grew less than 25% compared to the control. Therefore, the highest dose (1000 µg/mL) was chosen as discriminatory for CT.

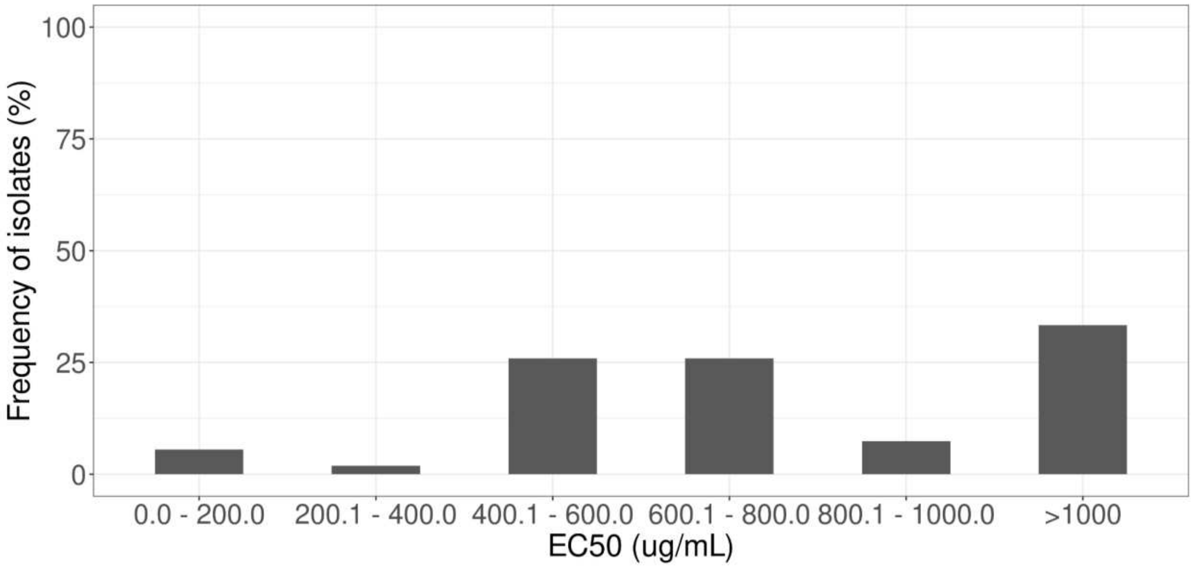


Figure 4. Frequency of isolates of *Alternaria grandis* (N=54) in different classes of effective concentration of chlorothalonil that inhibits 50% of the mycelial growth (EC50).

Assessment of CT and DC sensitivity using discriminatory doses

The sensitivity of 297 isolates of *A. grandis* was assessed to CT and DC using the discriminatory doses of 1000 and 5 µg/mL, respectively. There were no resistant isolates to either CT or DC, but there were isolates less sensitive to CT. The percent of inhibition varied from 39 to 64% and 69 to 92% for CT and DC, respectively.

Sensitivity to SDHI fungicides

For the 32 isolates used to define the EC50 and DD, 30, 26, 14, 9, and 2 isolates were insensitive (EC50 > 100 µg/mL) to BC, FX, PP, FP, and PF, respectively. The EC50 values of BC, FP, FX, PF and PP ranged from 0.3 to 0.8, 0.9 to 69.7, 0.6 to 11.3, 0.2 to 92.5, and 6.74 to 61.51 µg/mL, respectively (Fig. 5). There was no reduction in percent of germination to SDHI fungicides when assessing isolates collected in later years (Fig. 6).

Based on the EC50 values for all SDHIs, isolates were grouped in classes of sensitivity previously defined: sensitive (<5 µg/mL), moderately resistant (5 to 20 µg/mL), and highly resistant (>20 µg/mL) (Gudmestad et al. 2013). The dose of 100 µg/mL for BC; 25 µg/mL for FP, FX and PP; and 10 µg/mL for PF was chosen as discriminatory dose.

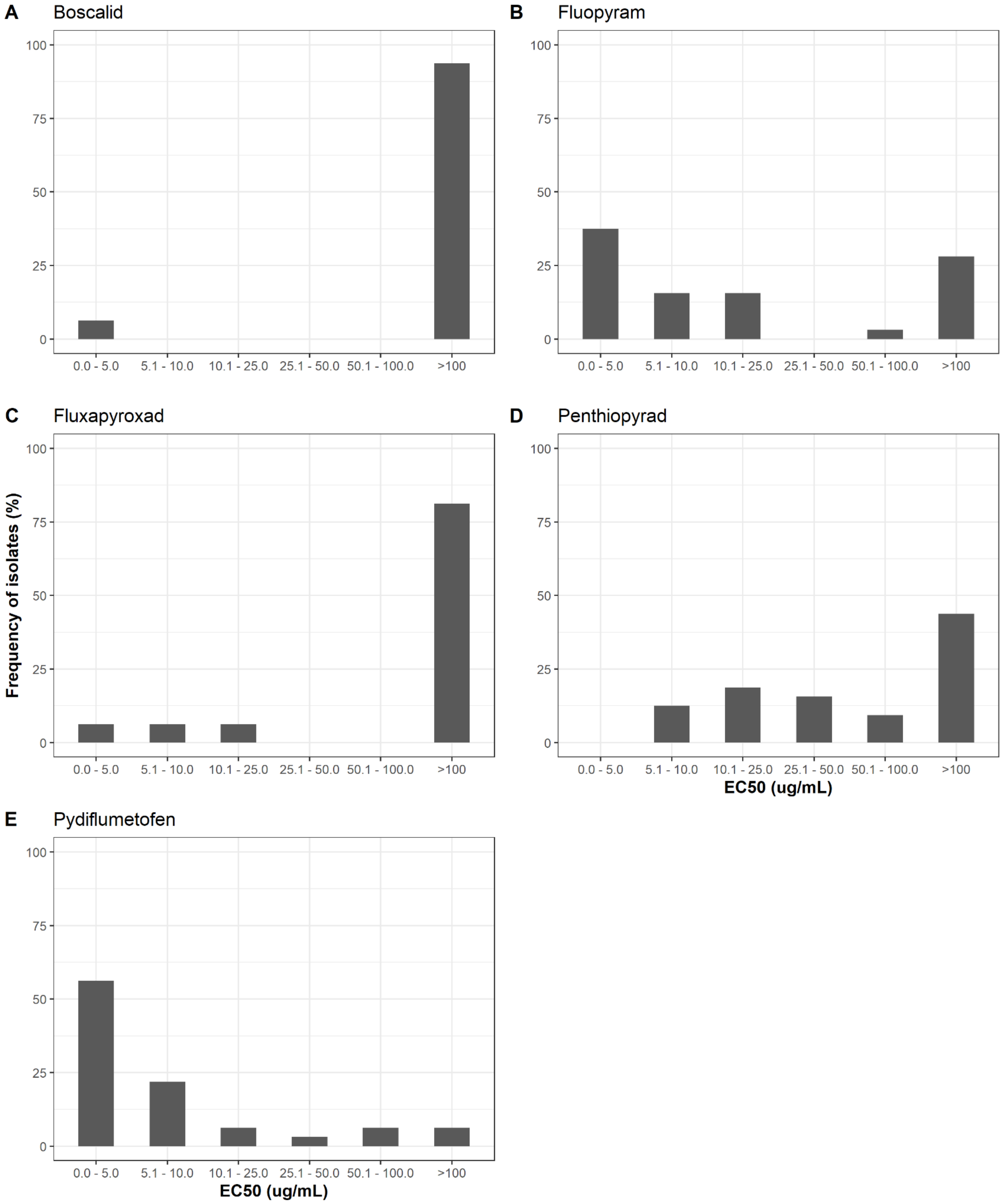


Figure 5. Frequency of isolates of *Alternaria grandis* (N = 32) in different classes of effective concentration of SHDIs that inhibits 50% of the germination (EC50). **A.** Boscalid; **B.** Fluopyram; **C.** Fluxapiroxad; **D.** Penthiopyrad; and **E.** Pydiflumetofen.

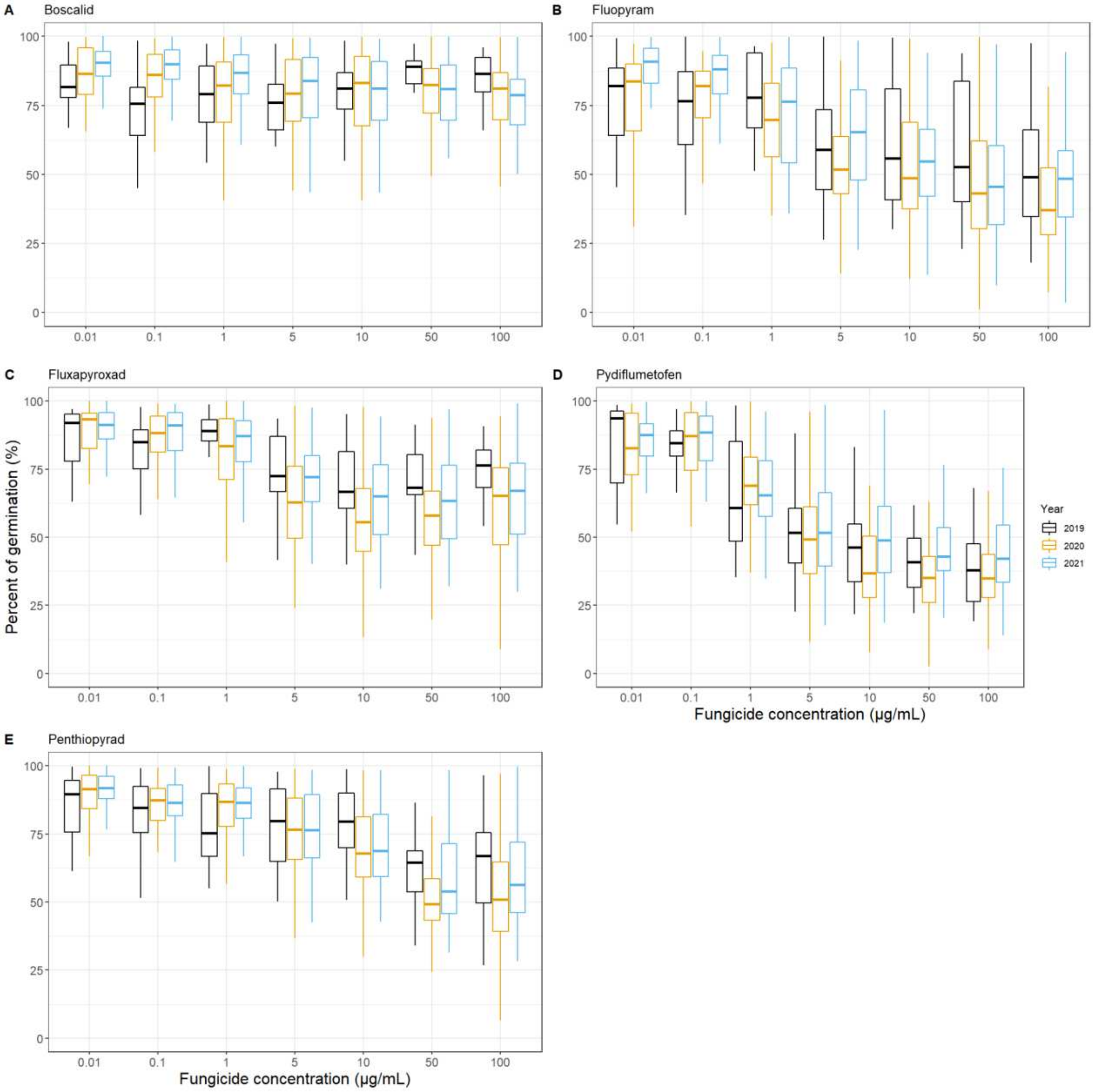


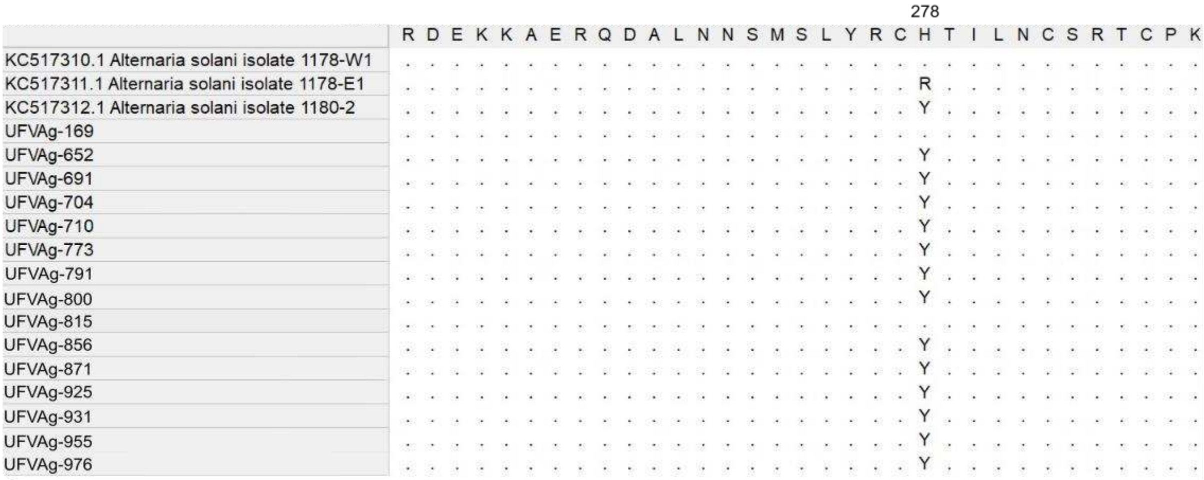
Figure 6. Boxplot showing the percent of germination compared to the control of the 30 isolates of *Alternaria grandis* collected in 2019 (N = 5 isolates), 2020 (N = 11), and 2021 (N = 14). **A.** Boscalid; **B.** Fluopyram; **C.** Fluxapiroxad; **D.** Penthiopyrad; and **E.** Pydiflumetofen.

Assessment of SDHIs sensitivity using discriminatory doses

The sensitivity of 113 isolates of *A. grandis* was assessed to BC, FP, FX, PP, and PF using the discriminatory doses. All isolates were insensitive to BC. For the other SDHIs, 106, 101, 71, and 69, isolates were insensitive to PP, FX, FP and PF, respectively.

Molecular detection of mutations associated with SDHI-resistant isolates

Sequencing of the *sdh* genes of 30 BC-resistant *A. grandis* isolates revealed the occurrence of one mutation each in subunits B or C compared with two sensitive isolates and sequences of the *A. solani sdh* genes deposited in GenBank database (accession numbers: KC517310 to KC517314). Fourteen resistant isolates had one transition mutation from cytosine (C) to thymine (T) at nucleotide position 990 in the *sdhB* gene that led to a change from H (codon CAC in sensitive isolates) to Y (codon TAC) at position 278 (B-H278Y) (Fig. 6).



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                                                                                       278
                                              R D E K K A E R Q D A L N N S M S L Y R C H T I L N C S R T C P K
KC517310.1 Alternaria solani isolate 1178-W1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
KC517311.1 Alternaria solani isolate 1178-E1  . . . . . . . . . . . . . . . . . . . . . R . . . . . . . . . . .
KC517312.1 Alternaria solani isolate 1180-2   . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-169                                     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
UFVAg-652                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-691                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-704                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-710                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-773                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-791                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-800                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-815                                     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
UFVAg-856                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-871                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-925                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-931                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-955                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
UFVAg-976                                     . . . . . . . . . . . . . . . . . . . . . Y . . . . . . . . . . .
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Figure 6. Mutation B-H278Y identified in the alignment of sdhB amino acid sequence from 13 isolates of *Alternaria grandis* (UFVAg-) Sequences of the *A. solani sdh* genes deposited in GenBank database: KC517310.1 = sensitive isolate; KC517311.1 and KC517312.1 isolates with H278R and H278Y mutation, respectively, used as reference. UFVAg-169 and 815 were sensitive isolates.

Sixteen resistant isolates had one transition mutation from adenine (A) to guanine (G) at nucleotide position 490 in the *sdhC* gene that caused the change from H (codon CAC in sensitive isolates) to R (codon CGC) at position 134 (C-H134R) (Fig. 7). No mutation was detected in the *sdhD*. Using PCR assays 46 isolates had the B-H278Y mutation and 89 isolates had the C-H134R mutation.

Figure 7. Mutation C-H134R identified in the alignment of sdhC amino acid sequence from 16 isolates of *Alternaria grandis* (UFVAg-). Sequences of the *A. solani sdh* genes deposited in GenBank database: KC517313.1 = sensitive isolate; KC517314.1 isolate with H134R used as reference. UFVAg-169 and 815 were sensitive isolates.

Isolates with B-H278Y and C-H134R exchange had reduced sensitivity to SHDI fungicides and the mean EC50 values were higher than wild-type isolates (Fig. 8). However, the effect of mutation varied among the different SDHIs and of the subunits. Isolates with any mutation were completely insensitive to BC. EC50 values of the other SDHIs (FP, FX, PF, and PP) were higher for isolates with C-H134R mutation.

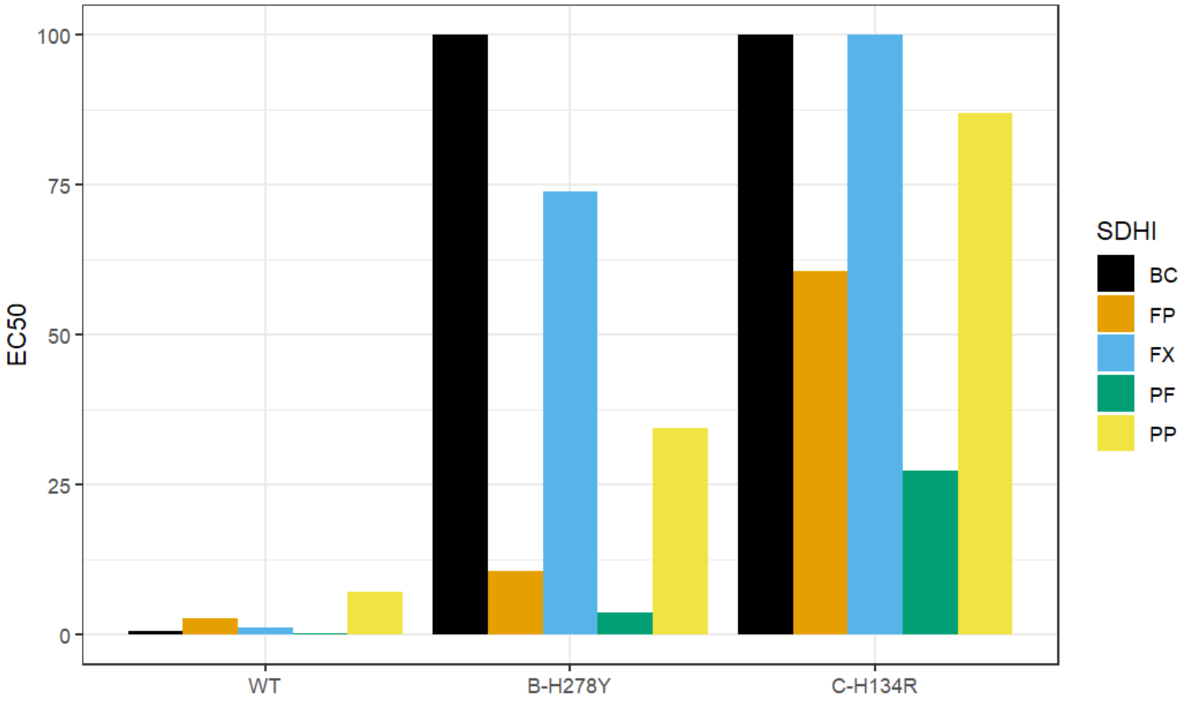

Figure 8. Comparison of the mean EC50 values of SDHI fungicides of sensitive isolates (WT) and resistant isolates (B-H278Y and C-H134R) of *Alternaria grandis.*

DISCUSSION

The present study is the first investigation ever conducted to assess the sensitivity of *A. grandis* to site-specific (SDHIs and DMIs) and multi-site (CT) fungicides. Additionally, the baseline sensitivity of *A. grandis* to two SDHI fungicides is being reported for the first time: PP and PF. Site-specific and multi-site fungicides are widely used to control EB of potato throughout Brazil. Therefore, the monitoring of the sensitivity of isolates of *A. grandis* is an important component of management programs aimed at the control of potato early blight under an applied perspective and the information presented herein can be useful to extend the lifetime of fungicide molecules. Finally, this study provided guidelines for discriminatory doses that can be used to high-throughput screening of resistance of *A. grandis* to the singe-site fungicides DC, BC, FP, FX, PP, PF, and the muti-site chlorothalonil.

Chlorothalonil is one the most commonly multi-site fungicides applied to control EB and to reduce the risk of developing resistance to site-specific fungicides. In Brazil, up to 8 applications are made per season. The risk of resistance development to CT is low (FRAC code M05). The sensitivity of 60 isolates of *A. solani* collected from tomato in the Jordan Valley was screened to CT and mancozeb (Abu-El Samen et al. 2016). The EC50 values ranged from 4.25 to 849.4 µg/ml and 9.05 to 712.65 µg/ml to CT and mancozeb, respectively. In the current study, the EC50 values (109.3 to 905.4 µg/mL) to CT of *A. grandis* were similar to those previously reported (Abu-El Samen et al., 2016), but Brazilian isolates seem less sensitive than isolates from the Jordan Valley. The EC50 values for CT were greater than 1000 µg/mL for 18 *A. grandis* isolates and some isolates grew more than 50% in DD compared to the control. One explanation for this is because perhaps these isolates were collected at the end of the season and became more adapted. This result was reported with isolates of *A. solani* collected from potato in EUA at different times of the season to assess the sensitivity of CT (Holm et al. 2003). Isolates collected at the end of season were less sensitive to chlorothalonil than isolates collected at the beginning.

Individuals of *A. grandis* from Brazil were highly sensitive to difenoconazole. The EC50 values reported here were similar to the values reported in a baseline study using isolates of *A. solani* from EUA in which the EC50 values for DC ranged from 0.02 to 0.3 µg/mL (Fonseka and Gudmestad 2016). Considering a different species, *A.*

alternata, the EC50 values for DC ranged from 0.02 to 0.82 µg/mL for isolates collected from pistachio in the United States (Avenot et al. 2016). The EC50 values ranged from 0.02 to 15.5 µg/mL for small-spored *Alternaria* spp. from China and because of its wide range, one resistant isolate to DC was reported to occur (Zhang et al. 2020). In this report, the resistant isolate had a mutation in the *cyp51* gene that led to exchange of arginine to tryptophan (R511W) at position 511. It was also found that some isolates also overexpressed this gene in the presence of DC (Zhang et al. 2020). In Brazil, there is no evidence of resistance of *A. grandis* to DC, but DMI fungicides must be used with caution because they are site-specific products with medium risk of resistance (FRAC code 3) and *Alternaria* spp. can develop resistance against fungicide with single modes of action relatively fast.

For approximately a decade, BC was the most commonly used SDHI to control EB in Brazil. This fungicide was registered to be used on potato crops in 2004, and the frequent use of this site-specific compound may have quickly selected resistant individuals in the field. The B-H278Y and C-H134R mutations were found in high frequency in isolates of *A. grandis*. Fortunately, no isolate had two *Sdh* mutations. The B-H278Y and C-H134R mutations seem to be pervasive and are the predominant mutations that confer resistance to SDHIs worldwide (Einspanier et al. 2022). A similar scenario apparently is taking place in Brazil since both mutations were the only ones identified in samples of *A. grandis* collected in the main Brazilian regions of potato production, from 2019 to 2021. Individuals with either mutation were completely insensitive to BC (EC50 > 100 ug/mL). These mutations were also the only ones found in Sweden (Mostafanezhad et al. 2022). The B-H278R, C-H134Q, D-D123E, and D-H133R mutations reported in isolates of *A. solani* from USA (Mallik et al. 2014), Germany (Metz et al. 2019), Belgium (Landschoot et al. 2017a) were not found associated with SDHI-resistant isolates in Brazil.

Penthyopirad is a fungicide not yet registered to control potato EB in Brazil. But, despite not being used by growers, reduced sensitivity was detected in 83% of the isolates (n=145). Also, 85% of the isolates were insensitive to FX. Both fungicides belong to the same chemical group (pyrazole-4-carboxamides) and FX is commonly used to control this disease. Cross-resistance among pyrazole-4-carboxamides is well-known (Sierotzki and Scalliet 2013).

Isolates of *A. grandis* were more sensitive to FP and PF than to BC, PP and FX regardless of the mutation. In Brazil, FP and PF were recently registered for use in potato and the use of carboxamides from a different chemical group (different structures) can influence the predominant mutation in the field population (Scalliet et al. 2012). Therefore, further screening for the sensitivity of *A. grandis* collected in the coming years will be important to monitor the shift in sensitivity to these fungicides and mutations in the *sdh* genes.

A. grandis is a high risk pathogen for developing fungicide resistance relatively fast because of its biological characteristics: the abundant production of assexual spores in one season leading to large effective population size with higher chances of large number of mutants resistant to the compounds. In addition, in some regions, potato crops are cultivated year-around in Brazil. Therefore, the monitoring of the sensitivity of *A. grandis* is very important to disease management and to increase the usage time of fungicides. Also, growers should follow the FRAC recommendation to SHDIs to reduce the risk for resistance development: preventive use, alternation of fungicide with different modes of action, and limitations on the total number of applications per season.

GENERAL CONCLUSIONS

In Brazil, *A. grandis* is the predominant species associated with early blight in potato. *A. linariae* was found to be associated with early blight on tomato in Minas Gerais state.

The population of *A. grandis* in Brazil is not structured by region or year and has high genetic variability. The evolutionary mechanisms that most likely shape the variability of the population are mutation and gene flow.

There is evidence of selection of individuals resistant to SDHIs. Difenoconazole and multi-site fungicides can be used in association with pydiflumetofen and fluopyram fungicides. Therefore, management of early blight must employ multi-site fungicides and site-specific products with different modes of action.

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