CARINE REZENDE CARDOSO

POTATO AND TOMATO EARLY BLIGHT: MOLECULAR IDENTIFICATION OF ALTERNARIA SPECIES, HOST RANGE AND EPIDEMICS

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

VIÇOSA
MINAS GERAIS – BRASIL
2014

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
Cardoso, Carine Rezende, 1984-
C268p Potato and tomato early blight: molecular identification of
2014 alternaria species, host range and epidemics / Carine Rezende Cardoso. – Viçosa, MG, 2014.
vii, 90f. : il. (algumas color.) ; 29 cm.

Orientador: Eduardo Seiti Gomide Mizubuti.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Fitopatologia. 2. Fungos patogênicos. 3. Tomate - Doenças e pragas. 4. Batata - Doenças e pragas. 5. Alternaria Solani - Identificação. 6. *Alternaria Solani*. 7. *Alternaria grandis*. 8. *Alternaria tomatophila*. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-graduação em Fitopatologia. II. Título.

CDD 22. ed. 571.92

CARINE REZENDE CARDOSO

POTATO AND TOMATO EARLY BLIGHT: MOLECULAR IDENTIFICATION OF *ALTERNARIA* SPECIES, HOST RANGE AND EPIDEMICS

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

APROVADA: 31 de julho de 2014.

Adalberto Côrrea Café Filho

Cleide Maria Ferreira Pinto

Emerson Medeiros Del Pontes

Luiz Antonio Maffia
(Coorientador)

Eduardo Seiti Gomide Mizubuti
(Orientador)

RESUMO

CARDOSO, Carine Rezende, D.Sc., Universidade Federal de Viçosa, Julho de 2014. **Pinta preta da batateira e do tomateiro: Identificação molecular das espécies de Alternaria gama de hospedeiro e epidemia.** Orientador: Eduardo Seiti Gomide Mizubuti. Coorientador: Luiz Antonio Maffia.

A pinta preta, doença comum em Solanum tuberosum L. e S. lycopersicum L. tem como agente causal Alternaria spp. Em 2000, algumas novas espécies associadas a pinta preta foram descritas. Alternaria grandis e A. tomatophila são as predominantes no Brasil. A identificação das espécies de Alternaria tem sido realizada principalmente pela caracterização morfológica. Neste estudo utilizou-se abordagem filogenética usando um conjunto de dados multilocos concatenados (gliceraldeído 3 fosfato desidrogenase, actina, ATPase e calmodulina) e análise de high-resolution melting (HRM) baseada nas regiões RPB2 e MCM7. A diferenciação de A. solani, A. grandis e A. tomatophila foi suportada pela filogenia. O ensaio baseado na análise de HRM permitiu a separação rápida e eficiente destas espécies. Alternaria spp. que causam pinta preta podem apresentar diferenças com relação a agressividade e em diferentes regiões do Brasil, produtores têm relatado maiores perdas de produtividade em função das epidemias. A disponibilidade de inóculo pode estar associada com a maior gama de hospedeiros dessas novas espécies. O método centrífugo-filogenético foi utilizado para determinar a gama de hospedeiros de A. solani, A. grandis, e A. tomatophila. Foram realizados testes de patogenicidade em 54 espécies de plantas. Das 28 espécies Solanaceae, 13 foram suscetíveis a espécies de Alternaria. Da família Asteraceae, Ageratumconyzoides e Erigeronbonariensis foram suscetíveis e da família Polygonaceae, Rumex acetosa,plantas até então não descritas como hospedeira de Alternaria spp. O desenvolvimento da pinta preta em pimentão e pimenta variou de acordo com a espécie do patógeno.Quando A. solani foi inoculado, o período de incubação (PI) foi de 14 dias em pimentão e 30 dias em pimenta. O PI para A. tomatophila foi de 30 dias em pimentão e de 14 dias em pimenta. Para A. grandis o PI foi de 7 dias, em ambos os hospedeiros. Aparentemente, a reação após a inoculação das diferentes espécies nestes dois hospedeiros pode ser usada para complementar os procedimentos de identificação de A. solani, A. grandis, e A. tomatophila. Estudou-se o progresso da pinta preta em batateira e tomateiro cultivados em Coimbra (720 m altitude) e Rio Paranaíba (1073m), Minas Gerais. Adicionalmente, avaliou-se a severidade das epidemias iniciadas por inoculação, em diferentes concentrações, de isolados de A. solani e A. grandis em parcelas pulverizadas com fungicida clorotalonil, em diferentes intervalos de tempo, em batateira. O progresso da pinta preta em Coimbra e Rio Paranaíba foi avaliado no verão e no inverno e em ambas as épocas, maiores valores de área abaixo da curva de progresso da doença (AACPD), taxa de progresso da doença e severidade aos 24 dias de epidemia ocorreram em Rio Paranaíba e para a cultura do tomate. Nesse estudo, A. tomatophila infectou batata em Coimbra, mas A. grandis não foi encontrado associado com pinta-preta em tomateiro em nenhuma das regiões pesquisadas. Em Rio Paranaíba, epidemias de pinta-preta em batateira foram causadas por A. grandis e, em tomateiro, por A. tomatophila. A especificidade por hospedeiro não é absoluta e a preferência pode ocorrer por diferenças na agressividade das espécies. Diferenças de agressividade também foram observadas entre A. grandis e A. solani, as variáveis epidemiológicas taxa de progresso da doença, AACPD e severidade no final da epidemia suportaram a existência de diferenças de agressividade entre as espécies. Epidemia de pinta preta causada por A. grandis foi muito severa até mesmo em condições não favoráveis, como baixa umidade, o que corrobora os relatos de produtores de batata sobre o aumento da intensidade da pinta-preta em qualquer época do ano.

ABSTRACT

CARDOSO, Carine Rezende, D.Sc., Universidade Federal de Viçosa, July, 2014. **Potato and Tomato Early Blight: Molecular identification of Alternaria species, host range and epidemics.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-adviser: Luiz Antonio Maffia.

Early blight, a common disease in Solanumtuberosum L. and S. lycopersicum L. is caused by Alternaria spp. New species associated with early blight, A. grandis and A. tomatophila, predominate in Brazil. The identification of Alternaria species commonly associated with early blight of potato and tomato has been mainly carried out by morphological characterization. In this study a phylogenetic approach was used combining a multilocus dataset (glyceraldehyde-3-phosphate dehydrogenase, actin, plasma membrane ATPase and calmodulin) and analysis of high-resolution melting (HRM) analysis based on the RPB2 and MCM7 regions was also carried out. The differentiation of A. solani, A. grandis and A. tomatophila was supported by phylogeny. The HRM analysis allowed rapid and efficient separation of these species. Alternaria spp. causing early blight may differ with respect to aggressiveness. In different regions of Brazil tomato and potato growers have reported increased yield losses due to early blight epidemics, concomitantly with reports of new species of Alternaria. The availability of inoculum may be associated with the wider host range of these new species. The centrifugal phylogenetic method was used to determine the host range of A. solani, A. grandis, and A. tomatophila. Pathogenicity tests were conducted on 54 plant species, 28 of which were species of Solanaceae. Thirteen Solanaceae species were susceptible to Alternaria. Ageratum conyzoides and Erigeron bonariensis in Asteraceae and Rumex acetosa in Polygonaceae were also susceptible and had not been previously described as hosts of Alternaria spp. The development of early blight in bell pepper and pepper varied according to the species of the pathogen. For A. solani, the incubation period (IP) was 14 days in bell peppers and 30 days in peppers. The IP for A. tomatophila in bell peppers and in pepper was 30 and 14 days, respectively. For A. grandis the IP was 7 days in both hosts. Apparently, the reaction after the inoculation of the different species in the two hosts can be used for identification purposes. Disease progression of early blight was studied in potato and tomato grown in Coimbra (altitude720m.a.s.l.) and Rio Paranaíba (1073 m.a.s.l.), Minas Gerais. In addition, we evaluated disease severity in potato plots inoculated with different spore concentrations of A. solani and A. grandis and sprayed with chlorothalonil at various time intervals.

The progress of early blight in Coimbra and Rio Paranaíba has been studied in summer and winter in two experiments. Higher values of area under the disease progress curve (AUDPC), rate of disease progress and severity at 24 days occurred in Rio Paranaíba and for tomato, with greater severity in the summer crops. In this study, A. tomatophila infected potato in Coimbra, but A. grandis was not associated with early blight on tomato in neither region. In Rio Paranaíba, epidemics of early blight in potatoes were caused by A. grandis whereas A. tomatophila was the causal agent in tomato, so it is possible to conclude at the field level, the host specificity is not absolute and that the preference for host occurs by differences in species aggressiveness. Differences in aggressiveness were also observed between A. grandis and A. solani. Collectively, the epidemiological variables, rate of disease progression, AUDPC and the severity at the end of the epidemic support the existence of differences in aggressiveness between A. grandis and A. solani. Epidemics of early blight caused by A. grandis were very severe, even in unfavorable conditions such as low humidity, the epidemic of early blight caused by A. grandis showed greater aggressiveness than A. solani, which corroborates reports of potato growers of increased intensity of early blight in any planting season.

ÍNDICE

INTRODUÇÃO GERAL

A pinta preta da batateira (Solanum tuberosum L.) e do tomateiro (Solanum lycopersicum L.), hortaliças de grande importância no mundo, era atribuída ser causada pelo fungo mitospórico Alternaria solani Sorauer (Rotem, 1994). Entretanto, em 2000,uma revisão do gênero Alternaria foi realizada e novas espécies foram propostas tomando por base estudos morfológicos (Simmons, 2000). Neste estudo, verificou-se que A. tomatophila Simmons era o agente causal predominante da pinta preta em tomateiro, permanecendo A. solani em batateira. Frazer & Zitter (2003), confirmaram os resultados encontrados por Simmons (2000). Esses trabalhos foram realizados nos Estados Unidos e a distribuição das novas espécies associadas à pinta preta não era amplamente conhecida. Estudos recentes realizados no Brasil empregando vários marcadores moleculares e técnicas analíticas de genética de populações constataram que a pinta preta em tomateiro é predominantemente causada por A. tomatophila Simmons e A. cretica Simmons, enquanto, em batateira, a espécie mais prevalente é A. grandis Simmons. Curiosamente, dentre os 28 isolados brasileiros analisados não se encontraram isolados de A. solani (Rodrigues, 2010).

Apesar de haver associação da população do patógeno com as espécies hospedeiras, a especificidade não é completa; isto é, isolados das espécies que afetam tomateiro podem infectar batateira e isolados de A. grandis, que afetam batateira, podem causar pinta preta em tomateiro (Rodrigues, 2010). No entanto, há evidência de que essas espécies são mais agressivas em seus hospedeiros de origem e diferem entre si quanto à agressividade; isolados de A. tomatophila foram mais agressivos do que isolados de A. solani quando inoculados em folhas, pecíolos e caules de tomateiro (Frazer & Zitter, 2003). A não identificação de isolados de A. solani no Brasil pode ter como possível explicação o fato de A. grandis ser mais agressivas em plantas hospedeiras do que A. solani.

Até o momento, somente um trabalho foi realizado a fim de detectar diferenças de agressividade entre as espécies de Alternaria associadas à pinta preta em batateira e tomateiro no Brasil. Por meio da quantificação de componentes epidemiológicos, observaram-se diferenças importantes principalmente quando as condições de temperatura e umidade foram limitantes (Cardoso, 2010). Acredita-se que tais diferenças de agressividade possam explicar relatos oriundos de várias regiões produtoras onde constatou-se aumento da intensidade da pinta preta mesmo com aplicações intensivas de fungicidas. Porém, até o presente, não se conduziram experimentos em campo com a finalidade de testar a hipótese de diferenças de agressividade entre os agentes causais de pinta preta e se essas traduzem em maiores intensidades de epidemias.

De modo amplo, inicialmente, é razoável propor a hipótese de que dois aspectos podem estar associados à maior intensidade das epidemias: aumento da gama de hospedeiros dos patógenos, o que contribuiria para o aumento do inóculo, e maior agressividade das novas espécies de Alternaria. Para A. solani, dispõe-se de informações sobre a gama de hospedeiros (Rotem, 1994), porém, não se conhecem os potenciais hospedeiros das espécies recentemente constatadas. Assim, a melhor forma de iniciar a avaliação da gama de hospedeiros é pela utilização do teste centrífugo-filogenético (Wapshere, 1974). Paralelamente, é necessário quantificar se as diferenças de agressividade, inferidas em testes sob condições controladas, são manifestadas em condições de campo. Para tal, aplicam-se técnicas de epidemiologia comparativa (Kranz, 2002) que são ferramentas poderosas e capazes de gerar informações importantes para o manejo da pinta preta.

Dessa forma, objetiva-se testar as hipóteses: i) As novas espécies de Alternaria têm maior gama de hospedeiros relativa àquela relatada para A. solani e ii) As epidemias de pinta preta ora registradas são de maior intensidade que aquelas causadas por A. solani devido a maior agressividade das novas espécies.

Antes de iniciar os estudos epidemiológicos, levando em consideração que diferentes espécies de Alternaria estão associadas à doença no Brasil, torna-se necessária aplicação de alguma ferramenta para identificação das espécies. Este trabalho tem como objetivo: i) Desenvolver metodologias de diagnose de base molecular, rápidas e robustas; ii) Determinar a gama de hospedeiros para A. solani,A. grandis e A. tomatophila; iii) Quantificar o progresso da doença em condições de plantios comerciais de batateira e tomateiro em duas regiões distintas; e iv) Quantificar o progresso das epidemias causadas por A. solani e A. grandis em batateira sob diferentes regimes de aplicação do fungicida protetor clorotalonil.

REFERÊNCIAS

Cardoso CR, 2010. Agressividade de Alternaria tomatophila, A. grandis e A. solani em batateira e tomateiro. Viçosa. Universidade Federal de Viçosa, dissertação de mestrado.

Frazer JT, Zitter, TA, 2003. Two species of Alternaria cause early blight of potato, (Solanum tuberosum) and tomato (Lycopersicon esculentum). Phytopathology (suppl. 3), **93**.

Kranz, J, 2002. Comparative epidemiology of plant diseases.Berlin, Springer, 206.

Rodrigues TTMS, Berbee M L, Simmons EG, Cardoso CR, Reis A, Maffia L A, Mizubuti ESG, 2010. First report of Alternaria tomatophila and A. grandis causing early blight on tomato and potato in Brazil.New Disease Reports**22**, 28.

Rotem J, 1994. The genus Alternaria.Biology, epidemiology, and pathogenicity. APS Press, St. Paul.

Simmons EG, 2000.Alternaria themes and variations (244-286) species on Solanaceae.Mycotaxon**75**, 1-115.

Wapshere A, 1974. A strategy for evaluating the safety of organisms for biological weed control.Annals of Applied Biology**77**, 201-11.

CHAPTER I - Identification of *Alternaria* species associated with potato and tomato early blight using high resolution melting (HRM) analysis

ABSTRACT

Potato and tomato early blight can be caused by at least three related Alternaria species, A. solani, A. grandis and A. tomatophila. Species sorting based on pathogen distribution, disease symptoms and pathogen life cycles cannot be achieved due to high similarities of these characteristics. Identification of Alternaria species is based on morphological characterization, but this is not an easy task to be accomplished by non-experts. In the present study, we have developed rapid and robust species-specific molecular-based diagnostic tools. DNA sequences of the Glyceraldehyde-3-phosphate dehydrogenase, Calmodulin, Actin, Plasma membrane ATPase from extype cultures of A. solani, A. grandis, A. tomatophila, A. cretica and also A. cyphomandrae were used in phylogenetic analysis. This approach provided strong support to distinguish the three species more widely distributed in potato and tomato crops, A. solani, A. grandis and A. tomatophila. Species identification based on the molecular analysis concurred with morphology analysis. Additionally, a HRM assay based on the RNA polymerase II beta subunit and the mini-chromosome maintenance protein genes was developed for rapid identification of Alternaria spp. Distinct melting profiles for each of the three species allowed proper identification of the Alternaria spp.

Key words: Alternaria solani, molecular phylogenetics, phylogeny, Solanum tuberosum, Solanum lycopersicum, etiology, resistance.

1.1 INTRODUCTION

The genus Alternaria is a large taxon, diverse and economically important (Rotem, 1994). Although this group has been extensively studied by many taxonomists, no comprehensive systematic treatment has been adopted to fully understand its evolutionary biology and classification (Simmons, 1992; Rotem 1994). Alternaria species have been commonly identified and classified based on colony and conidial morphology (Elliott, 1917; Neergaard, 1945). Identification based on morphological characteristics can be subjective once spore morphology can be highly variable even within species (Neergaard, 1945; Rotem, 1994). A thorough revision of the genus Alternaria was conducted and careful analysis of spore morphology was recommended by Simmons (2000). Nevertheless, identifying and correctly interpreting taxonomically relevant structures can be time-consuming and sometimes non-reliable.

Alternaria solani Sorauer is a well known destructive pathogen that affects Solanaceae (Elliot, 1917). For several years, A. solani was considered the sole species causing early blight in potato (Solanum tuberosum L.) and tomato (S. lycopersicum L.). However, after the revision of the Alternaria spp. associated with Solanaceae, it was found that A. grandis Simmons and A. tomatophila Simmons can also cause early blight on potato and tomato, respectively (Simmons, 2000).

In Brazil, a thorough sampling was implemented in many potato and tomato producing areas aiming at determining the genetic structure of the population of the causal agent of early blight (Lourenço Jr. et al., 2009). Molecular analyses based on the coalescent process with population subdivision pointed to subpopulations associated with host. More detailed studies at the sub-population and individual levels were conducted using molecular and morphological tools. Early blight on tomato was found to be caused predominantly by A. tomatophila and A. cretica, while in potato, the most prevalent species is A. grandis (Rodrigues, 2009). Interestingly, among the 28 Brazilian isolates examined no A. solani was found (Rodrigues et al., 2010).

Two facts related to the Alternaria spp. population deserve special attention: 1. A. solani was not identified in Brazil; and 2. more than one species of Alternaria can cause early blight. Currently, there is no well-supported explanation for the no detection or an apparently very low frequency of A. solani associated with early blight in Brazil. Among several possible scenarios, the sampling scheme could have interfered in the recovery of isolates. The increase in the number of analyzed specimens and improvement of the sampling scheme could have led to detect rare species.

Accurate identification of Alternaria species based on morphological features is time-consuming and prone to a high degree of subjectiveness, mainly for the untrained mycologists. The subtle differences among species that affect Solanaceae make it difficult to analyze a high number of samples. Etiology of early blight would benefit from efficient methods based on more objective criteria.

Molecular-based methods for fast and accurate identification of plant pathogens would be useful for many studies involving potato and tomato early blight. Molecular phylogeny has shown its usefulness in fungal characterization and identification (Berbee et al., 1999; Weir et al., 2012;Brun et al., 2013;Chen et al., 2014; Deng et al., 2014). Molecular phylogenetic analysis of Alternaria spp. has been studied since 2000 and has been extensively reported (Pryor and Gilbertson, 2000; Hoog and Horré, 2002; Pryor and Bigelow, 2003; Hong et al., 2005; Andrew et al., 2009; Lawrence et al., 2012; Lawrence et al., 2013; Woudenberg et al., 2013; Brun et al., 2013; Deng et al., 2014). Phylogenetic analyses have been used as complementary to the morphological analysis. The addition of new gene sequences to the databases (Lawrence et al., 2013; Woudenberg et al., 2013), it allows designing new PCR primers to identify the species commonly associated with Solanaceae: A.grandis, A.solani, A.tomatophilaand A.cretica.

Real-time PCR is known to be a rapid, reliable and quantitative detection method that is more sensitive than conventional PCR (Bester et al., 2012). However an extension to real-time PCR, the high-resolution melting (HRM) analysis, has been an attractive alternative to other molecular assays for identification and differentiation of plant pathogens. It provides a rapid, high-throughput, cost effective and single tube approach without labelled fluorescence probes or post amplification processing (Vossen et al., 2009; Bester et al., 2012; Wong et al., 2013). This technique has been used extensively (Jaakola et al., 2010; Luchi et al., 2011; Ganopoulos, 2012; Goldschmidt et al., 2012; Wong et al., 2013) and applied in diagnostics of plant diseases. It has been more robust and cost-effective than other methods (Wong et al., 2013).

The correct identification of a plant pathogenic fungus is required for the proper implementation of disease management (Chilvers, 2012). Epidemiological studies conducted under both controlled and field conditions found that early blight epidemics vary according to the causing species (Frazer and Zitter, 2003;Cardoso unpublished data). Therefore, the effectiveness of planting resistance cultivars, screening genotypes for early blight resistance, forecast systems, and fungicides can be affected by the species of the pathogen. To date there are no species-specific primers that allow separation of closely related Alternaria species, i.e. those causing potato and tomato early blight. This can be achieved after sequencing, but this method is laborious, expensive and cannot be performed routinely for daily diagnosis(Chilvers, 2012). This study describes a HRM assay developed for the rapid diagnosis of Alternaria species that cause early blight.

1.2 MATERIAL AND METHODS

1.2.1 Isolates, DNA extraction and quantification

Sixty-one isolates of Alternaria spp. including the reference isolates were carefully examined morphologically to be used in the experiments (Tables 1 and 2). DNA extraction was performed using Wizard Genomic DNA Purification Kit (Promega) from mycelia macerated in liquid nitrogen. DNA was quantified with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). DNA was diluted to a concentration of 20 ng/µL in ultrapure water and stored at -20°C.

1.2.2 DNA phylogeny

All phylogenetic analyses were performed using actin, plasma membrane ATPase and calmodulin regions. Partial sequences of these genomic regions were amplified by the following primer pairs ACTDF1/ACTDR1, ATPDF1/ATPDR1 and CALDF1/CALDR1, respectively (Lawrence et al., 2013). For partial sequence of the glyceraldehyde-3-phosphate dehydrogenase gene the gpd1/gpd2 primer pair was used (Berbee et al., 1999).

The polymerase chain reaction (PCR) was done with a mixture containing 20 ηg of DNA, 0.2 µM of each primer and 1X of DreamTaq™ DNA Polymerase Master mix as described by the manufacturer (Thermo Fisher Scientific). PCR cycles were carried out in a PTC100 thermal cycler (MJResearch, Incline Village, NV) and consisted of a 5 min denaturation step at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC for all primer combinations and 1min at 72 ºC with a final extension of 10 min at 72 ºC. PCR products were visualized by ultra-violet fluorescence following 1% agarose gel electrophoresis in 1X TBE buffer and GelRed™ (Biotium) staining. DNA concentration was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). PCR amplicons were purified using ExoSAP-IT (USB) according to the manufacturer`s protocols. The same primers used for PCR amplification were used for the sequencing reactions using the Macrogen, Inc service. Sequences were manually edited with The Staden Package, ver. 1.6.0 (Staden, 1996) to generate a consensus sequence. Additional sequences used in the analysis were obtained from GenBank (Table 1). Sequences were aligned with the Muscle® v. 3.6 software (Edgar, 2004) implemented in the Mega 5.0 program (Tamura et al., 2007).

Maximum parsimony analysis was performed using PAUP* 4.0b10 (Swofford 2002) for the concatenated dataset (actin, plasma membrane ATPase, calmodulin and GPD). Gaps were treated as missing data. Analyses were conducted by heuristic searches consisting of 1000 stepwise random addition replicates with branch swapping by the tree bisection-reconnection algorithm. Branch stability for individual datasets and the concatenated dataset were evaluated by 1000 bootstrap replications using a heuristic search with simple sequence addition to produce a majority rule consensus tree with nodal support values.

Bayesian phylogeny was derived from the same concatenated dataset. The MrModeltest v.2.2 (Nylander, 2004) selected the best nucleotide substitution model for each partition. Bayesian analysis was conducted with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The Markov Chain Monte Carlo (MCMC) analysis used four chains that started from a random tree topology and lasted 10 million generations. Trees were saved at each 100 generations, resulting in 100,000 saved trees. Burn-in was set at 1,000,000 generations after which the likelihood values were stationary, leaving 90,000 trees from which the 50 % majority rule consensus trees and posterior probabilities were calculated. Quality of mixing and convergence to the stationary distribution were assessed from three independent runs using Tracer v1.5 (Rambaut and Drummond, 2007). The resulting phylogenetic trees were prepared using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Phylogeny at the species level was conducted using sequences from fourteen isolates (Table 1). Five isolates were obtained in Brazil and were previously characterized by morphological criteria as A. grandis (As185, As260 and As352) and A. tomatophila (As232 and As420). These isolates were used in the analyses together with isolates obtained from CBS culture collection and from E. G. Simmons personal culture collection. Actin, plasma membrane ATPase, calmodulin and GPD sequences from A. dauci, available in GenBank were included in the phylogenetic analysis to be used as outgroup (Table 1).

1.2.3 Molecular diagnostic by high resolution melting (HRM) analysis.

Sequences of A. tomatophila (CBS 109156) and A. grandis (CBS 109158) available in GenBank were analyzed to design primer pairs using the Primer 3 program (Untergrasser et al., 2012). The partial sequence of RNA polymerase II beta subunit (RPB2) gene (Genbank accession numbers: JQ646521, JQ646500) showed a polymorphic region between the two species. This region was amplified using the following primer pairs: External_AlHRM_RPB2F (5’ ACTTCATCGCYAAGCGTGGT 3’) and External_AlHRM_RPB2R (5’ GTRTTYGTTCGACGCAAATG 3’) for isolates A. tomatophila (EGS 42156) and A. grandis (EGS 44108) as previously described. For the distinction of A. grandis and A. solani, the partial sequences of nuclear genes such as the mini-chromosome maintenance protein (MCM7) were amplified using the primers Mcm7-709for and Mcm7-1384rev (SCHMITT et al., 2009). The primer pairs AlHRM_RPB2F (5’ CACGCAAGGCCTACTTCTTC 3’)/AlHRM_RPB2R (5’ TTGCCAGRGAGTAYTTCAGG 3’) and AlHRM_MCM7F (5’ TGCAAGAGCAACAAGACCAA 3’)/AlHRM_MCM7R (5’ GGGAGGAAGATACCAGCACA 3’) were designed for amplification of these RPB2 and MCM7 regions for subsequent HRM analysis.

The HRM PCR analysis was performed on an HRM-equipped real-time rotary analyser (Rotor-Gene Q). We performed PCR reactions with Type-it HRM PCR Kit as described by the manufacturer (Qiagen). The final reaction volume was 10 µL containing 1x of HRM PCR Master mix, 2 ηg of DNA and 0.7 µM primer mix. The PCR program consisted of an initial denaturation of 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. For the HRM analysis, the tubes were heated from 60°C to 95°C allowing 10 data acquisitions per 1°C. All analyses were done in duplicates. Positive (DNA of Ex-type and authentic isolates of A. tomatophila EGS 42-156, A. grandis EGS 44-108 and A. solani EGS 44-098) controls and non-template controls were included in each run.

The generation and analysis of melting profiles as well as genotyping of isolates were conducted using the Rotor-Gene Q associated software, Rotor-Gene v. 2.0.2.4, applying the default parameters recommended by the manufacturer. Genotypes of test samples (morphologically characterized isolates) were defined by selecting a representative sample for each species. To achieve more consistent and reliable results, only samples with amplification threshold cycle values of less than 30 and generating sigmoid to near sigmoidal curve shape were considered for use in this current study. A confidence threshold of 90% was applied (Kirkpatrick et al., 2009; Ganopoulos et al., 2012).

To verify the HRM analysis results, a new set of analysis was also performed with uncharacterized samples. Twenty potato and tomato leaves with typical symptoms of early blight, collected in two geographic locations were analyzed to identify the causal agent (Table 2). A reference isolate was included for each Alternaria species in the same run, allowing all samples to be genotyped by comparison to the closest reference species.

1.3 RESULTS

1.3.1 DNA Phylogeny

Similar tree topologies were obtained with both maximum-parsimony and bayesian analyses (Figures 1 and 2). Maximum-parsimony analysis of the four regions studied resulted in two most-parsimonious trees. For bayesian trees, the nucleotide substitution model GTR was used for actin, calmodulin and GPD. For these three genes 926, 712 and 563 characters were constant, 4, 16 and 5 were parsimony-informative and 9, 19 and 6 were singletons, respectively. GTR+I was the nucleotide substitution model used for plasma membrane ATPase, in total 1198 characters were used, 1157 were constant, 13 were parsimony-informative and 28 were singletons.

The OTUs formed well-supported clades for A. grandis, A. solani and A. tomatophila. The support values for the different phylogenetic methods vary with the Bayesian posterior probabilities being higher than the maximum-parsimony support values for clade A. grandis and A. solani, however there was weak support for separate A. tomatophila and A. cretica.

1.3.2 Molecular diagnostic by high resolution melting (HRM) analysis.

Primers AlHRM_RPB2F/AlHRM_RPB2R and AlHRM_MCM7F/ AlHRM_MCM7R amplified a 271 and 206-bp region for separation of A. tomatophila/A. grandis and A. solani/A. grandis, respectively. The region RPB2 has a transversion (T/G) and two transitions of type A/G and three of C/T among A. tomatophila and A. grandis (Figure 3A). The region MCM7 has two transitions of type C/T among A. solani/A. grandis (Figure 3B). Each amplicon produced a similar curve shape but with different melt profiles running parallel to each other (Figure 4A-B). Two curves were distinguished by a shift on the temperature axis, with lower melting temperature for A. grandis than A. tomatophila for the RPB2 region (Figure 4C-E). Additional primer pair of the MCM7 region was used in a subsequent HRM analysis and differentiated A. grandis the A. solani (Figure 4D-F).

Ex-types isolates EGS of A.grandis, A. solani and A. tomatophila plusthesamespecies,butthe collectionCBS, were used as the reference genotype for identification and validation of HRM analysis assay using morphologically characterized isolates. Two distinct melt profiles were obtained for HRM analysis based on the RPB2 region with the 21 isolates previously characterized morphologically as seen in Figure 5.The two genotypes were separated by incremental shifts on the temperature axis with A. grandis to the left, and A. tomatophila to the right (Figure 5A). Subsequently, in orderto confirm thespecies found inpotato, isolates putatively identified as A. grandis based on the analysis with RPB2 were analyzed with the MCM7 region to separate from A. solani (Figure 5B). When normalized HRM curves were autocalled to genotype by the software, 13 and 8 samples of isolates characterized morphologically were correctly typed to A. grandis and A. tomatophila, respectively with a confidence threshold greater than 90%, with most having 99%. All samples were successfully genotyped, with the results agreeing to those obtained by morphologicalcharacteristics.

1.3.3 Evaluation of HRM analysis assay with uncharacterized samples

All twenty isolates from potato plants from Coimbra grouped with A. tomatophila and the ten isolates from potato plots from Rio Paranaíba grouped with A. grandis (Table 2). All isolates from tomato plants sampled from the two regions grouped with A. tomatophila (Table 2). Each of these specimens was genotyped with a confidence level greater than 92%.

1.4 DISCUSSION

The identification of the species of *Alternaria* commonly associated with potato and tomato early blight is primarily done by morphological characterization. In this study we used phylogenetic approaches using concatenated multilocus dataset and developed primers for HRM useful for the assays diagnostic of *A. solani*, *A. grandis* and *A. tomatophila*. Thesethree species are more widely distributed in potato and tomato production regions (Simmons, 2000; Rodrigues et al., 2010). HRM-based assay allowed fast and efficient separation of these species from each other without any undesired cross-reaction.

Four genomic regions can be useful to assess molecular phylogeny of *Alternaria* species: Actin, ATPase, Calmodulin and GPD. While separation of *Alternaria* spp. has been achieved with protein-coding loci, such as GPD, identification of lineages of a species is possible by analyzing actin, plasma membrane ATPase and calmodulin regions. Results from each individual locus and the combined dataset support the grouping of previously described species-groups of the genus*Alternaria*based on molecular data and morphological characteristics (Lawrence et al., 2013). In the current study, the phylogenetic analysis using concatenated multilocus dataset, differentiated the three *Alternaria* species that cause early blight, with most branches supported by high bootstrap values. The species identifications based on these four regions concurred with those based on morphology.

The HRM was a useful tool for the identification and differentiation of the three *Alternaria* species associated with early blight of potato and tomato. The HRM analysis technique has already been used to genotype closely related species of plant pathogenic fungi causing similar disease symptoms on the same host species (Wong et al., 2013). Variations in the RPB2 and MCM7 regions of *Alternaria* spp. generate distinct melting profiles for each of the three species, indicating its discriminatory capacity as valuable tool for diagnosis. Identification of these pathogens to the species level would facilitate not only an improved understanding of disease etiology and epidemiology, but also potentially improve management recommendations given the significant differences in pathogenicity, aggressiveness and fungicide sensitivity between species (Chilvers, 2012).

The identification of *Alternaria* species using HRM analysis can be concluded in less than 3 h, whereas for nucleotide sequence analysis more than 2 days are required. Other studies that validated the technique for identification of plant pathogens also demonstrated that little time is required to complete the diagnosis (Jaakola et al., 2010; Wong et al., 2013). HRM analysis would also be particularly useful for plant pathogens that are difficult to isolate or are slow-growing in *vitro* (Wong et al., 2013).

All 61 samples tested were identified and differentiated with a level of confidence of 90–99%. HRM analysis performed for the 21 isolates used by Rodrigues et al., (2010) revealed two *Alternaria* genotypes among the samples. The clustering of genotypes was in agreement with morphological characterization and all isolates from tomato were identified as *A. tomatophila* and those from potato as *A. grandis*. *A. solani* was not detected in the samples. In the HRM analysis assay, a reference genotype is included for each *Alternaria* species in the same run, allowing all samples to be genotyped by comparison to the closest reference species. The results of 40 uncharacterized samples further confirmed the capability of the assay. Each sample, possessed identical sequences to those of the reference isolate.While results have practical implications for identifying the individual species, future studies should be conducted to verify the efficiency of identifying mixtures of these species co-occurring in the same lesion.

The molecular-based detection and tools developed and optimized in this study are substantial for the correct classification of the species of *Alternaria*, as causal agents of early blight disease on potato and tomato.Management actions can be dependent on the prevalent species of the pathogen and correct classification is a crucial first step.

REFERENCES

Andrew M, Peever TL, Pryor BM, 2009. An expanded multilocus phylogeny does not resolve species among the small-spored Alternaria species complex. Mycologia**101**, 95-109.

Berbee ML, Pirseyedi M, Hubbard S, 1999. Cochliobolus phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia**91**, 964-97.

Bester R, Jooste AEC, Maree1 HJ, Burger, JT, 2012. Real-time RT-PCR high-resolution melting curve analysis and multiplex RT-PCR to detect and differentiate grapevine leafroll-associated virus 3 variant groups I, II, III and VI. Virology Journal**9**, 219-229.

Brun S, Madrid H, Van Den Ende BG, Andersen B, Marinach-Patrice C, Mazier D, De Hoog G S, 2013.Multilocus phylogeny and MALDI-TOF analysis of the plant pathogenic species Alternaria dauci and relatives.Fungal Biology**117**, 32-40.

Chen SF, Morgan DP, Hasey JK, Anderson K, Michailides TJ, 2014.Phylogeny, morphology, distribution, and pathogenicity of Botryosphaeriaceae and Diaporthaceae from English walnut in California.Plant Disease**98**, 636-652.

Chilvers MI, 2012. Molecular Diagnostics in Plant Disease Diagnostic Clinics... What’s the Status? Fungal Genomics and Biology**2**, e102. doi:10.4172/2165-8056.1000e102

Deng JX, Sun Cho H, Paul NC, Lee HB, Hun Yu S, 2014.A novel Alternaria species isolated from Peucedanumjaponicum in Korea. Mycobiology**42**, 12-16.

Edgar RC, 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research**32**, 1792-1797.

Elliott JA,1917. Taxonomic characters of the genera Alternaria and Macrosporium. American Journal of Botany**4**, 439-476.

Frazer JT, Zitter TA, 2003. Two species of Alternaria cause early blight of potato, (Solanum tuberosum) and tomato (Lycopersicon esculentum). Phytopathology (suppl. 3) 93.

Ganopoulos I, Madesis P, Zambounis A, Tsaftaris A, 2012. High-resolution melting analysis allowed fast and accurate closed-tube genotyping of Fusarium oxysporum formae speciales complex. FEMS Microbiology Letters**334**, 16-21.

Goldschmidt P, Degorge S, Che Sarria P, Benallaoua D, Semoun O, Borderie V, Laroche L, Chaumeil C, 2012. New strategy for rapid diagnosis and characterization of fungal infections: The example of corneal scrapings. PLoS ONE **7**, e37660.

Hong SG, Cramer RA, Lawrence CB, Pryor BM, 2005. Alt a1 allergen homologs from Alternaria and related taxa: analysis of phylogenetic content and secondary structure. Fungal Genetics and Biology**42**, 119-129.

Hoog GS, Horré R, 2000.Molecular taxonomy of the Alternaria and Ulocladium species from humans and their identification in the routine laboratory. Mycoses **45**, 259-276.

Jaakola L, Suokas M, Häggman H, 2010. Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analysis of berry species. Food Chemistry**123**, 494-500.

Kirkpatrick NC, Blacker HP, Woods WG, Gasser RB, Noormohammadi AH, 2009. A polymerase chain reaction-coupled high-resolution melting curve analytical approach for the monitoring of monospecificity of avian Eimeria species.Avian Pathology**38**, 13-19.

Lawrence DP, Park MS, Pryor BM, 2012.Nimbya and Embellisia revisited, with nov. comb. forAlternaria celosiae and A. perpunctulata. Mycological Progress**11**, 799-815.

Lawrence DP, Gannibal PB, Peever TL, Pryor BM, 2013. The sections of Alternaria: Formalizing species-groups concepts. Mycologia**105**, 530-546.

Lourenço Junior V, Moya A, González-Candelas F, Carbone I, Maffia LA, Mizubuti ESG, 2009. Molecular diversity and evolutionary processes of Alternaria solani in Brazil inferred using genealogical and coalescent approaches. Phytopathology**99**, 765-774.

Luchi N, Pratesi N, Simi L,Pazzagli, M, Capretti, P, Scala, A, Slippers, B, Pinzani, P, 2011. High-Resolution Melting Analysis: a new molecular approach for the early detection of Diplodia pinea in Austrian pine. Fungal Biology**115**, 715-23.

Neergaard P, 1945. Danish Species of Alternaria and Stemphylium. London: Oxford Univ. Press. 560 p.

Nylander JAA, 2004. MrModeltest v 2.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

Pryor BM, Gilbertson RL. 2000. Molecular phylogenetic relationships amongst Alternaria species and related fungi based on analysis of nuclear ITS and mtSSU rDNA sequences. Mycological Research**104**:1312-1321.

Pryor BM, Bigelow DM, 2003. Molecular characterization of Embellisia and Nimbya species and their relationship to Alternaria, Ulocladium and Stemphylium.Mycologia**95**, 1141-1154.

Rambaut A, Drummond AJ, 2007. Tracer v1.4, Available from [http://beast.bio.ed.ac.uk/Tracer].

Rodrigues TTMS, 2009. Morphological and molecular characterization, with inference about recombination, for species of Alternaria related to early blight of potato and tomato. Viçosa: FederalUniversity ofViçosa, PhD thesis.

Rodrigues TTMS, Berbee ML, Simmons EG, Cardoso CR, Reis A, Maffia LA, Mizubuti ESG, 2010. First report of Alternaria tomatophila and A. grandis causing early blight on tomato and potato in Brazil.New Disease Reports**22**, 28.

Ronquist F, Huelsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics**19**, 1572-1574.

Rotem J, 1994. The genus Alternaria.Biology, epidemiology, and pathogenicity. St. Paul: APS Press. 326 p.

Simmons EG, 1992.Alternaria taxonomy: current status, viewpoint, challenge. In: Chelkowski J, Visconti A, eds. Alternaria biology, plant diseases and metabolites. Amsterdam, the Netherlands: Elsevier Science Publishers.

Simmons EG, 2000.Alternaria themes and variations (244-286) species on Solanaceae.Mycotaxon**75**, 1-115.

Staden R, 1996. The Staden sequence analysis package.Molecular Biotechnology**5**, 233-241.

Swofford, DL, 2002. PAUP.Phylogenetic Analysis Using Parsimony.Version 4.0b10. Sunderland, MA: Sinauer Associates.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. Molecular Biology Evolution**24**, 1596-1599.

Untergrasser A, Cutcutache I, Koressaar TYJ, Faircloth BC, Remm M, Rozen SG, 2012. Primer3 - new capabilities and interfaces. Nucleic Acids Research**40**(15), e115.

Vossen RHAM, Aten E, Roos A, den Dunnem JT, (2009) High-Resolution Melting Analysis (HRMA)—More than just sequence variant screening. Human Mutation**30**, 860-866.

Weir BS, Johnston PR, Damm U, 2012.The Colletotrichum gloeosporioides species complex.Studies in Mycology**73**, 115-180.

Wong MH, Henderson J, Drenth A, 2013. Identification and differentiation of Phyllosticta species causing freckle disease of banana using high resolution melting (HRM) analysis. Plant Pathology**62**, 1285-1293.

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW, 2013. Alternaria redefined. Studies in Mycology**75**, 171-212.

TABLES

Table 1.Alternaria isolates used for phylogenetic study. Sequences that were determined in the course of this study appear in bold.

			GenBank accession			
Isolate -Source	**Species**	**Location**	**Actin**	**Calmodulin**	**Plasma membrane ATPase**	**GPD**
CBS 109164	Alternaria cretica	Greece	JQ671741.1	JQ646250.1	JQ671916.1	JQ646342.1
CBS 109155	A. cyphomandrae	New Zealand	JQ671761.1	JQ646270.1	JQ671936.1	JQ646360.1
ATCC 36613	A. dauci		JQ671732.1	JQ646241.1	JQ671907.1	AY278803.1
CBS 109158*	A. grandis	USA, Pennsylvania	JQ671740.1	JQ646249.1	JQ671915.1	JQ646341.1
EGS 44108*	A. grandis	USA, Pennsylvania				
CBS 116651*	A. solani	USA, California	JQ671723.1	JQ646232.1	JQ671898.1	AY278807.1
EGS 44098*	A. solani	USA, Washington				
CBS 109156*	A. tomatophila	USA, Indiana	JQ671748.1	JQ646257.1	JQ671923.1	JQ646347.1
EGS 42156*	A. tomatophila	Indiana-USA				

Abbreviations for sources are as follows: ATCC, American Type Culture Collection, Manassas, VA 20108; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933.* Isolates used also in HRM analysis.

Table 2. Isolates usedfor HRM analysis.

					Confidence threshold (%)	
Isolate-Source	**Host of origin**	**Species**	**Location**	**HRM genotyping**	**RPB2**	**MCM7**
AS012	Potato	A. grandis	Araxá/MG-BR	A. grandis	99.12	98.86
AS013	Potato	A. grandis	Araxá/MG-BR	A. grandis	98.02	99.42
AS169	Potato	A. grandis	B. Brandão/MG-BR	A. grandis	99.25	99.51
AS185*	Potato	A. grandis	Bom Repouso/MG-BR	A. grandis	98.54	98.93
AS203	Potato	A. grandis	Bom Repouso/MG-BR	A. grandis	94.40	97.14
AS216	Potato	A. grandis	Camanducaia/MG-BR	A. grandis	95.89	99.04
AS220	Potato	A. grandis	Camanducaia/MG-BR	A. grandis	98.01	99.03
AS248	Potato	A. grandis	Cons. Lafaiete/MG-BR	A. grandis	99.04	94.92
AS260*	Potato	A. grandis	Cristalina/GO-BR	A. grandis	99.01	98.52
AS308	Potato	A. grandis	Contenda/PR-BR	A. grandis	99.01	96.30
AS313	Potato	A. grandis	S. Franc.Paula/RS-BR	A. grandis	98.35	97.11
AS352*	Potato	A. grandis	Brasília/DF-BR	A. grandis	99.40	96.79
AS394	Potato	A. grandis	M. Izidro/MG-BR	A. tomatophila	97.20	[a]nd
AS094	Tomato	A. tomatophila cfr	Coimbra/MG-BR	A. tomatophila	99.08	Nd
AS095	Tomato	A. tomatophila cfr	Coimbra/MG-BR	A. tomatophila	99.01	Nd
AS109	Tomato	A. tomatophila	Vassouras/RJ-BR	A. tomatophila	99.86	Nd
AS232*	Tomato	A. tomatophila cfr	Cons. Lafaiete/MG-BR	A. tomatophila	98.20	Nd
AS420*	Tomato	A. tomatophila cfr	Jaíba/MG-	A. tomatophila	92.84	Nd
AS422	Tomato	A. tomatophila cfr	Jaíba/MG-	A. tomatophila	94.93	Nd
AS423	Tomato	A. tomatophila cfr	Jaíba/MG-BR	A. tomatophila	99.64	Nd
AS452	Tomato	A. tomatophila cfr	Planaltina/DF-BR	A. grandis	99.50	98.59
RP1P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	97.80	97.49
RP2P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	91.57	96.73
RP3P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	95.89	94.34
RP4P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	93.05	93.25
RP5P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	98.42	96.10
RP6P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	92.92	98.76
RP7P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	98.23	97.60
RP8P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	92.20	96.92
RP9P	Potato	not defined	Rio Paranaíba-MG-BR	A. grandis	93.47	99.53
RP10P	Tomato	not defined	Rio Paranaíba-MG-BR	A. grandis	96.31	98.15
RP1T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	96.07	Nd
RP2T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	97.09	Nd
RP3T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	94.76	Nd
RP4T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	95.99	Nd
RP5T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	98.70	Nd
RP6T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	99.03	Nd

Table 2.Cont.

Isolate - Source	Host of origin	Species	Location	HRM genotyping	Confidence threshold (%)	
					RPB2	MCM7
RP7T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	98.40	Nd
RP8T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	95.95	Nd
RP9T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	97.50	Nd
RP10T	Tomato	not defined	Rio Paranaíba-MG-BR	A. tomatophila	98.44	Nd
C1P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	98.25	Nd
C2P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	93.54	Nd
C3P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	96.12	Nd
C4P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	97.12	Nd
C5P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	94.81	Nd
C6P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	93.76	Nd
C7P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	98.48	Nd
C8P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	97.82	Nd
C9P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	97.06	Nd
C10P	Potato	not defined	Coimbra/MG-BR	A. tomatophila	97.32	Nd
C1T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	96.28	Nd
C2T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	92.38	Nd
C3T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	92.97	Nd
C4T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	97.66	Nd
C5T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	97.04	Nd
C6T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	98.75	Nd
C7T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	98.82	Nd
C8T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	92.98	Nd
C9T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	95.70	Nd
C10T	Tomato	not defined	Coimbra/MG-BR	A. tomatophila	98.50	Nd

* Isolates used also in phylogeny.[a]nd=not determined

FIGURE

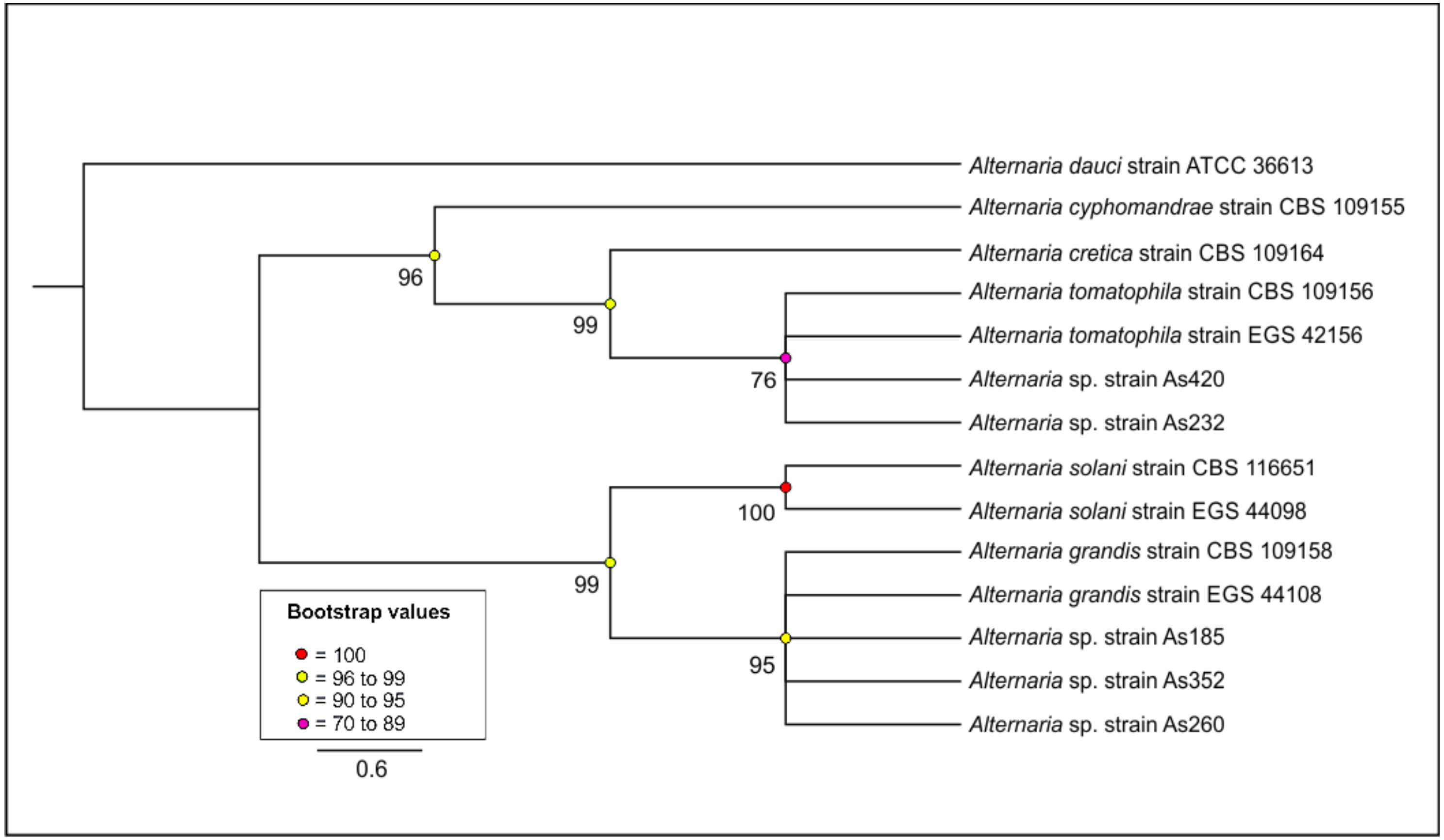


Figure 1. Consensus parsimonious trees generated from maximum parsimony analysis of the four-gene combined dataset. Parsimony bootstrap values from 1000 replicates are given at the nodes and coded according to the legend. The black line scale bar shows 0.6 expected nucleotide substitutions. The tree was rooted with Alternaria dauci.

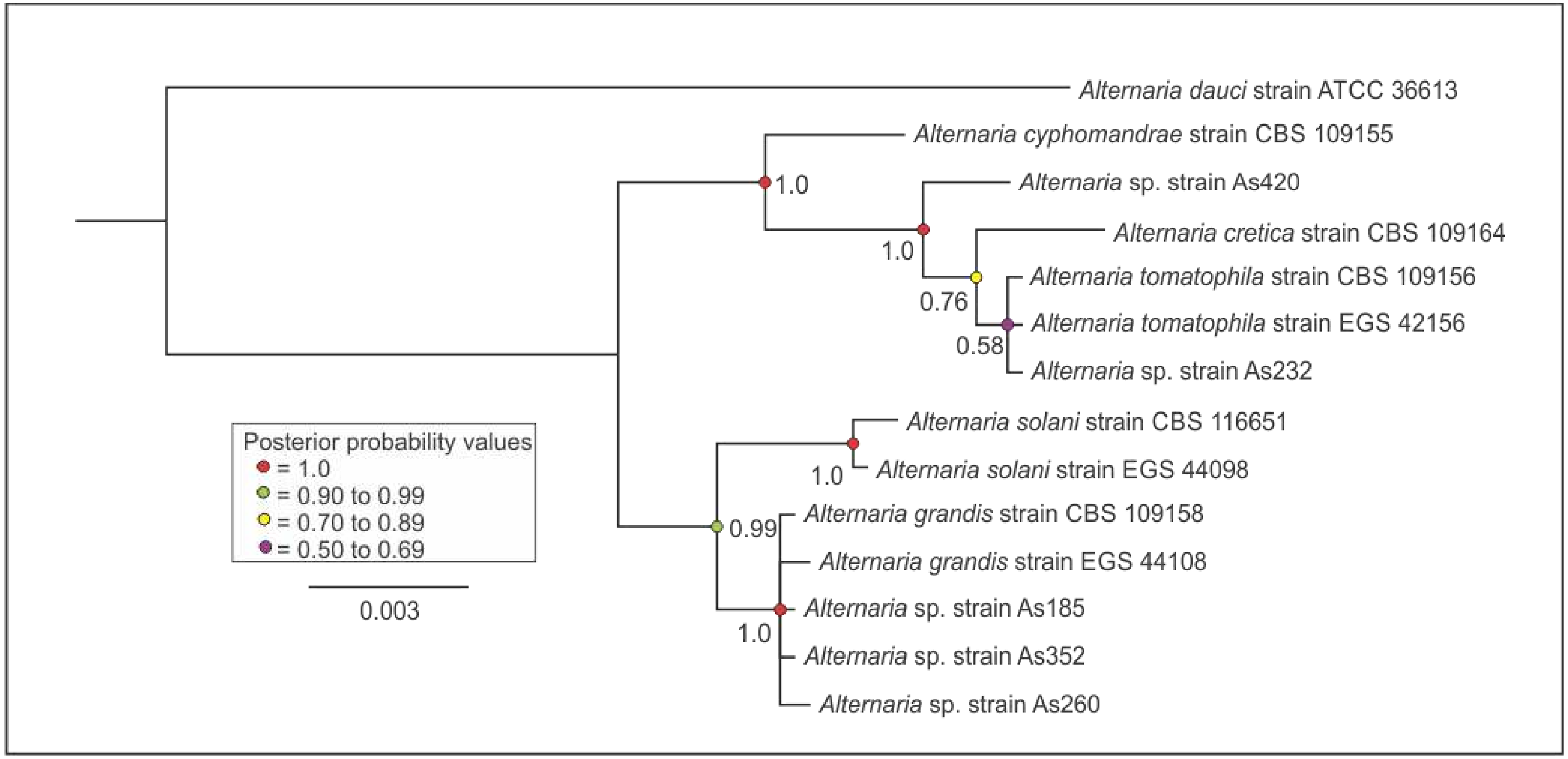
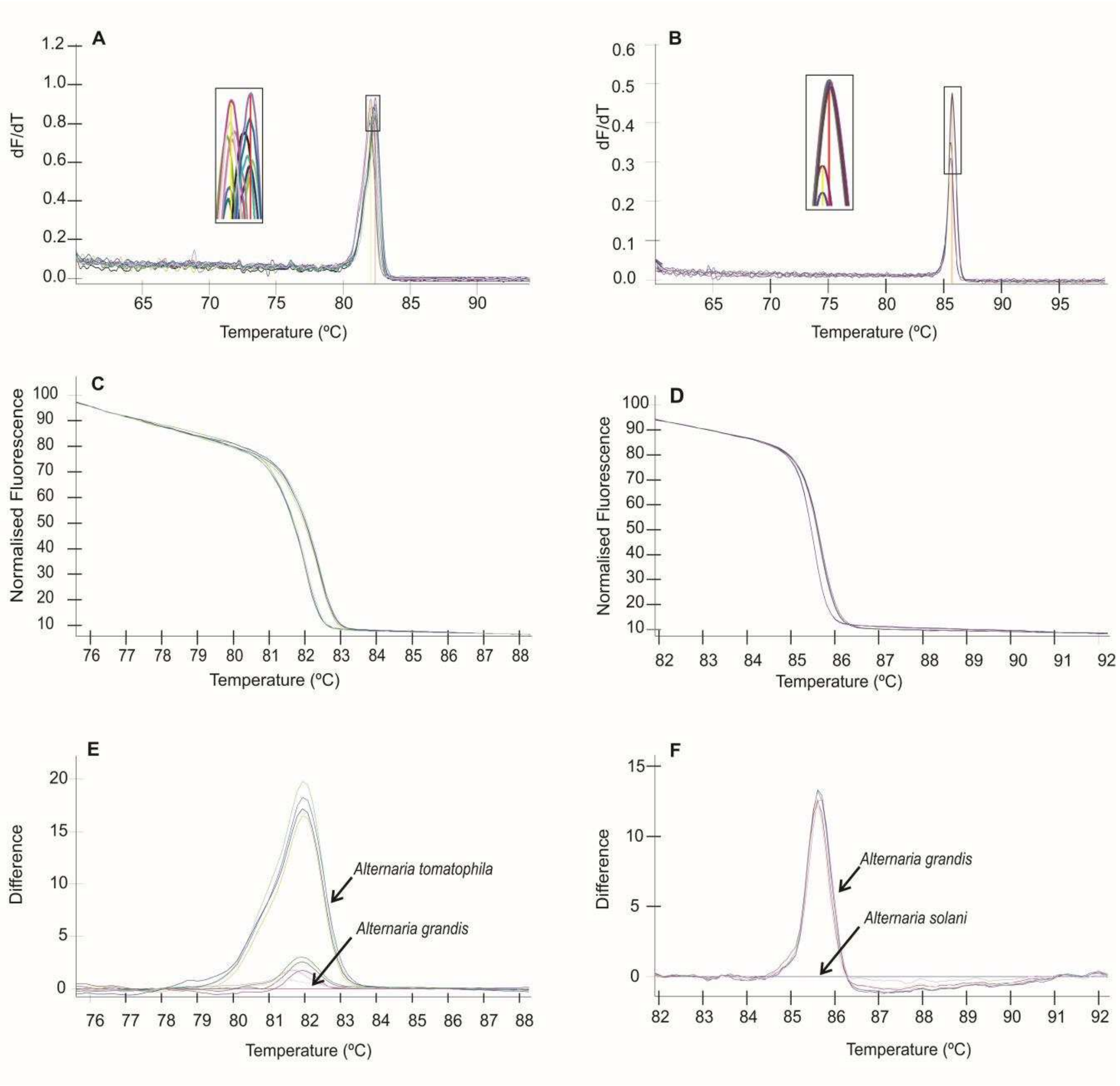


Figure 2. Bayesian analysis of Alternaria species associated with tomato and potato lesions. Tree was constructed based on the combined data set of partial sequences of actin, plasma membrane ATPase, calmodulin and GPD genes. Bayesian posterior probabilities are given at the nodes and coded according to the legend. The black line scale bar shows 0.003 expected changes per site. The tree was rooted with Alternaria dauci.

```
A
A. tomatophila EGS 42156  CGCAAGGCCTACTTCTTCGGCTACATGATCCACCGCCTCTTGCAGTGTGTGCTGGGTCGCCGCGACGAGGACGATCGT
A. tomatophila CBS 109156 ..............................................................................
A. grandis EGS 44108      .......................................................................T......

A. tomatophila EGS 42156  GATCACTTCGGTAAGAAGCGTCTGGATCTGGCTGGACCCTTGGTCGCCAACTTGTTCCGTATCCTCTTCCTGAAGCTC
A. tomatophila CBS 109156 ..............................................................................
A. grandis EGS 44108      ..............................................................................

A. tomatophila EGS 42156  ACCAAGGACGTATACAAGTACCTCCAGCGGTGTGTTGAGAACAACCAGGATTTCAACGTCCAGATGGCTGTCAAGGCC
A. tomatophila CBS 109156 ..............................................................................
A. grandis EGS 44108      .............................A.....G.......................T..................

A. tomatophila EGS 42156  AGCATCATCACAAACGGCCTGAAGTACTCTCTGGCAA
A. tomatophila CBS 109156 .....................................
A. grandis EGS 44108      .......................A.....C.......

B
A. grandis EGS 44108  TGCAAGAGCAACAAGACCAAGGGCCAGCTCTTCCTCTCGACGCGCGCCTCCAAGTTCCTTCCGTTCCAAGAAGTCAAGAT
A. grandis CBS 109158 ................................................................................
A. solani EGS 44098   ................................................................................
A. solani CBS 116651  ................................................................................

A. grandis EGS 44108  CCAAGAAATGGCCGACCAAGTCCCTGTTGGCCATATTCCGAGACAGCTGACTATCCACTGCCATGGCGCGCTCGTCAGGC
A. grandis CBS 109158 ................................................................................
A. solani EGS 44098   .....................T..............C...........................................
A. solani CBS 116651  .....................T..............C...........................................

A. grandis EGS 44108  AGATCAACCCCGGCGATGTCATCGACTGTGCTGGTATCTTCCTCCC
A. grandis CBS 109158 ..............................................
A. solani EGS 44098   ..............................................
A. solani CBS 116651  ..............................................
```

Figure 3. Alignment of the RPB2 (A) and MCM7 (B) regions showed the SNPs used for species separations in HRM analyzes.

Figure 4. High resolution melting (HRM) analysis curves generated from the Alternaria tomatophila/A. grandis and A. solani/A grandis species. A. Melting profile for RPB2 region with A. tomatophila/A. grandis.B. Melting profile for MCM7 region with A.solani/A. grandisC. Normalized HRM curves showing differentmelting profiles of A. tomatophila/A. grandis. D. Normalized HRM curves showing differentmelting profiles of A. solani/A. grandis. E. The corresponding difference plots for (C), using A. grandis as the reference genotype. F. The corresponding difference plots for (D), using A. solani as the reference genotype.



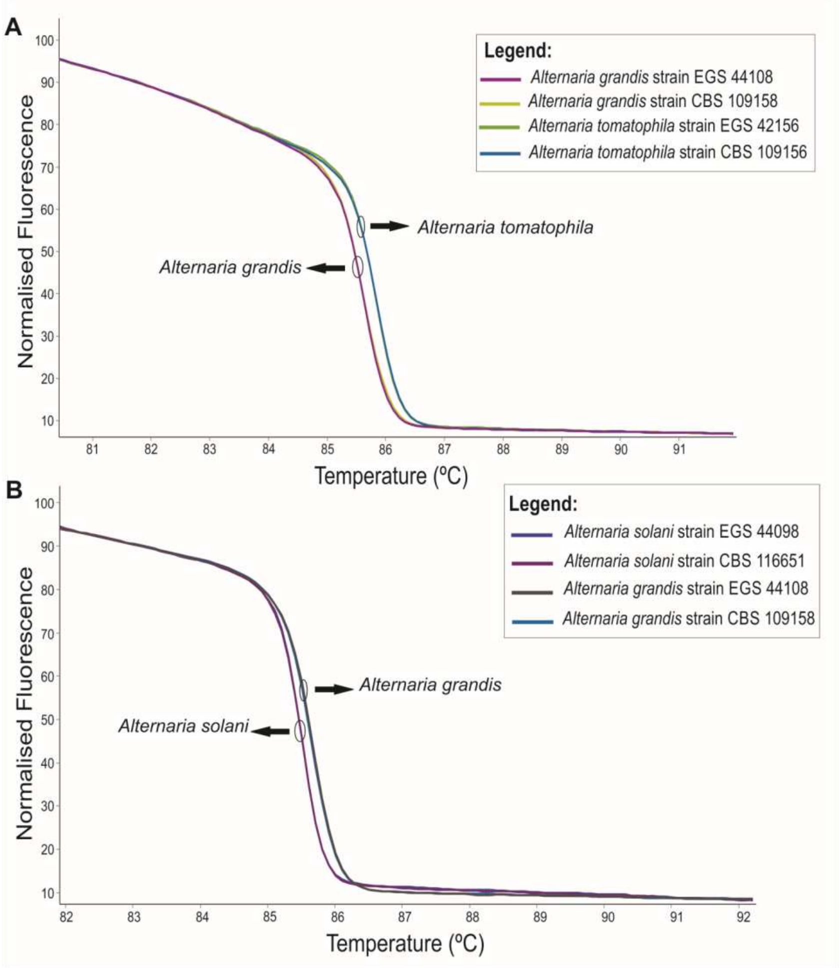


Figure 5. Normalized HRM curves showing differentmelting profiles of reference isolates EGS and CBS. A. HRM melting profiles for A. tomatophila/A. grandis. B. HRM melting profiles for A. solani/A. grandis.

CHAPTERII - Experimental host range of Alternaria solani,A. grandis andA. tomatophila

ABSTRACT

Early blight epidemics caused by different species of Alternaria have been a serious recurring problem in potato and tomato crops worldwide. Inoculum availability may be associated with an increase in the host range of the new Alternaria species. The centrifugal phylogenetic method was used to determine the host range of A. solani, A. grandis, and A. tomatophila. The method was used so as to emphasize Solanaceae species and plants cited in the literature as hosts of A. solani. A total of 54 plant species were assessed including 28 Solanaceae species. The experiment was performed twice with similar results. Besides Solanum tuberosum andS. lycopersicum, 13 Solanaceae species were susceptible to Alternaria spp. A. solani caused mild symptoms in Capsicum annuum var. annuum, C. annuum var. grossum and C. annuum var. Casca Dura Ikeda. In C. frutescens, C. chinense and C. baccatum, lesions were noticed 30 days after inoculation. A. tomatophila grew less actively on C. frutescens, C. chinense and C. baccatum. In Capsicum annuum var. annum, C. annuum var. grossum and C. annuum var. Casca Dura Ikeda the lesions were noticed 30 days after inoculation**.**Inoculation of host plants can complement other identification procedures used for A. solani, A. grandis and A. tomathophila.The new updated host list for Alternaria spp. includes Ageratumconyzoides, Erigeronbonariensis, S. capsicoides, S.lycocarpum, S.viarum and Rumexacetosa, which hitherto have not been described as hosts. This suggests that alternative host surrounding potato and tomato fields can serve as inoculum sources that may constitute an important element in the epidemiology of Alternaria species that cause early blight.

Key words: Early blight, centrifugal phylogenetic, potato, tomato, Solanum tuberosum, Solanum lycopersicum.

2.1 INTRODUCTION

The genus Alternaria is one of the most widely distributed and ecologically diverse (Elliot, 1917, Rotem 1994). This genus was first described by Nees von Esenbeck in 1816, with A. tenuis as the type (Elliot, 1917) and has been extensively reviewed (Elliot, 1917; Neergaard, 1945; Simons, 2000; Lawrence et al., 2013). Many species of Alternaria cause destructive plant disease epidemics and early blight, caused by A. solani Sorauer (Sorauer, 1896, Rotem, 1994), is an example of a severe yield-limiting factor for potato (Solanum tuberosum L.) and tomato (S. lycopersicum L.) crops worldwide (Reifschneider et al., 1984; Rotem 1994; Guenthner et al., 1999; Van Der Walls et. al., 2004; Shuman and Christ, 2005).

For decades A. solani has been considered the causal agent of early blight on both potato and tomato. However, based on morphological studies Simmons (2000) reported that more than one species can cause early blight on these hosts. In Brazil, at least two species are widely distributed and cause severe early blight epidemics in potato and tomato fields: A. grandis Simmons and A. tomatophila Simmons, respectively (Rodrigues et al., 2010). Interestingly, no isolates of A. solaniwere detected among several samples collected from different regions in Brazil.

The three species ofAlternaria,A. solani, A. grandis, and A. tomatophila, were able to colonize both potato and tomato plants (Rodrigues et al., 2010). Isolates of A. grandis and A. tomatophila cause the same foliar symptoms on potato and tomato as those caused by A. solani: necrotic lesions on leaves, stems, tubers and fruits (Rodrigues et al., 2010). The lesions are dark, elongated or circular, with concentric rings (Jones, 1991). At high severity, the disease causes defoliation, thereby decreasing yield and the quality of fruits or tubers (Rotem, 1994).

Epidemics of polycyclic diseases, such as early blight, can achieve high intensity because large amount of secondary inoculum (conidia) is produced in infected plants. Conidia can be easily dispersed reaching healthy plants located far away from the source of inoculum (Strandberg, 1992). Therefore, both locally produced (host plants, alternative hosts), and external (from distant places) inocula are potentially important for the epidemic of early blight. Lately, an increase in the severity of early blight epidemics, mainly in potato producing areas, has been reported in Brazil. According to many growers epidemics are highly destructive even under intensive fungicideuse in the fields. One aspect that may be associated with higher disease pressure is a wider host range of the pathogen, which would contribute to increase spore production.

Broad host ranges of plant pathogens can challenge disease management. The host range of A. solani is somewhat limited to Solanaceae plants (Rotem, 1994). Plants of nine Solanaceae genera were inoculated with A. solani and 14 species of Solanum, Lycopersicon, Hyoscyamus and Nicandra,were infected (Rands, 1917). A. solani can infect potato, tomato, eggplant, nightshade and gilo (African garden egg) (S. aethiopicum L.) (Neergaard, 1945, Fancelli, 1991) as well as a number of non-cultivated plant species(Rands, 1917). Recently, A. solani was reported causing a severe early blight outbreak in tomato in Pakistan where the pathogen was also observed infecting Convolvulus arvensis (Akhtar et al., 2011). All these plant species may act as inoculum sources for early blight epidemics.

Several economically and scientifically important issues require predicting the likely host range of plant pathogens, among these, the study of plant disease epidemics (Garrett et al. 1999).For newly described species of pathogens, the determination of host range is an important component for disease management. The host range of A. grandis and A. tomatophila is unknown. Nevertheless, unrecognized host plants may serve as unmonitored sources of inoculum that may contribute to disease on potato or tomato.

Usually, the host range of a pathogen is determined by inoculation tests on different plants. However, a more systematic procedure to design this type of experiment may contribute to enhance the detection of potential hosts. The basic idea is to inoculate phylogenetic relatives of the target host plant and progressively unrelated species. This approach was proposed to assess the specificity and safety of organisms intended to be used as a biological control agent of weeds and was named as the centrifugal phylogenetic method (Wapshere, 1974). Host-range test of Phytophthora infestans followed the ‘‘centrifugal phylogenetic method’’ (Lima et. al., 2009).Using this method we now seek to determine the host range of the new species of Alternaria, A. grandis and A. tomatophila that cause potato and tomato early blight.

2.2 MATERIAL AND METHODS

2.2.1 Choice of the plant species for the centrifugal phylogenetic method

The choice of plant species to be tested as hosts followed the centrifugal phylogenetic method (Wapshere, 1974). Fifty four plant species were tested, 28 were Solanaceae species. The other 26 species were chosen based on the decreasing phylogenetic affinity in relation to S. tuberosum and S. lycopersicum, but species officially reported as host to A. solani were also included in the test (Table 1). The plants were chosen based on the official reports by EMBRAPA – Cenargen (National Center for Genetic Resources), which is in charge of quarantine in Brazil (Mendes and Urben, 2010) and on the database of Fungus-Host Distributions maintained by the United States Department of Agriculture (USDA) Agricultural Research Service (Farr and Rossman, 2011).Priority was given to plant species native to Brazil.

2.2.2 Plant material and inoculation

All plants were grown in 4L-plastic pots under greenhouse conditions. Plants varied in age because the time to develop a suitable leaf canopy varied between species. One week before inoculation, all plants were thoroughly inspected, confirmed to be healthy and transferred to field conditions. The field was located at Universidade Federal de Viçosa in Viçosa-MG.

Nine isolates of Alternaria spp. were used in the experiments. Three isolates of A. tomatophila (As 109, As 422 and As 423) and three of A. grandis (As 169, As 260 and As 220) were previously identified morphologically and by molecular tools (Rodrigues, et al, 2010). Additionally, Ex-type and authentic isolates of A. tomatophila (EGS42-156), A. grandis (EGS 44-108), and a representative isolate of A. solani (EGS 44-098) were included as positive controls in all experiments. All isolates are kept at the Culture Collection of the VIC Herbarium at Universidade Federal de Viçosa.

For inoculum production, sporulation was induced according to previously described procedures (Simmons, 2007). Shortly, 5-day-old colonies of Alternaria species grown in V8 $CaCO_3$ agar (175mL of V8 juice, 3g $CaCO_3$, 20g agar, 1L) at 25 ± 2^oC, 12h-photoperiod, were used. Superficial mycelium was removed using 10mL of sterile distilled water (SDW) and a clean paintbrush. The suspension was discarded. The Petri plates were then kept uncovered at 22 ± 2^oC, under near-UV light for 8h and 16h in the dark to induce sporulation. After 24h, conidia were removed as above and a suspension was adjusted for 10^4 conidia/mL.

The plants were sprayed until runoff with the inoculum suspension of each species. Susceptible tomato plants of cv. Santa Clara and potato plants of cv. Ágata were used as controls. After inoculation, plants were kept for 12h in a moist chamber at 25°C, 12h-photoperiod. After this time, plants were transferred to field conditions.

Daily and up to 30 days after planting, inoculated plants were evaluated for the disease symptoms. When symptoms were observed, microscopic slides were prepared to verify the pathogen structures. In the absence of sporulation on lesions, indirect isolation was performed. Alternaria species were considered established only when conidia were observed in the necrotic tissue or when a characteristic colony grew after indirect isolation.

The experiments were conducted twice in a completely randomized design, with four replications. One pot with one plant was considered as an experimental unit. Each Alternaria isolate was inoculated in four independent experimental units. Experiment 1 was conducted between February and March 2012 and experiment 2 between September and October 2013. Meteorological variables were obtained from Universidade Federal de Viçosa Main Weather Station, located 1.5 km from the experimental area.

2.3 RESULTS

Of the 54 plant species inoculated, 19 were susceptible to Alternaria spp.,about half of the (15)solanaceous species were susceptible (Figure 1). Three species of Asteraceae, Ageratum conyzoides, Erigeron bonariensis,Galinsoga parviflora, and one of Polygonaceae, Rumex acetosa were infected by all three species of Alternaria (Figure 1). Typical early blight symptoms, necrotic lesions with "target board" pattern, were seen on tomato and potato plants, as well as in the other susceptible species. Plants previously described in the literature as hosts of A. solani, such as Amaranthusretroflexus, A. spinosus, Apiumgraveolens, Ipomoeabatatas, Citrullusvulgaris, Cucumissativus, Phaseolusvulgaris, Nicotianarustica, Solanumcapsicastrum and Zingiberofficinale, were not infected by the Alternaria spp. in the present study (Table 1).

Symptoms caused by all Alternaria species developed faster in potato and tomato plants than in any other of the Solanaceae hosts. Spots were noticed on potato and tomato plants two days after inoculation in all replicates of both runs. Leaf symptoms were as evident in Solanum melongena, S. aethiopium, S. viarum, S. lycocarpum, S. americanum, S. capsicoides, and Cyphomandra betaceae as in potato and tomato, all members of the Solanaceae; and also in Ageratumconyzoides, Erigeronbonariensis and Galinsoga parviflora, members of the Asteraceae. In the Astearaceae, plants lesions appeared seven days after inoculation. For Rumex acetosa, Polygonaceae, lesions appeared 10 days after inoculation.

Fast lesion growth of A. solani was observed in solanaceous hosts, except on Capsicum annuum var. annuum, C. annuum var. grossum and C. annuum var. Casca Dura Ikeda in which spots were noticed 14 days after inoculation in all replicates of both runs. The A. solani isolate was capable of infecting C. frutescens, C. chinense and C. baccatum, however, small spots were noticed 30 days after inoculation and the disease was of low intensity. A. tomatophila grew less actively on C. frutescens, C. chinense and C. baccatum and was capable of infecting C. annuum var. annuum, C. annuum var. grossum and C. annuum var. Casca Dura Ikeda but, spots were noticed 30 days after inoculation and disease developed at low intensity, with small lesions. A. grandis was capable of infecting all these plant species and the spots were noticed 7 days after inoculation, being so aggressive as in other susceptible Solanaceae species.

In the first experiment the minimum, average and maximum temperatures were 16.7^{o}C, 20.8^{o}C, and 27.2^{o}C, respectively. The average relative humidity was 80.2% and the precipitation was 136mm. In the second experiment, the minimum, average, and maximum temperatures were 15.4°C, 19.6°C, and 26.4°C, respectively. The average relative humidity was 75.9% and the accumulated precipitation was 117.7mm (Figure 2).

2.4 DISCUSSION

Early blight was considered to be easily managed, but lately epidemics have become a serious threat to these crops. The Alternaria spp. affecting potato and tomato have a short life cycle, and several cycles may occur during the crop season characterizing a typical polycyclic disease (Rotem, 1994). For polycyclic diseases the amount of initial inoculum is considered to be not so influential on epidemic development. However, under an integrated disease management system, the delay of disease outbreak can contribute to reduce yield losses (Berger, 1977).Thus, identifying the inoculum sources for the disease constitutes critical knowledge for a better understanding of the epidemiology of early blight. Ultimately, this knowledge may help reduce the risks of infection by elimination of potential sources of inoculum in the surroundings of potato and tomato fields. This is the first study conducted to determine the host range of A. grandis and A. tomatophila, species that were recently reported as causing early blight in potato and tomato in Brazil. New plant species are reported as hosts of A. solani.

Almost half of the Solanaceae were infectedby Alternaria spp. Since the beginning of the 20th century it is known that isolates of A. solani obtained from potato plants infected Solanum tuberosum, Datura stramonium; Lycopersicumesculentum (S. lycopersicum), S. nigrum, all solanaceous hosts (Elliot, 1917). Susceptibility of these plants to Alternaria spp.as well as of wild or ornamental plants may be relevant toearly blight management because small farmers commonlygrow several solanaceous plants in a single area (Becktell et al., 2005).

Overall, the host range determined for A. solani in the present study is in agreement with that recorded for A. grandis and A. tomatophila. The host range of Alternaria species is primarily comprised by Solanaceae species, but some members of the Asteraceae and Polygonaceae were also susceptible to the pathogens. Leaf symptoms were as evident in other plant species belonging to the Solanum genus as in potato and tomato. In potato and tomato the development of symptoms was rapid and necrotic cells in the inoculation sites were seen 12h after inoculation. Asteraceae species such as Ageratumconyzoides, Erigeronbonariensis and Galinsoga parviflora were susceptible to early blight.The incubation period (IP) in these plants was short (7 days). In Rumex acetosa the IP was 10 days. Lower IP contributes to increase the number of conidia, the intensity of early blight, and epidemic progress.

The updated list of hosts of A. solani now includes plant species reported for the first time as susceptible to early blight: Ageratum conyzoides,Erigeron bonariensis, Solanum capsicoides, Solanum lycocarpum, Solanum viarum and Rumex acetosa. On the other hand, some plant species reported early as susceptible to the infection by A. solani did not show any symptoms after inoculation in the current study. For instance, in our work, species of the genus Amaranthus were not susceptible to Alternaria spp. It is not easy to point a single factor that can explain this result. Probably, the amount of inoculum that was used, the physiological conditions, the environmental variables and the genetic background of the hosts that were tested can be some of the reasons that might explain the differences in the results.

Potato and tomato are both cultivated throughout the year in several production regions, so infected plants are likely to provide enough conidia to start or develop early blight epidemics. We have identified 17 hosts of Alternaria species. It is necessary to verify if these plants can act as inoculum sources to potato and tomato crops and whether the host from where the inoculum comes affects the aggressiveness ofAlternaria spp. Isolates of Phytophthora infestans from alternative hosts had higher aggressiveness than those from potato, indicating that an alternative host affects aggressiveness, which could lead to more severe potato late blight epidemics (Grönberg et al., 2011).

We have shown that both bell pepper and pepper were infected by A. solani, A. grandis and A. tomatophila, however these plants were infected to a limited degree by all three species and the severity of early blight was lower than in the other susceptible plants. Early blight development in bell pepper and pepper varied according to the pathogen species. When A. solani was inoculated the IP was 14 days in bell pepper and 30days in pepper. The IP for A. tomatophila was 30 days in bell pepper and 14 days in pepper. For A. grandis the IP was 7 days in both hosts. Apparently, the reaction after inoculation of the different pathogen species in these two host plants can be used to complement the identification procedures used for A. solani, A. grandis and A. tomathophila. Bell pepper and pepper are easy to grow and might be useful to use as differential plants.

The use of a phenotypic test can be helpful and easier to implement than genotypic analysis when aiming at the identification of plant pathogens. When standardized and well-conducted the phenotypic tests can safely and effectively distinguish between different Alternaria species. The influence of factors associated with inoculation (inoculum concentration) and incubation (environment) must be taken into consideration so that the results are consistent. This technique is exploited for the identification of races and pathotypes (Sartorato, 2002; Gutiérrez et al., 2007; Goates et al., 2012). Variation in the responses of a wide range of accessions of Glycine to infection by isolates of Phakopsorapachyrhizi allowed to generate a set of differential hosts useful in the identification of different races of the pathogen (Burdon and Speer, 1984).

To identify a plant pathogen, the host range can be used as an auxiliary tool to molecular and morphological characterization. The identification of a Puccinia coronata population from Smooth bromegrass was possible based on the host range, and morphology of the urediniospores and teliospores (Delgado et al., 2001). Elsinoë fawcettii and E. australis are important pathogens of citrus and fungal colonies and pigments vary substantially among isolates, providing no reliable traits for distinguishing E. fawcettii from E. australis. In addition to molecular tests the two species can be separated by host range (Chung, 2011). For early blight, to complement the morphological analysis and to reduce the chance of errors, performing the differential host test may be an interesting alternative.

Furthermore, phylogenetic relationships of the plant species tested may offer some insight into the different host range of the each Alternaria species. New species may have a broader or different host range. Based on these results, further studies of host range should focus on non-Solanum species because the three species of Alternaria differed in the ability to infect them, which contributes to establish a different host range for each Alternaria spp.

This study was conducted to determine an experimental host range for Alternaria spp. and several plant species could serve as hosts. The increased range of hosts of A. solani, A. tomatophila, A. grandisand also the presence of more species causing the disease, may be contributing to the greater intensity of the epidemic in the country and consequently with increased fungicide usage to control early blight. We have shown that bell pepper in combination with pepper can be used to separate Alternaria species causing early blight. Furthermore our results are particularly useful for other studies that will investigate the role of these plants in early blight epidemics in major (potato and tomato) and in minor crops (bell pepper and pepper).

REFERENCES

Akhtar KP, Sarwar N, Saleem MY, Asghar M, 2011.Convolvulus arvensis, a new hosp for Alternaria solani causing early blight of Solanum lycopersicum in Pakistan.Australasian Plant Disease Notes**6**, 84-6.

Becktell MC, Daughtrey ML, Fry WE, 2005. Epidemiology and management of petunia and tomato late blight in the greenhouse.Plant Disease **89**, 1000–8.

Berger RD, 1977. Application of Epidemiological Principles to Achieve Plant Disease Control.Annual Review of Phytopathology**15**, 165-81.

Burdon JJ, Speer SS, 1984. A set of differential Glycine hosts for the identification of races of Phakopsorapachyrhizi. Euphytica**33**, 891- 96.

Castro MEA, Zambolim L, Chaves GM, Cruz CD, Matsuoka K, 2000. Variabilidade patogênica de Alternaria solani, agente causal da pinta-preta do tomateiro. Summa Phytopathologica**8**, 24-28.

Chung K-R, 2011. Elsinoë fawcettii and Elsinoë australis: the fungal pathogens causing citrus scab. Molecular plant pathology **12**, 123-35.

Delgado NJ, Grau CR, and Casler MD, 2001.Host range and alternate host of a Puccinia coronata population from smooth brome grass.Plant Disease**85**, 513-16.

Elliott JA,1917. Taxonomic characters of the genera Alternaria and Macrosporium.American Journal of Botany**4**, 439-76.

Fancelli MI, 1991. Comparação patogênica, cultural, serológica e eletroforética entre isolados de Alternaria solani do tomate e da batata e variabilidade patogênica de A. solani f. sp. lycopersici N. F. Piracicaba, Brasil: Escola Superior de Agricultura Luiz de Queiroz, tese de doutorado.

Farr DF, Rossman AY, 2011. Fungal Databases, Systematic Mycology and Microbiology Laboratory, (Agric Res St/US Departement of Agriculture). [http://nt.ars-grin.gov/fungaldatabases/]. Accessed 15 may 2014.

Garrett KA, Mundt CC, 1999. Epidemiology in mixed host populations.Phytopathology**89**, 984-90.

Goates BJ, 2012.Identification of new pathogenic races of common bunt and dwarf bunt fungi, and evaluation of known races using an expanded set of differential wheat lines.Plant Disease**96**, 361-69.

Grönberg L, Andersson B, Yuen J, 2012. Can weed hosts increase aggressiveness of Phytophthorainfestans on potato? Phytopathology**102**, 429-33.

Guenthner JF, Wiese MV, Pavlista AD, Sieczka JB, Wyman J, 1999. Assessment of pesticide use in the US potato industry.American Journal of Potato Research**76**, 25-9.

Gutiérrez WA and Mila AL, 2007.A rapid technique for determination of races of Phytophthora nicotianae on tobacco.Plant Disease**91**, 985-89.

Jones JB, 1991. Jones, J. P., Stall, R. E.& Zitter, T. A. Compendium of Tomato Diseases. St. Paul. APS Press.

Lawrence DP, Gannibal PB, Peever PB, Pryor BM. 2013. The sections of Alternaria: formalizing species-group concepts. Mycologia105, 530-46.

Lima MA, Maffia L A, Barreto RW, Mizubuti ESG. 2009. Phytophthora infestans in a subtropical region: survival on tomato debris, temporal dynamics of airborne sporangia and alternative hosts. Plant Pathology**58**, 87-99.

Lourenço Jr. V, Moya A, González-Candelas F, Carbone I, Maffia L A, Mizubuti ESG, 2009. Molecular diversity and evolutionary processes of Alternaria solani in Brazil inferred using genealogical and coalescent approaches. Phytopathology**99**, 765-74.

Mendes MAS, Urben AF, 2010. Fungos relatados em plantas no Brasil, Laboratório de Quarentena Vegetal. Brasília, DF. In: Embrapa Recursos Genéticos e Biotecnologia. [http://pragawall.cenargen.embrapa.br/aiqweb/michtml/fgbanco01.asp]. Accessed 11 May 2010.

Neergaard P, 1945. Danish species of Alternaria and Stemphylium. London: Oxford Univ. Press. 560p.

Rands RD, 1917. Early blight of potato and related plants.Wisconsin Agricultural Experimental Station Research Bulletin**42**, 1-48.

Reifschneider F J B, Furumoto O, Filgueira FAR, 1984. Illustrated key for the evaluation of early blight of potatoes. Plant Protection Bulletin**32**, 91-94.

Rodrigues TTMS, Berbee M L, Simmons EG, Cardoso CR, Reis A, Maffia L A, Mizubuti ESG, 2010. First report of Alternaria tomatophila and A. grandis causing early blight on tomato and potato in Brazil.New Disease Reports**22**, 28.

Rotem J, 1994. The genus Alternaria.Biology, epidemiology, and pathogenicity. APS Press, St. Paul.

Sartorato A, 2002. Identification of Phaeoisariopsis griseola pathotypes from five states in Brazil.Fitopatologia Brasileira**27**, 78-81.

Shuman JL, Christ B J, 2005. Integrating a host-resistance factor into the Fast system to forecast early blight of potato.American Journal of Potato Research**82**, 9-19.

Simmons EG, 2000.Alternaria themes and variations (244-286) species on Solanaceae.Mycotaxon**75**, 1-115.

Sorauer, Z, 1896. Auftreten einer dem amerikanischen “Early Blight” entsprechenden Krankheit an den deutschen Kartoffeln. Zeitschrift für Pflanzenkrankheiten**6**, 1-9.

Strandberg JO, 1992. Alternaria species that attack vegetable crops: biology and options for disease management. In: Chelkowski, J. & Visconti, A.Alternaria biology, plant disease and metabolites. Amsterdam: Elsevier, 175-208.

Van Der Walls JE, Korsten L, Slippers B, 2004. Genetic diversity among Alternaria solani isolates from potatoes in South Africa. Plant Disease**88**, 959-64.

Wapshere A, 1974. A strategy for evaluating the safety of organisms for biological weed control.Annals of Applied Biology**77**, 201-11.

TABLE

Table 1. Reaction of plants from 54 species to Alternaria solani, A. grandis and A. tomatophila isolates from different regions evaluated in two field runs.

Family	Species	Reaction[a]		
		Alternariatomatophila	Alternariagrandis	Alternariasolani
Solanaceae	**Lycopersicon esculentum***	+	+	+
Solanaceae	**Solanum tuberosum***	+	+	+
Solanaceae	**Brugmansia suaveolens**	-	-	-
Solanaceae	**Capsicum annuum***	+	+	+
Solanaceae	**Capsicum annuum var Casca Dura**	+	+	+
Solanaceae	**Capsicum annuumvar. grossum(Capsicum angulosum) ***	+	+	+
Solanaceae	**Capsicum baccatum**	+	+	+
Solanaceae	**Capsicum chinense**	+	+	+
Solanaceae	**Capsicum frutescens***	+	+	+
Solanaceae	**Cestrum sp.**	-	-	-
Solanaceae	**Cyphomandra betaceae**	+	+	+
Solanaceae	**Datura metel**	-	-	-
Solanaceae	**Datura suaveolens**	-	-	-
Solanaceae	**Nicotianarustica ***	-	-	-
Solanaceae	**Nicotiana tabacum**	-	-	-
Solanaceae	**Solanum americanum ***	+	+	+
Solanaceae	**Solanum capsicastrum ***	-	-	-
Solanaceae	**Solanum capsicoides**	+	+	+
Solanaceae	**Solanum cernuum**	-	-	-
Solanaceae	**Solanum erianthun**	-	-	-
Solanaceae	**Solanum gilo***	+	+	+
Solanaceae	**Solanum lycocarpum**	+	+	+
Solanaceae	**Solanum mauritianum**	-	-	-
Solanaceae	**Solanum melongena***	+	+	+
Solanaceae	**Solanum paniculatum**	-	-	-
Solanaceae	**Solanum sisymbrifolium**	-	-	-
Solanaceae	**Solanum viarum**	+	+	+

* plants described in literature as hosp of Alternaria solani

[a] + or – = early blight symptoms/pathogen structures present or absent, respectively.

Phylogenetic of representation via grayscale

Table 1. Cont.

Family	Species	Reaction		
		Alternariatomatophila	Alternaria grandis	Alternaria solani
Solanaceae	**Vassobia breviflora**	-	-	-
Convolvulaceae	**Ipomoea batatas ***	-	-	-
Convolvulaceae	**Ipomoea purpurea**	-	-	-
Apiaceae	**Apium graveolens ***	-	-	-
Apiaceae	**Daucus carota var. sativa**	-	-	-
Apocynaceae	**Plumeria rubra**	-	-	-
Asteraceae	**Ageratum conyzoides**	+	+	+
Asteraceae	**Calendula officinalis**	-	-	-
Asteraceae	**Emilia sonchifolia**	-	-	-
Asteraceae	**Erigeron bonariensis**	+	+	+
Asteraceae	**Galinsoga parviflora ***	+	+	+
Asteraceae	**Tagetes minuta**	-	-	-
Bignoniaceae	**Pyrostegiavenusta**	-	-	-
Polemoniaceae	**Phlox drummondii**	-	-	-
Plantaginaceae	**Plantagomajor**	-	-	-
Rubiaceae	**Coffeaarábica**	-	-	-
Verbenaceae	**Lantana câmara**	-	-	-
Amaranthaceae	**Amaranthus retroflexus ***	-	-	-
Amaranthaceae	**Amaranthus spinosus ***	-	-	-
Cucurbitaceae	**Citrullus vulgaris ***	-	-	-
Cucurbitaceae	**Cucumis sativus***	-	-	-
Cucurbitaceae	**Momordica charantia**	-	-	-
Geraniaceae	**Pelargoniumhortorum**	-	-	-
fabaceae	**Phaseolus vulgaris ***	-	-	-
Polygonaceae	**Polygonumhydropiper**	-	-	-
Polygonaceae	**Rumexacetosa**	+	+	+
Zingiberiaceae	**Zingiber officinale ***	-	-	-

* plants described in literature as hosp of Alternaria solani

[a]+ or − = early blight symptoms/pathogen structures present or absent, respectively.

Phylogenetic of representation via grayscale

FIGURES

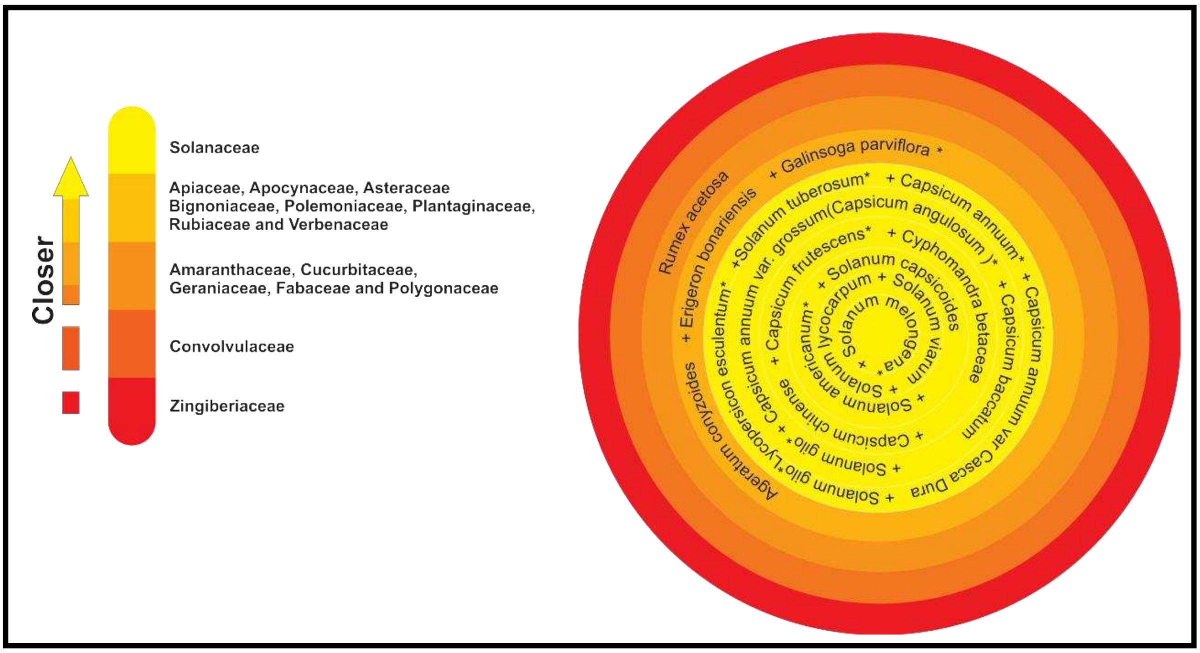


Figure 1. Phylogenetic of representation via gradient of species of plants susceptible to Alternaria spp. that cause early blight. + = early blight symptoms/pathogen strutures present for A. solani, A. grandis, A. tomatophila. * plants described in literature as hosp of A. solani.

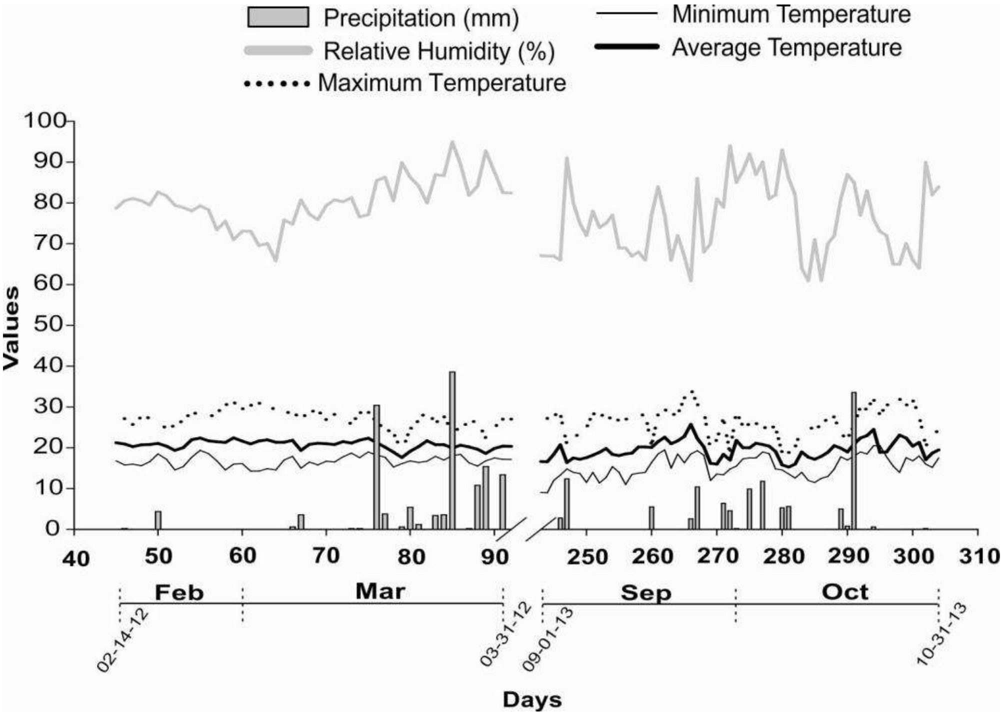


Figure 2. Average values of meteorological variables during the experiments.

CHAPTERIII - A comparative study of early blight epidemics in potato and tomato

ABSTRACT

Epidemics of early blight of potato and tomato have reached high intensity, causing substantial yield loss. Alternaria grandis and A. tomatophila were reported to be more frequently associated with the disease. Environmentalconditions and the host-pathogen combination interfere withtheepidemic. So far,there are noepidemiological studiesto support theseobservations. This work was aimed to studythe development ofearly blightepidemicsin Coimbra(720m.a.s.l.) and Rio Paranaíba(1073 m.a.s.l.). Threeexperiments were conductedin each region: potatoesin the summer 2012, potatoes and tomatoesin the winter 2012and summer 2013. Epidemics were initiated by natural inoculation and disease severity was estimated at every four days. The disease progress rate (r), the values of disease severity at day 24 (Y_{24}) and the standardized area under the disease progress curve (AUDPC) were calculated. Monosporic isolates were obtained and later identified using SNPs in the RPB2 and MCM7 regions by high-resolution dissociation curve using qPCR. In Coimbra, in summer 2012 and 2013, all isolates were A. tomatophila. Isolates of A. tomatophila were more aggressive in tomato. In winter, isolates of A. grandis were detected in potato and A. tomatophila in tomato. Similar result was recorded in Rio Paranaíba in the summer, however, in the winter, A. grandis prevailed and there was no infection in tomato. The epidemiological descriptors supported that epidemic of early blight varied by region, species and hosts. Highest values of epidemic descriptors were obtained in tomato in Rio Paranaíba, in all experiments. Temperature differences influenced the occurrence and intensity of epidemics.

Key words: Alternaria solani, Alternaria grandis, Alternaria tomatophila, aggressiveness, specialization

3.1 INTRODUCTION

In a review of the taxonomy of the species of the genus *Alternaria* associated with Solanaceae, *A. tomatophila* Simmons was commonly found as the causal agent of early blight on tomato (*Solanumlycopersicum* L.) and *A. solani*on potato (*S. tuberosum* L.) (Simmons, 2000). Subsequently, Frazer and Zitter (2003) confirmed the morphological and cultural differences reported by Simmons, concluding the early blight of potato and tomato is caused by at least two species of *Alternaria*. In Russia, it was found that early blight epidemics in potato and tomato are caused by *A. solani* and *A. tomatophila*, respectively (Gannibal et al., 2014). In Brazil, apparently two species of *Alternaria* are often reported as the causal agent of early blight:*A. tomatophila* in tomato and *A. grandis* in potato (Rodrigues et al., 2010). Interestingly, among several Brazilian isolates that were examined none was *A. solani* (Rodrigues et al., 2010).

Early blight epidemics can cause considerable yield losses, especially if control measures are not properly adopted (Reifschneider et al., 1984; Rotem, 1994; Guenthner et al., 1999; Van Der Walls et al., 2004; Shuman and Christ, 2005). The disease can be observed at any stage of plant development, however, it is more severe in mature and senescent tissues (Johnson and Teng, 1990; Dita Rodriguez et al, 2006). Regardless of the *Alternaria* species and the host, necrotic lesions on leaflets, stems, fruits or tubers are typical symptoms of early blight (Rotem, 1994). The lesions are dark, slightly depressed, which may present as elongated and circular shape, with marked concentric rings (Jones et al., 1991). At high severity early blight epidemics cause defoliation of the plant, reducing yield and quality of fruits and tubers (James et al., 1972; Jones et al., 1991; Andersen et al., 2008). In Brazil, epidemics of early blight can occur at any time of the year (Batista et al., 2006) however, the disease is more severe in the summer, when the humidity and temperature are higher. Under conducive environment, the use of fungicides is intensive (Guenthner et al., 1999; Mantecón, 2007; Shuman and Christ, 2005; Töfoli et al., 2003).

Isolates of *A. grandis* and *A. solani* can cause early blight in tomato and isolates of *A. tomatophila* can infect potato (Rodrigues et al, 2010; Gannibal et al, 2014). The hosts range is primarily comprised by Solanaceae species, but some members of the Asteraceae and Polygonaceae were also susceptible to the pathogens (Cardoso unpublished data) as well as other non-solanaceous plants (Akhtar et al, 2011).As host-pathogen specificity is not absolute, differences in aggressiveness may explain the differentialprevalence ofAlternaria species in the host. Variations in the aggressiveness of isolates of A. solani both derived from tomato (Bonde, 1929) and potato (Henning and Alexander, 1959) were reported. Differences inaggressiveness amongBrazilian isolates ofA.solani were reported and isolates were more aggressive when inoculated in their respective hosts of origin (Fancelli, 1991; Castro et al, 2000; Martínez et al, 2004). Isolates of A. tomatophila were more aggressive than isolates of A. solani when inoculated on tomato (Frazer and Zitter, 2003; Gannibal et al., 2014). It is believed that such differences in aggressiveness will be reflected in different intensity of epidemics in the field. In many producing regions, there has been an increase in the intensity of early blight even with intensive application of fungicides.

Several epidemiologicalstudieshave been conducted toinvestigate early blightcaused byA.solani,but, so far,none werecarried forepidemics causedby the “new” species of Alternaria. Differences in aggressiveness based on the combinationof host – pathogen specieshavenot beenquantified yet.Nevertheless, quantificationof aggressivenessunder theepidemiological point of viewis importanttoplanbetter control strategies, since the increased aggressivenessof isolatesof a pathogenresults in higherconsumption offungicides(Katoet al.,1997).

In addition todifferences inaggressiveness, it is possibleto havedistinct responsesofAlternaria speciesto climatic variablesand that thesemay affectthe preference forhosts, as demonstratedforlate blight, caused by Phytophthorainfestans (Maziero et al., 2009). In Brazil, tomato plants are grown in broad climatic conditions while potato crops are concentrated in regions of altitude generally above 800 m. The presence of "ecotypes", or ecological races, i.e. populations ecologically distinct which are strictly subject to periodic selection events (Ward et al., 2008), could explain the distribution of species. Apparently, epidemics caused by the new species respond similarly to those caused by A. solani. However, noepidemiological studies onearly blightcaused byA.grandisand A.tomatophila were conducted intropical regions. The objectivesof this studywere: (i) determine the occurrence of speciesof Alternaria associated with early blight intwo distinctregions and(ii) to compare epidemics inrelation to pathogen species,hosts andregions.

3.2 MATERIAL AND METHODS

3.2.1 Feature of tomato and potato producer regions.

Three experiments were conducted in the municipalities of Coimbra and Rio Paranaíba, both in Minas Gerais State, Brazil (Figure 1). Coimbra (20º51’24”S and 42º48’10”W) is at 720 m altitude and is a tomato production area. Rio Paranaíba (19°11’38” S and 46°14’49” W) is at 1073 m altitude and is one of the largest producers of potato in Brazil, however, there are small areas of tomato, among other crops. Table 1 shows the above mentioned information, in detail, for each experiment.

3.2.2 Field experiments.

Tomato cultivar “Santa Clara” and potato cultivar “Ágata” were planted in experimental plots. Plants were fertilized based on soil analyses and cultural practices were as recommended (Filgueira, 2000; Ribeiro et al., 1999). Tomato and potato spacing consisted of rows measuring 1.0 m x 0.50 m and 0.8 m x 0.3 m, respectively. In the case of tomato, we used the traditional staking system (or inverted V) and plants were conducted with a single stem until the formation of the sixth fruit bunch. In Coimbra, the potato plots were sprinkler-irrigated with an average of 10 mm water/irrigation date. In Rio Paranaíba, potato plots were irrigated by center pivot applying 12 mm/day. In both regions plots were irrigated twice a week, except on rainy periods. Tomato plots, in both regions, were drip-irrigated.

The herbicide metribuzin (0.36 Kg.ai.ha^{-1}, for potato and 0.48 Kg.ai.ha^{-1}, for tomato) was applied to control weeds in the experimental area. The insecticides cypermethrin + profenofos (0.22 Kg.ai.ha^{-1} for potato and 0.44 kg.ai. ha^{-1}for tomato) and abamectin (0.18 Kg.ai.ha^{-1} for potato and 0.144 Kg.ai.ha^{-1}for tomato) were used to control pests. In both crops dimethomorph fungicide (0.4 Kg.ai.ha^{-1} on potato and 0.95 Kg.ai.ha^{-1} on tomato) was sprayed to prevent late blight epidemics and thiophanate-methyl (0.21 kg.ai. ha^{-1}) was sprayed on tomato plants to control septoria leaf spot.

During the experiments in Coimbra, relative humidity and temperature values were recorded at every 30 minutes in an automatic data logger (Hobo Pro RH / Temp) installed at 1.50 m above the crop canopy in the experimental field. Daily precipitation values were obtained from the Irriplus E1000 meteorological station. In Rio Paranaíba, we used the Davis Vantage Pro 2 weather station located 5 m from the tomato experimental area and 4 km from the potato experimental area. For regions, average daily temperature (^{o}C), relative humidity (%) and average daily rainfall accumulated (mm) were used.

For the potato trials, the plot measured of 4.2 (length) x 4.0 m (width). The plot had six lines with 13 plants per row, for a total of 78 plants per plot. For the tomato trials the plots measured 5.0 (length) x 6.0 m (width), with six rows with 10 plants in each. In both trials involving both crops, the plots were spaced 2 m from each other. In Coimbra potato trials were spaced 80 m from tomato and in Rio Paranaíba they were located 1.3 Km apart.

3.2.3 Disease assessment

Early blight epidemics started by natural inoculum. Weekly visual assessments of disease severity were performed starting at the emergence of the potato crop or after the first bloom in the tomato crop. After the first symptoms were observed, assessments of early blight severity (SEV) were performed at every four days until the end of the crop cycle for potatoes, and until 104 days after transplanting (DAT) or when early blight severity reached 100 % in any crop. In each plot, eight plants in the two central rows were marked and SEV was visually assessed with the aid of diagrammatic scales previously described for early blight in tomato (Boff et al., 1991) and potato (Duarte et al., 2013).

3.2.4 Molecular identification of Alternaria species associated with potato and tomato early blight

Typical lesions of early blight were collected in each trial during the epidemics in both crops. Twenty monosporic isolates were obtained. Isolatesamples were collected at the start, middle or at the end of the epidemics. DNA extraction was performed using Wizard Genomic DNA Purification Kit (Promega) from mycelia macerated in liquid nitrogen. DNA was quantified with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). DNA was diluted to a concentration of 20 ng/µL in ultrapure water and stored at -20°C.

The molecular identification of Alternaria species was based on high resolution melting (HRM) analysis using the RPB2 and MCM7 regions (Cardoso et al., unpublished data). The HRM PCR analysis was performed on an HRM-equipped real-time rotary analyser (Rotor-Gene Q). The PCR reactions were done using Type-it HRM PCR Kit as described by the manufacturer (Qiagen). The final reaction volume was 10 μL and contained 1x of HRM PCR Master mix, 20 ηg of DNA and 0.7 μM primer mix (AlHRM_RPB2F/AlHRM_RPB2R and AlHRM_MCM7F/ AlHRM_MCM7R). The PCR program consisted of an initial denaturation of 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. For the HRM analysis, the tubes were heated from 60°C to 95°C allowing 10 data acquisitions per 1°C. All analyses were done in duplicates. Positive (DNA of Ex-type and authentic isolates of A. tomatophyla EGS 42-156, A. grandis EGS 44-108 and A. solani EGS 44-098) controls and non-template controls were included in each run.

The generation and analysis of melting profiles as well as genotyping of isolates were conducted using the Rotor-Gene Q associated software program, Rotor-Gene v. 2.0.2.4 applying the default parameters recommended by the manufacturer. Genotypes of test samples (morphologically characterized isolates) were defined by selecting a representative sample for each species. To achieve more consistent and reliable results, only samples with amplification threshold cycle values of less than 30 and generating sigmoid to near sigmoidal curve shape were considered. A confidence threshold of 90% was applied throughout the experiments (Kirkpatrick et al., 2009; Ganopoulos et al., 2012).

3.2.5 Data Analysis

Severity data were plotted to obtain disease progress curves and the area under the disease progress curve (AUDPC) was calculated (Shaner and Finney, 1977). The duration of the epidemic was considered from the appearance of first symptoms until the end of the crop cycle or when SEV reached 100%. When the epidemics had different durations, the AUDPC was standardized as proposed by Fry (1978). The AUDPC valueswere compared by 95% confidence interval.

To perform the comparative study of the progress of the disease in the two regions, we standardized the first twenty-four days of the epidemic to determine the disease progress rate (r) for all experiments. Different progress models were evaluated and model fitness was based on the criteria described in Campbell and Madden (1990). Disease progress rates were compared by the 95% confidence interval of the difference of the estimates (Campbell and Madden, 1990). The SEV values at the 24th day of the epidemic (Y_{24}) were chosen for analysis because all epidemics reached this duration. Additionally, the Y_{24} valueswere compared by 95% confidence interval.

Microclimate variables recorded during the crop cycle were plotted in relation to the disease progress curve. The evaluated meteorological data were: the average daily relative humidity, cumulative precipitation during a day, daily maximum average temperatures (MaxT), daily minimum average temperatures (MinT) and daily medium temperatures (MT).

3.3 RESULTS

3.3.1 Molecular characterization of Alternaria spp.

Based on the HRM analyses A. tomatophila was the most prevalent species in potato in Coimbra and A. grandis in Rio Paranaíba, during summer 2012 (Table 2). During winter 2012, in Coimbra, A. grandis prevailedin potato and A. tomatophila in tomato. In Rio Paranaíba, A. grandis was identified as the causal agent of early blight in potato and there was no epidemic in tomato. During summer 2013 in Coimbra, A. tomatophila was identified in both hosts. In Rio Paranaíba, early blight in potato and tomato was caused by A. grandis and A. tomatophila, respectively.

3.3.2 Meteorological data

3.3.2.1 Summer 2012

In Coimbra the MaxT was 29.6 ± 2.8 ^oC, MinT of 17.7 ± 1.3 ^oC and MT was 23.4 ± 1.4 ^oC. In Rio Paranaíba, the values of these variables were 27.1 ± 2.2 ^oC, 16.7 ± 1.0 ^oC and 21.0 ± 1.2 ^oC, respectively. During the epidemics the temperature in Coimbra ranged from 16 to 36.6 ^oC with average of 22.7 ^oC. In Rio Paranaíba, the average temperature was 21.2 ^oC and varied from 14.2 to 30.7 ^oC (Figure 2C and D).

The precipitation was more concentrated in Coimbra, totaling 241 mm rainfall, 136 mm in the first 5 days of planting, 84.2 mm from 50 DAP until the end of the experiment, and 20.8 mm during planting. In Rio Paranaíba total precipitation was 230 mm, but distributed throughout the experiment (Figure 2C and D). The average RH during the experiment was 78% in Coimbra and 81% in Rio Paranaíba. During the epidemic in Coimbra (45-73 DAP), the average relative humidity was 82.5% with 95% RH at 60 DAP. In Rio Paranaíba, during the epidemic (40-72 DAP), the average relative humidity was 78.1% and between 61 and 73 DAP, when the disease was in its exponential phase, the average relative humidity was 89.1% (Figure 2D).

3.3.2.2 Winter 2012

During the growing of potatoes, the values of MaxT, MinT and MT in Coimbra were 24.3 ± 2.0 ^{o}C, 11.3 ± 2.3 ^{o}C and 18.0 ± 1.4 ^{o}C. In Rio Paranaíba these values were 24.8 ± 2.7 ^{o}C, 12.0 ± 2.1 ^{o}C and 17.8 ± 2.0 ^{o}C, respectively. When early blight epidemic in potato was assessed in Coimbra, the temperature ranged from 7.1 ^{o}C to 29.9 ^{o}C with average of 18.4 ^{o}C. In Rio Paranaíba the maximum temperature recorded was 33.2 ^{o}C and the minimum was 10.1 ^{o}C. The average temperature was 19.5 ^{o}C (Figure 3C and F). The total precipitation during the experiment was 30.7 mm in Coimbra, with 13 mm during the epidemics. In Rio Paranaíba, the precipitation was 147.4 mm concentrated during the beginning of planting (Figure 3C and F). The RH was 79.5 % in Coimbra and 72.8 % in Rio Paranaíba. The average relative humidity during the epidemics (68 to 92 DAP) was 80.8 % in Coimbra. During the exponential growth of disease severity (84 to 92 DAP), the average relative humidity was 76 %. In Rio Paranaíba, the average relative humidity during the epidemics was 58.2 %. During 108 to 116 DAP the average relative humidity was 48.7 %.

During the growing of tomatoes in Coimbra, the values of MaxT, MinT and MT were 26.2 ± 3.5 ^{o}C, 12.7 ± 3.2 ^{o}C and 19.6 ± 2.8 ^{o}C, respectively. In Rio Paranaíba, the values of these variables were 26.7 ± 3.3 ^{o}C, 13.7± 3.1 ^{o}C and 19.6 ± 2.8 ^{o}C, respectively. In Coimbra, during the epidemics, the temperature ranged of 7.5 ^{o}C to 35.0 ^{o}C with average of 21.1^{o}C. The values of total precipitation and average relative humidity in Coimbra and Rio Paranaíba were 121.4 and 640.7 mm and 77.1 ± 7.5 % and 68.9 ± 14.9 % respectively (Figure 3C and F).

3.3.2.3 Summer 2013

During the growing of potatoes, the values of MaxT, MinT and MT in Coimbra were 35.0 ± 5.0 ^{o}C, 18.0 ± 2.0 ^{o}C and 24.0 ± 2.0 ^{o}C, respectively (Figure 4C). In Rio Paranaíba, the values of MaxT, MinT and MT were 27.0 ± 3.0 ^{o}C, 18.0 ± 1.0 ^{o}C and 22.0 ± 2.0 ^{o}C, respectively (Figure 4F). When early blight epidemic in potato was assessed in Coimbra, the temperature ranged from 15.6 ^{o}C to 42.0 ^{o}C with average of 23.8 ^{o}C. In Rio Paranaíba, the temperature ranged from 16.0 ^{o}C to 30.8 ^{o}C with average of 22.0 ^{o}C. The total precipitation was 301.0 mm in Coimbra, 137.7 mm during the epidemics. In Rio Paranaíba, the precipitation was 739.3 mm, 343 mm during the early blight epidemics. The UR was 52.0 % and 71.2 % in Coimbra and in Rio Paranaíba, respectively. The average relative humidity during the epidemics in Coimbra (68 to

92 DAP) and in Rio Paranaíba (42 to 78) was 43.0 % and 54.3%, respectively (Figure 4C and F).

During the growing of tomatoes in Coimbra, the values of MaxT, MinT and MT in Coimbra were 33.1 ± 5.5 ^{o}C, 16.0 ± 3.4 ^{o}C and 22.4 ± 2.9 ^{o}C, respectively (Figure 4C). In Rio Paranaíba, the values of these variables were 26.6 ± 2.9 ^{o}C, 16.8 ± 2.4 ^{o}C and 20.9 ± 2.3 ^{o}C, respectively (Figure 4F). During the epidemic in Coimbra the temperature ranged from 7.8 ^{o}C to 38.8 ^{o}C with average of 20.5 ^{o}C. In Rio Paranaíba, the minimum and maximum temperatures registered were 10.5 ^{o}C and 30.8 ^{o}C, respectively with average of 20.7 ^{o}C (Figure 4B and C). The total precipitation was 307.3 and 490.9 mm, respectively in Coimbra and Rio Paranaiba. The average relative humidity was 54.3 ± 15.4 % in Coimbra and 69.6 ± 12.7 % in Rio Paranaíba (Figure 4C and F).

3.3.3 Progress of early blight in potato and tomato

3.3.3.1 Summer 2012

Potatowere cultivated in Coimbra and Rio Paranaíba for 73 and 72 days, respectively. In Coimbra, the epidemics started at 45 DAP and severity at the end of the epidemics (28 days after epidemics started) was 5 % (Figure 2A). In Rio Paranaiba, SEV reached 100% after 32 days. The disease started at 40 DAP and in 60 DAP the severity was approximately 10% (Figure 2B).

In Coimbra, the progress rate in potato at the 24th day of the epidemic (69 DAP) was estimated as 0.11 and Y_{24} was 4% (Table 3). The progress rate and Y_{24} were significantly lower than those obtained in Rio Paranaíba: r= 0.36 and Y_{24}=25% (64 DAP) (Table 3).

3.3.3.2 Winter 2012

In Coimbra epidemics on potato started at 68 DAP and lasted 24 days. The final severity was 1.9% at 92 DAP (Figure 3A). In Rio Paranaíba, the epidemics started at 88 DAP and lasted 28 days. At 92 DAP the severity was 0.08 % and reached 7.3 % at 116 DAP (Figure 3D).

In Coimbra, epidemics in tomato started at 60 DAP with total duration of 44 days. The average final severity was 36.0 %. Considerable increase in SEV occurred between 100 to 104 DAP (Figure 3B). In Rio Paranaíba there was no early blight epidemic in tomato (Figure 3E).

There was no difference between the progress rate during the 24 day period of epidemics in potato in Coimbra and Rio Paranaíba (Table 5). However, the Y_{24} values were greater in Rio Paranaíba than in Coimbra. The progress rate and Y_{24} values were greater in tomato than in potato plots in Coimbra (Table 3). Significant differences were found in progress rate and Y_{24} for exp1 and exp2 in potato in Rio Paranaíba (Table 5). The rate in exp1 was greater than in exp2 (Table 3).

3.3.3.3 Summer 2013

In Coimbra, the early blight epidemics in potato started at 43 DAP and lasted 24 days. The final severity (67 DAP) reached 5 % (Figue 4A). In Rio Paranaíba, the epidemics lasted 36 days. At 62 DAP, the severity was 7 %, and at 78 DAP SEV reached 100 % (Figure 4D). There were significant differences for the disease progress rate and Y_{24} in potato in Coimbra and Rio Paranaíba. The rate and Y_{24} were greater in Rio Paranaíba ($r= 0.02$; $Y_{24}= 12.7\%$). Significant differences occurred between progress rate and Y_{24} values in exp2 and exp3 conducted in Rio Paranaíba, with greater values in exp3. Significant differences were detected for Y_{24} between exp1 and exp3 and greater values were found in exp1 (Table 3).

In Coimbra, early blight epidemics in tomato started at 52 DAT and finished at 104 DAT. The average final severity was 65 %. Substantial severity increase occurred between at 68 and 80 DAT and between 88 and 104 DAT (Figure 4B). In Rio Paranaíba the epidemics started at 21 DAT and SEV reached 70% at 45 DAT and 100% at 73 DAT (Figure 4E). The progress rate of tomato early blight in Rio Paranaíba ($r= 0.22$; $Y_{24}= 72.2\%$) was higher than in Coimbra (Table 5).In Coimbra both variables, progress rate and Y_{24} in tomato differed when compared between exp2 and exp3 (Table 5). Higher values of progress rate and Y_{24} were obtained in exp3 (Table 3). In Rio Paranaíba, the progress rate of early blight and Y_{24} were greater in tomato than in potato (Table 3).

3.3.4 Area under disease progress curve of early blight in potato and tomato

In exp1, the AUDPC for potato in Coimbra (0.02) was lower than that obtained in Rio Paranaíba (0.20) (Table 4). Similarly, for exp2 in potato, the AUDPC in Coimbra was 0.006, which is lower than that found in Rio Paranaíba (0.02). There was no tomato early blight epidemic in Rio Paranaíba (Table 4).

In exp3 in potato, the AUDPC in Coimbra was 0.02, less than the epidemics in Rio Paranaíba (0.19). The same was observed in tomato. There were no significant differences for AUDPC values obtained in potato in Coimbra for exp1 and exp3. The same was observed in Rio Paranaíba. However, when comparing values AUDPC in potato from both exp1 and exp3 with exp2, in both regions, the smallest value was obtained for epidemics in exp2 (Table 3).

In tomato, higher AUDPC values were observed in exp3 for both regions. Considering the exp3, for both regions AUDPC values were higher in tomato than in potato (Table 3).

3.4 DISCUSSION

The correct use of fungicides and other control measures depend on basic information of epidemiology and biology of the pathogen. When considering potato and tomato early blight caused by distinct species of Alternaria there is considerable lack of information notably with respect to aggressiveness factors, which have not yet been generated. In this work, experiments were conducted in two regions and the results indicate that early blight epidemics can develop rapidly and reach high severity depending on weather conditions as well as the relation between the prevalent Alternaria species and the host plant. Alternaia grandis and A. tomatophila were found as prevalent species in potato and tomato, respectively (Rodrigues et al., 2010), and in this study, these species also were the prevalent in the samples from potato and tomato.

In the interaction between pathogen and host, there are several factors that influence the amount of disease: the environment, the traits of the pathogen, and the traits of the host (Grönberg et al., 2012). Despite the fact that experiments were performed in the summer, when there are favorable environmental conditions for disease development in both regions, early blight epidemics in potato had lower intensity in Coimbra than in Rio Paranaíba. This is attributed to the prevalent species in each region. While A. tomatophila prevailed in Coimbra, A. grandis was isolated in Rio Paranaíba. To date there is no comparative epidemiology study for early blight caused by these species, but there is evidence for differences in aggressiveness towards the host. A recent study concluded that A. tomatophila was able to infect potato leaf fragments, but it was less aggressive than in tomato (Gannibal et al, 2014). Furthermore, aggressiveness differences has been found between A. solani and A. tomatophila (Frazer and Zitter, 2003; Andersen et al., 2008, Cardoso, unpublished data). The difference in epidemic intensity between regions can be explained by the fact that isolates of A. tomatophila have been more aggressive on tomato than on potato. In the summer, weather conditions are more favorable to epidemics of early blight, and may have favored A. tomatophila in potato. Diversity within A. tomatophila in terms of aggressiveness has been established. Frazer (2002) found that isolates of A. tomatophila are more aggressive totomato, however, a group of A. tomatophila, characterized by the production of dark mycelium, was so virulent as A. solani on potato and of intermediate virulence on tomato. The diversity within A. tomatophila in terms of aggressiveness can therefore be found in a restricted area but also in a larger spatial scale. However, despite its detection in potato in Coimbra, isolates of A. tomatophila were more aggressive to tomato.

Until now, under natural conditions, isolates of A. tomatophila had not been found in potato neither of A. grandis in tomato (Rodrigues et al., 2010). In this study, A. tomatophila infected potato in Coimbra, but A. grandis was not found associated with early blight in tomato in any of the regions studied. In Rio Paranaíba, early blight epidemics in potato was caused by A. grandis and in tomato plants by A. tomatophila in all experiments, so it is possible to conclude that, at the field level, host specificity is not absolute and host preference occurs by differences in aggressiveness, which explains the occurrence of distinct species causing early blight on tomato and potato in areas where these vegetable crops are deployed in the same micro-region. For other Alternaria species, including A. solani, there are reports in relation to host preference (Fancelli, 1991; Weir et al., 1998; Castro et al., 2000; Martinez et al., 2004) and, as pointed out previously, differential aggressiveness on a particular host species can be considered as an indicator of host specificity (Suassuna et al., 2004), thus we can conclude that A. grandis has specificity to potato and A. tomatophila to tomato.

Despite the widespread occurrence of early blight, climatic conditions during cultivation influenced the intensity of the epidemic and the process of infection of the pathogens. In the winter 2012 experiment disease development in both regions was slower than in warmer planting seasons, however there were also differences in disease development between regions and hosts. In Coimbra, early blight in potato caused by A. grandis was less severe than epidemics caused by A. tomatophila in tomato. Also, early blight severity in potato in Coimbra was less intense than in Rio Paranaiba. Coimbra region has several commercial tomato fields throughout the year. Thus, this condition may imply in greater inoculum concentration of A. tomatophila than A. grandis, which enabled the higher disease intensity in tomato, even during unfavorable weather conditions. Greater severity of early blight was observed when rainfalls were more frequent and temperatures were between 25 and 30^{o}C (Andrade, 1997; Salustiano, 2000). However, Rotem (1994) reported that the minimum temperature for infection may be reduced from 20 to 10^{o}C, and the maximum may increase from 30 to 35^{o}C and optimum temperatures of 25^{o}C extend to a range of 20 to 30^{o}C if the inoculum is present and if the leaf wetness period is favorable, conditions found in Coimbra.

In Rio Paranaíba, during winter 2012 season, the weather conditions were similar to those found in Coimbra, except the relative humidity, which during the epidemic in potatoes was lower than that recorded in Coimbra. A. grandis has been reported causing disease even in conditions of low relative humidity (Cardoso, 2010; Cardoso, unpublished data). Thus, high disease intensity in potatoes in Rio Paranaíba is probably due to high inoculum availability in this region,once potato is commonly cultivated in this municipality.

Contrary to what was observed in the winter 2012 season, the non-detection of A. grandis in potato samples collected in the 2012 and 2013 seasons in Coimbra, does not indicate its absence. Symptoms caused by A. grandis and A. tomatophila develop fast. Necrosis in the inoculated leaflets can be visualized after 12 h of incubation (Cardoso, 2010). The greater amount of inoculum of A. tomatophila in the area increases the chances of its detection at the infection sites. Thus, A. tomatophila was more often detected than A. grandis and the species identification may have been biased by the isolation of the pathogen that caused larger lesions.

Epidemics of early blight in tomato were also less severe in periods of low temperature. In Coimbra, duringJuly to September (winter 2012), epidemics of early blight in tomato had a delay of 8 days, slower progress and reduced severity at the end of the epidemic. In Rio Paranaíba, no early blight developed on tomato from July to November. Low temperatures, lack of rain or short periods of leaf wetnessresulted in low early blight incidence in tomato (Salustiano, 2000). In addition to less conducive environmental conditions, the reduced area with tomato plantations in Rio Paranaíba possibly contributed to a reduced amount of inoculum available for early blight epidemics. Commonly, the concentration ofsporesin the airis related to the intensity of diseasein the field (Jeger, 1999).

In the summer, early blight on tomato was very severe in Rio Paranaíba. The disease started at 21 DAT, reaching 100% at 73 DAT, being more severe than in Coimbra. Despite both regions having commercial tomato crops, in Coimbra planting is cyclical, i.e., new plantings start after the old ones are finished and the new plantings are set in a new area.This practice contributes to reduce the aumont of inoculum of the area.In Rio Paranaíba planting is sequential, new area can be located near old plantations and this favors the higher amount of inoculum in the field.The occurrence of infected crop residues with A. solani in the field can anticipate epidemics of early blight (Patterson, 1991). The highest intensity of the disease in Rio Parnaíba could have been due to the lack ofproper management of crop residues. The incorporation of crop residues of tomato at 20 cm depth reduced the viability of propagules of A.

solani(Batista, 2006), and this should be used for the management of early blight.

Gilbert and Webbs (2007) hypothesized that some isolates of A. tomatophila and A. grandismay be equally aggressive on both hosts, especially under favorable conditions for infection; i.e. high amount of inoculum and favorable weather. Both species are phylogenetically close relatives and their two host species are also closely related to one another. However, this hypothesis was not supported based on our study. We found that despite finding A. tomatophila in potato, this pathogen was more aggressive to tomato than to potato. Additionally, even under favorable conditions for both species in Rio Paranaíba, A. grandis was not associated with early blight on tomato or A. tomatophila on potato.

Proper knowledge about the host-pathogen association and the epidemiology of early blight in different sites is important for the establishment of new strategies and the development of new technologies for decision making. Additionally, differences in aggressiveness in Alternaria spp. detected here are important for some practical issues such as the durability of resistant cultivars and the effectiveness of fungicides used in chemical control program.

REFERENCES

Akhtar KP, Sarwar N, Saleem MY, Asghar M, 2011.Convolvulus arvensis, a new hosp for Alternaria solani causing early blight of Solanum lycopersicum in Pakistan.Australasian Plant Disease Notes**6**, 84-6.

Andersen B, Dongo A, Pryor BM, 2008. Secondary metabolite profiling of Alternaria dauci, A. porri, A. solani, and A. tomatophila.Mycological Research**112,** 241-250.

Andrade DFAA, 1997. Previsão e controle químico da pinta preta (Alternaria solani Sorauer), sob dois sistemas de condução do tomateiro (Lycopersicon esculentum Mill.). Viçosa: Universidade Federal de Viçosa, tese de mestrado.

Batista DC, Lima MA, Haddad F, Maffia LA, Mizubuti ESG, 2006. Validation of decision support systems for tomato early blight and potato late blight, under Brazilian conditions.Crop Protection**25**, 664-70.

Boff P, Zambolim L, Vale FXR, 1991. Escalas para a avaliação da severidade da mancha de estenfílio (Stemphyliumsolani) e da pinta preta (Alternariasolani) em tomateiro. Fitopatologia Brasileira**16**, 280-83.

Bonde R, 1929. Physiological strains of Alternaria solani. Phytopathology**19**, 533-48.

Campbell CL, Madden LV, 1990 Introduction to Plant Disease Epidemiology. New York: John Willey & Sons.

Cardoso CR, 2010. Agressividade de Alternaria tomatophila, A. grandis e A. solani em batateira e tomateiro. Viçosa. Universidade Federal de Viçosa, dissertação de mestrado.

Castro MEA, Zambolim L, Chaves GM, Cruz CD, Matsuoka K, 2000. Variabilidade patogênica de Alternaria solani, agente causal da pinta-preta do tomateiro. Summa Phytopathologica**8**, 24-28.

Dita Rodriguez MA, Brommonschenkel SH, Matsuoka K, Mizubuti ESG, 2006. Components of resistance to early blight in four potato cultivars: effect of leaf position. Journal of Phytopathology**154**, 230-35.

Duarte HSS, Zambolim L, Capucho AS, Nogueira Junior AF, Rosado AWC, Cardoso CR, Paul PA, Mizubuti ESG, 2013. Development and validation of a diagrammatic scale for assessment of potato early blight.European Jornal of Plant pathology**137**, 249-257.

Fancelli MI, 1991. Comparação patogênica, cultural, serológica e eletroforética entre isolados de Alternaria solani do tomate e da batata e variabilidade patogênica de A. solani f. sp. lycopersici N. F. Piracicaba: Escola Superior de Agricultura Luiz de Queiroz, tese de doutorado.

Filgueira FAR, 2000. Novo manual de Olericultura: Agrotecnologia moderna na produção e comercialização de hortaliças. 2. Ed. Viçosa:Editora UFV, 402p.

Frazer JT, 2002. Two species of Alternaria cause early blight of potato, (Solanum tuberosum) and tomato (Lycopersicon esculentum). Faculty of the Graduate School of Cornell University, Master's thesis.

Frazer JT, Zitter, TA, 2003. Two species of Alternaria cause early blight of potato, (Solanum tuberosum) and tomato (Lycopersicon esculentum). Phytopathology (suppl. 3), **93**.

Fry WE, 1978. Quantification of general resistance of potato cultivars and fungicide effects for integrated control of potato late blight.Phytopathology**68**,1650-55.

Gannibal PB, Orina AS, Mironenko NV, Levitin MM, 2014. Differentiation of the closely related species, Alternaria solani and A. tomatophila, by molecular and morphological features and aggressiveness.European Journal of Plant Pathology DOI 10.1007/s10658-014-0417-6.

Ganopoulos I, Madesis P, Zambounis A, Tsaftaris A, 2012. High-resolution melting analysis allowed fast and accurate closed-tube genotyping of Fusarium oxysporum formae speciales complex. FEMS Microbiology Letters**334**, 16-21.

Gilbert, G.S., Webbs, C.O. 2007. Phylogenetic signal in plant pathogen–host range.
Proceedings of the National Academy of Sciences 104: 4979–4983.
Guenthner JF. Wiese MV, Pavlista AD, Sieczka JB, Wyman J, 1999. Assessment of pesticide use in the US potato industry.American Journal of Potato Research**76**, 25-29.

Grönberg L, Andersson B, and Yuen J, 2012. Can weed hosts increase aggressiveness of Phytophthora infestans on potato? Phytopathology**102**, 429-433.

Henning RG and Alexander LJ, 1959.Evidence of existence of physiologic races of Alternariasolani.Plant Disease Reporter**43**, 298-308.

James WC, Shih CS, Hodgson WA, Callbeck LC, 1972. The quantitative relationship between late blight of potato and loss in tuber yield. Phytopathology**62**, 92-96.

Jeger MJ, 1991. Improved understanding of dispersal in crop pest and disease management: current status and future directions. Agricutural and Forest Meteorology**97**, 331-349.

Johnson KB, Teng PS, 1990. Coupling a disease progress model for early blight to a model of potato growth.Phytopathology **80**, 416-25.

Jones JB, Jones JP, Stall RE., Zitter, TA, 1991. Compendium of Tomato Disease. St. Paul. APS Press.

Kato M, Mizubuti ES, Goodwin SB, Fr, WE, 1997.Sensitivity to protectant fungicides and pathogenic fitness of clonal lineages of Phytophthora infestans in the United States.Phytopathology**87**, 973-78.

Kirkpatrick NC, Blacker HP, Woods WG, Gasser RB, Noormohammadi AH, 2009. A polymerase chain reaction-coupled high-resolution melting curve analytical approach for the monitoring of monospecificity of avian Eimeria species.Avian Pathology**38**, 13–19.

Mantecón JD, 2007. Potato yield increases due to fungicide treatment in Argentinian early blight (Alternaria solani) and late blight (Phytophthora infestans) field trials during the 1996-2005 Seasons. Plant Health Progress online, doi:10.1094/PHP-2007-0202-01-RS.

Martínez SP, Snowdon R, Pons-Kuhnemann J, 2004. Variability of Cuban and international populations of Alternaria solani from different hosts and localities: AFLP genetic analysis. European Journal Plant Pathology**110**, 399-409.

Maziero MJ, Maffia LA, Mizubuti ESG, 2009. Effects of temperature on events in the infection cycle of two clonal lineages of Phytophthora infestans causing late blight on tomato and potato in Brazil. Plant Disease**93**, 459-466.

Patterson CL, 1991. Importance of chlamydospores as primary inoculum of Alternaria solani, incitant of collar rot and early blight on tomato.Plant disease**75**, 274-278.

Reifschneider FJB. Furumoto O, Filgueira FAR, 1984. Illustrated key for the evaluation of early blight of potatoes. Plant Protection Bulletin**32**, 91-94.

Ribeiro AC, Guimarães PTG, Alvarez VH, 1999. Recomendações parao uso de corretivos e fertilizantes em Minas Gerais– 5ª Aproximação. Viçosa: CFSEMG, 359 p.

Rodrigues TTMS, Berbee ML, Simmons EG, Cardoso CR, Reis A, Maffia LA, Mizubuti ESG, 2010. First report of Alternaria tomatophila and A. grandis causing early blight on tomato and potato in Brazil.New Disease Reports**22**, 28.

Rotem J, 1994. The genus Alternaria.Biology, epidemiology, and pathogenicity.APS Press, St. Paul. 326.

Salustiano ME, 2000. Progresso da pinta-preta do tomateiro em cultivares de tomate em diferentes épocas de plantio. Viçosa: Universidade Federal de Viçosa, tese de doutorado.

Shaner G, and Finney RE, 1977.The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat.Phytopathology**67**, 1051-56.

Shuman JL, Christ BJ, 2005. Integrating a host-resistance factor into the Fast system to forecast early blight of potato.American Journal of Potato Research**82**, 9-19.

Simmons EG, 2000.Alternaria themes and variations (244-286) species on Solanaceae.Mycotaxon**75**,1-115.

Suassuna ND, Maffia LA, Mizubuti ESG, 2004. Aggressiveness and host specificity of Brazilian isolates of Phytophthora infestans. Plant Pathology**53**, 405-13.

Töfoli JG, Domingues RJ, Garcia Junior O, Kurozawa C, 2003. Controle da pinta preta do tomateiro por fungicidas e seus impactos na produção. Summa Phytopathologica**29**, 225-33.

Van Der Walls JE, Korsten L, Slippers B, 2004. Genetic diversity among Alternaria solani isolates from potatoes in South Africa. Plant Disease**88**, 959-64.

Ward DM, Cohan FM, Bhaya D, Heidelberg JF, Kuhl M, Grossman A, 2008. Genomics, environmental genomics and the issue of microbial species.Heredity**100**, 207–19.

Weir TL, Huff DR, Christ BJ, Romaine CP, 1998. RAPD-PCR analysis of genetic variation among isolates of Alternaria solani and Alternaria alternata from potato and tomato. Mycologia**90**, 813-21.

TABLE

Table 1- Description of the experiments conducted in two different locations, crops and experimental realization period.

Experiment	Location	Crops	Date (Month/Day)
Summer 2012	Coimbra	Potato	01/25 to 04/07 (2012)
	Rio Paranaíba	Potato	01/12 to 03/24 (2012)
Winter 2012	Coimbra	Potato	06/06 to 09/05 (2012)
		Tomato	07/16 to 10/14 (2012)
	Rio Paranaíba	Potato	05/21 to 10/14 (2012)
		Tomato	07/13 to 11/23 (2012)
Summer 2013	Coimbra	Potato	01/22 to 04/03 (2013)
		Tomato	01/29 to 05/13 (2013)
	Rio Paranaíba	Potato	01/16 to 04/04 (2013)
		Tomato	02/15 to 04/29 (2013)

Table 2- Isolates obtained in experiment 1, 2 and 3 in Coimbra and Rio Paranaiba, Minas Gerais state.

Experiment	**Location**	**Potato**	**Tomato**	**commercial cultivation**
Summer 2012	Coimbra	A. tomatophila	-*	Tomato
	Rio Paranaíba	A. grandis	-	Potato and Tomato
Winter 2012	Coimbra	A. grandis	A. tomatophila	Tomato
	Rio Paranaíba	A. grandis	A. tomatophila	Potato
Summer 2013	Coimbra	A. tomatophila	A. tomatophila	Tomato
	Rio Paranaíba	A. grandis	A. tomatophila	Potato and Tomato

* No planting tomato.

Table 3- Epidemiological descriptors: progress rate of early blight and disease severityon potato andtomato at 24 days of the epidemic (Y_{24}). Confidence interval (CI) for Y_{24} (in parenthesis).

Experiment	Location	Crops					
		Potato			**Tomato**		
		Rate	**Y_{24}**	**Time to twenty-fourth day of epidemic (DAP)**	**Rate**	**Y_{24}**	**Time to twenty-fourth day of epidemic (DAP)**
Summer 2012	Coimbra	0.107	3.69 (2.1 - 5.3)	69	-*	-	-
	Rio Paranaíba	0.359	25.2 (18.2 - 3.2)	64	-	-	-
Winter 2012	Coimbra	0.138	1.89 (1.4 - 2.4)	92	0.085	6.78 (5.2 - 8.4)	84
	RP	0.128	3.69 (2.9 - 4.4)	112	-**	-	-
Summer 2013	Coimbra	0.136	5.04 (4.513 - 5.567)	67	0.178	24.79 (21.044 - 28.543)	76
	RP	0.179	12.69 (7.769 - 17.607)	66	0.22	72.23 (66.368 - 78.101)	45

* No planting tomato. ** No symptoms of early blight.

Table 4- Standardized area under the disease progress curve of early blight (AUDPC) on potato and tomato, in Coimbra-MG and Rio Paranaiba-MG for experiments 1, 2 and 3 and confidence interval (in parenthesis).

Region/ AUDPC*	**Experiment 1**		**Experiment 2**		**Experiment 3**	
	Potato	**Tomato**	**Potato**	**Tomato**	**Potato**	**Tomato**
Coimbra	0.019 (0.0132 - 0.0238)	-**	0.006 (0.0049 - 0.0068)	0.091 (0.0896 - 0.9308)	0.016 (0.0149 - 0.0161)	0.270 (0.2566 - 0.2840)
Rio Paranaíba	0.190 (0.0170 - 0.0203)	-	0.0190 (0.0179 - 0.2012)	--***	0.187 (0.1818 - 0.1915)	0.591 (0.5847 - 0.5974)

* Mean of four replications.
** Was not grown tomatoes in experiment1.
*** No known symptoms of early blight.

Table 5- Confidence interval at 95% probability of the estimative b2, for comparisons between progress rate early blight on potato and tomato in Coimbra and Rio Parnaíba.

Variables/Coimbra (CO)	**Rate**	**Variables/ Rio Paranaíba (RP)**	**Rate**
exp1_potato_CO x exp3_potato_CO	(-0.0643- 0.0061)	exp1_potato_RP x exp2_potato_RP	(0.1697 - 0.2914) *
exp2_potato_CO x exp2_tomato_CO	(0.0242 - 0.0814) *	exp1_potato_RP x exp3_potato_RP	(0.1140 - 0.2456) *
exp2_tomato_CO x exp3_tomato_CO	(-0.121 --0.0648) *	exp2_potato_RP x exp3_potato_RP	(-0.0935- -0.0079) *
exp3_tomato_CO x exp3_potato_CO	(0.0154- 0.0679) *	exp3_tomato_RP x exp3_potato_RP	(0.0018- 0.0796) *
Variables/Coimbra (CO)X Rio Paranaíba (RP)			
exp1_potato_CO x exp1_potato_RP	(-0.3165- -0.1865) *		
exp2_potato_CO x exp2_potato_RP	(-0.0188- 0.03845)		
exp3_potato_CO x exp3_potato_RP	(-0.0793- -0.0059) *		
exp3_tomato_CO x exp3_tomato_RP	(-0.0709- -0.0124) *		

*When confidence intervals do not include zero, rates of progress of early blight are statistically different at 5% probability (we reject H0: A = 0).

FIGURES

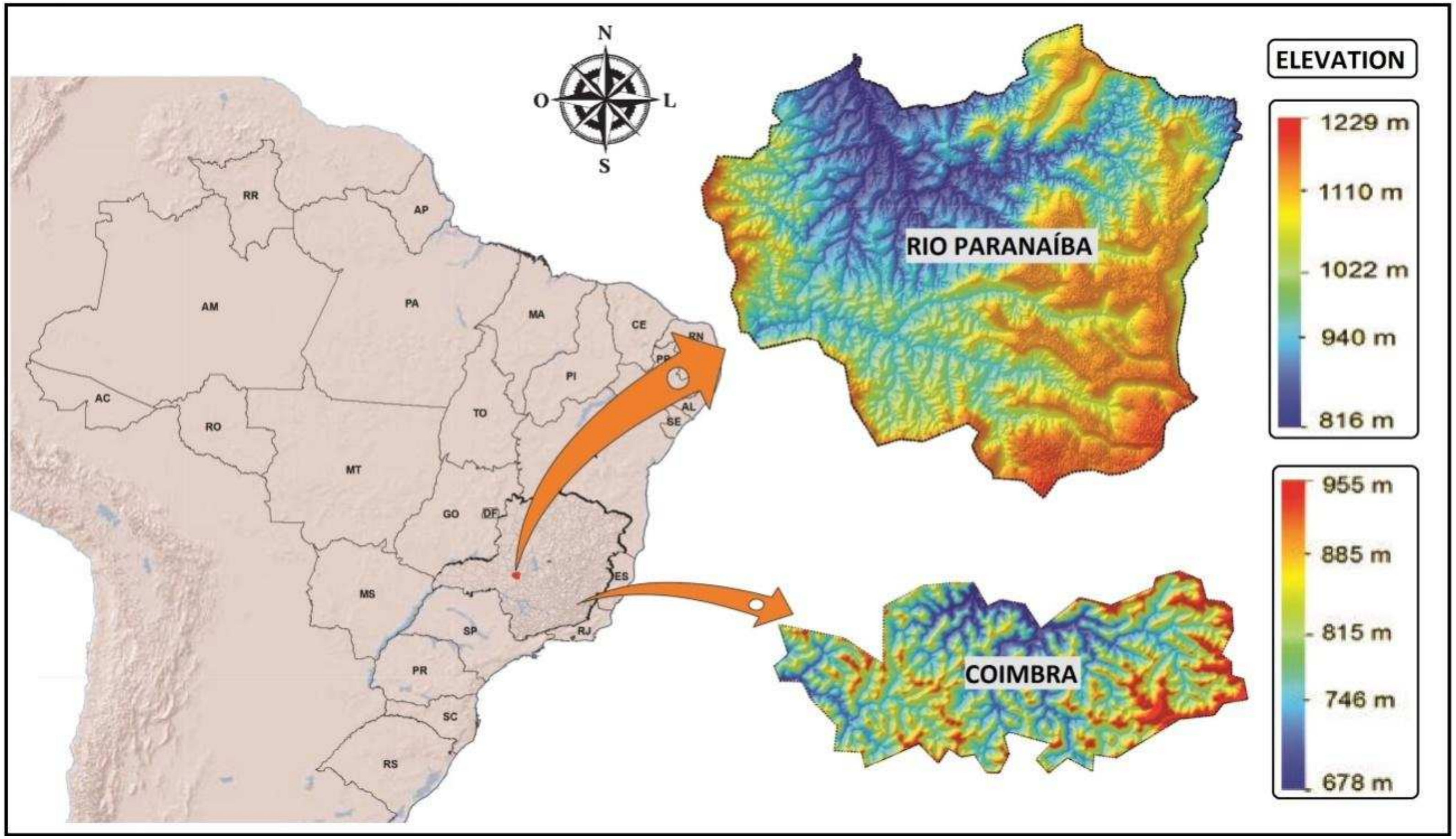


Figure 1 - Experimental area (location and elevation) in Rio Paranaiba and Coimbra, MG, Brazil

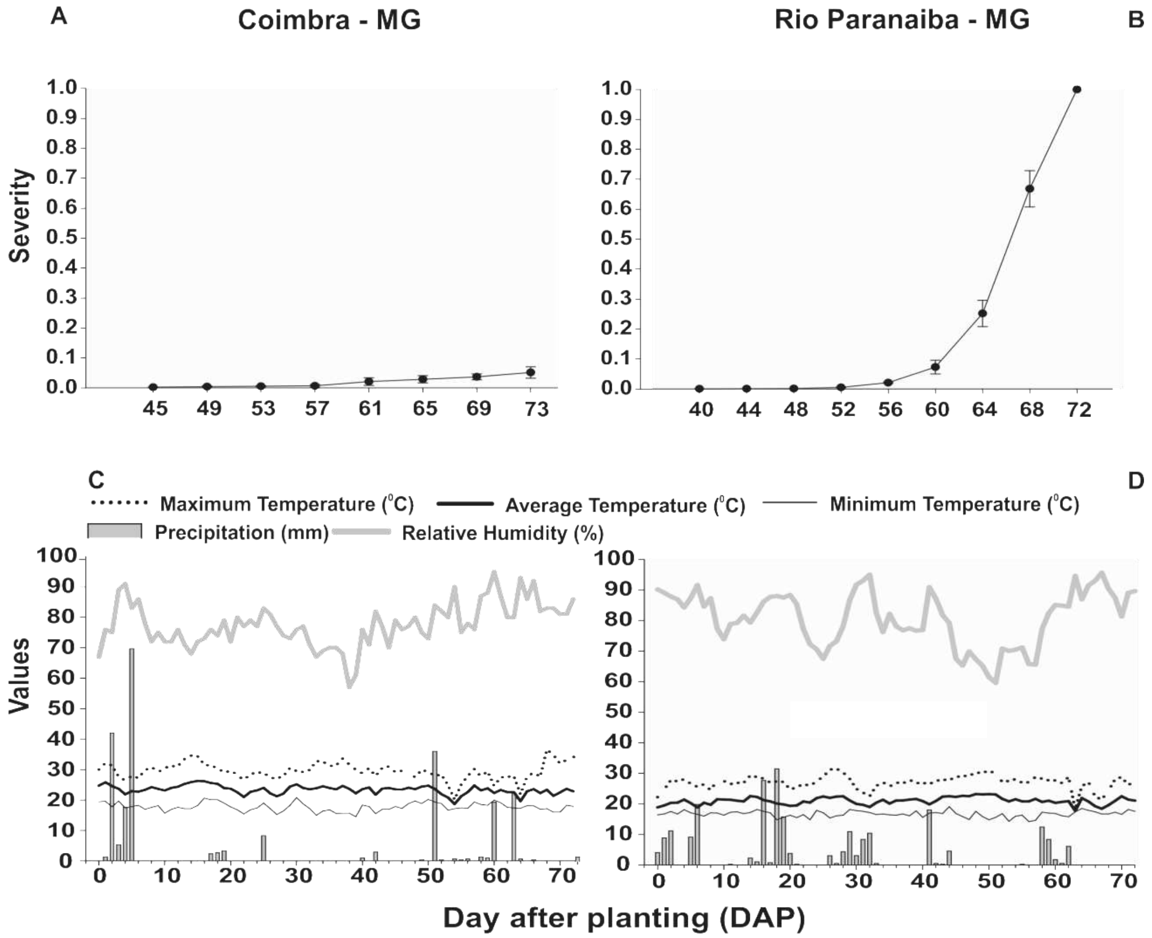


Figure 2 – Early blight on potato in Coimbra-MG and Rio Paranaíba-MG in summer 2012 (A and B, respectively), bars represent the standard deviation of the mean. Weather variables recorded during the experiment in Coimbra-MG (C) and Rio Paranaíba-MG (D) during the experiment 1 (summer 2012).

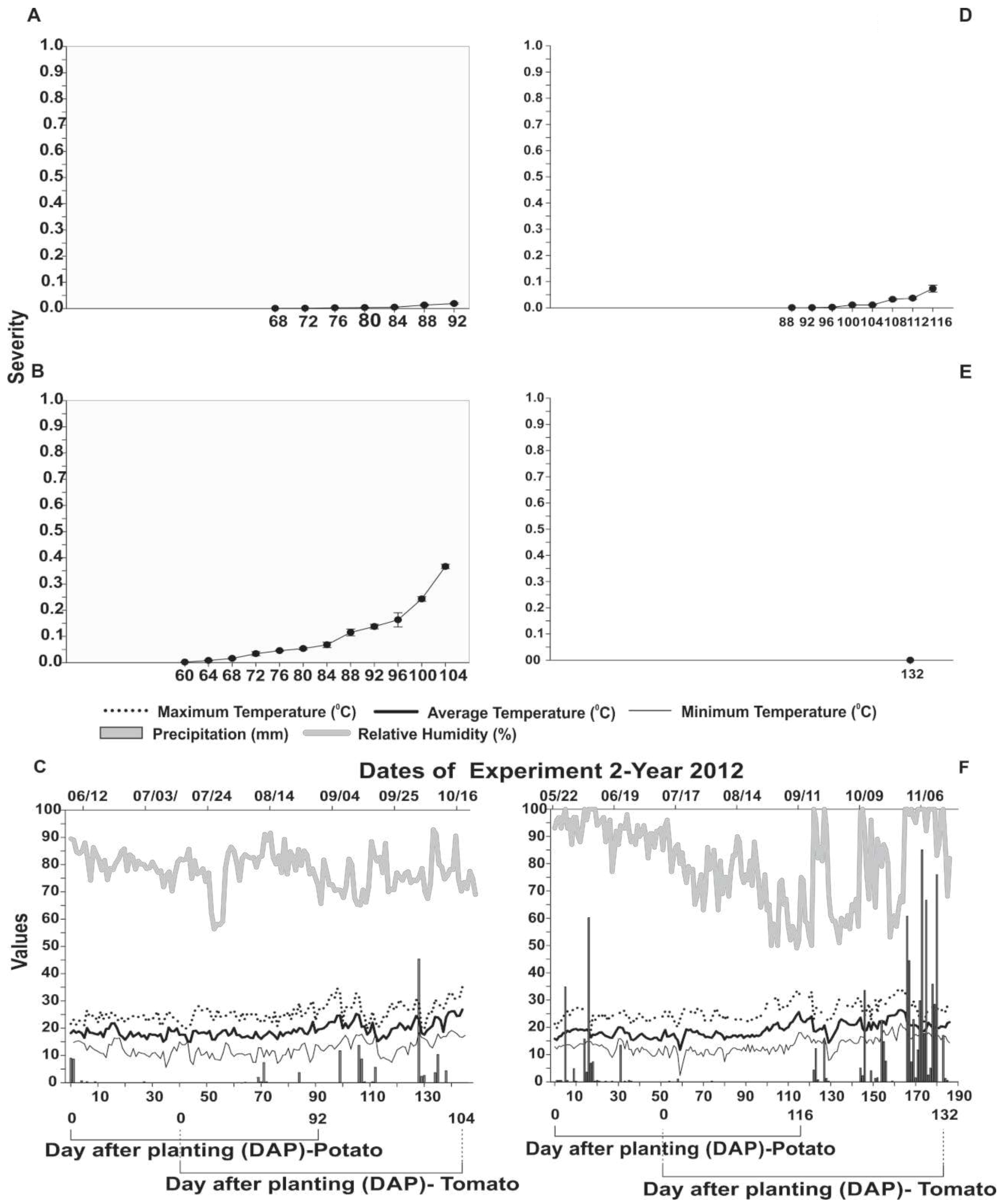


Figure 3 - Early blight on potato and tomato in Coimbra-MG (A and B, respectively) and Rio Paranaíba-MG (D and E, respectively) in winter 2012, bars represent the standard deviation of the mean. Weather variables recorded during the experiment in Coimbra-MG, using potato (June-September) and tomato (July-October) (C) and Rio Paranaíba-MG in potato (May-September) and tomato (July-November) (F).

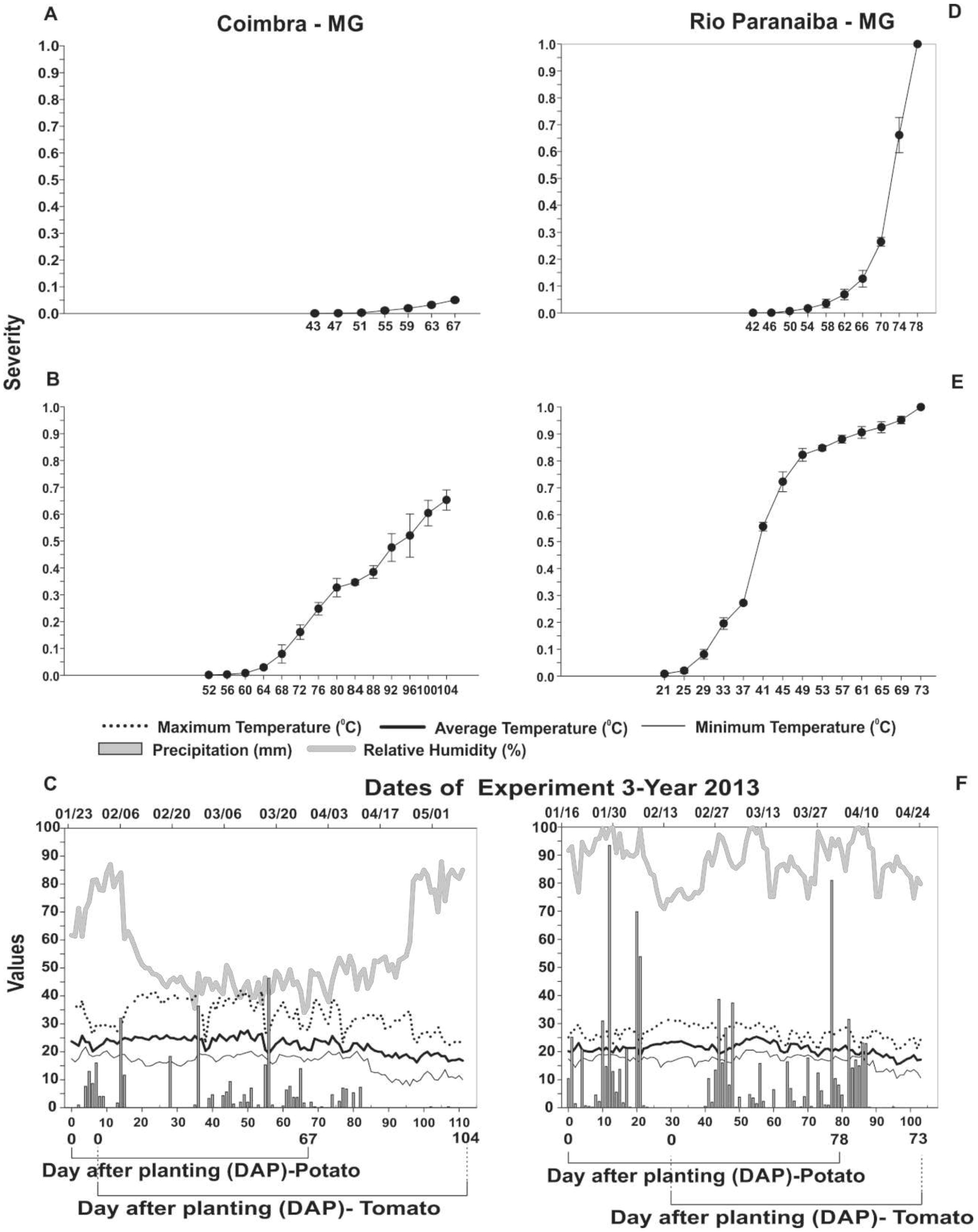


Figure 4 -Early blight on potato and tomato in Coimbra-MG (A and B, respectively) and Rio Paranaíba-MG (D and E, respectively) in summer 2013, bars represent the standard deviation of the mean. Weather variables recorded during the experiment in Coimbra-MG, using potato (January-April) and tomato (January-May) (C) and Rio Paranaíba-MG in potato (January-April) and tomato (February-April) (F).

CHAPTERIV - COMPARATIVE EPIDEMIOLOGY OF POTATO EARLY BLIGHT CAUSED BY *ALTERNARIA SOLANI* AND *A. GRANDIS*

ABSTRACT

Several species of *Alternaria* were recently reported causing early blight in potato and tomato in Brazil. *A. grandis* Simmons is the most prevalent in potato fields. Two experiments were carried out aiming to compare potato early blight epidemics caused by *A. grandis* and *A. solani* under different concentration of inoculum and fungicide applications. Plants were artificially inoculated 50 days after planting with a spore suspension at $5x10^2$ (C1) or 10^4 conidia/mL (C2) and subjected to either weekly application of chlorothalonil (P1) or at every three days (P2). Early blight severity was quantified every three days, and the disease severity data were used to calculate the disease progress rate, area under the disease progress curve and severity at the end of the epidemic. For all epidemiological variables quantified, *A. grandis* was more aggressive than *A. solani*.Early blight epidemics caused by *A. grandis* were severe. Plots inoculated with higher inoculum concentration reached 100% severity.Even under unfavorable conditions such as low humidity, epidemics caused by *A. grandis* had high severity.

Keywords: *Solanum tuberosum*; early blight; management; control.

4.1 INTRODUCTION

Early blight is a foliar disease that affects solanaceous crops worldwide (Neergaard, 1945; Rotem, 1994; Van der Waals et al., 2004; Kumar et al., 2008).Early blight epidemics can cause considerable losses in potato production worldwide and in short period of time, especially if control measures are not taken properly (Shuman and Christ 2005; Van Der Walls et. al., 2004, Guenthner et al. 1999; Rotem 1994, Reifschneider et al., 1984). One of its causal agents, Alternaria solani Sorauer, has been more commonly reported in association with the disease in potato (Solanum tuberosum L.) (Rotem, 1994). In addition to A. solani, A. grandis (Simmons) was reported as the causal agent of early blight in some regions in the United States (Simmons, 2000). In Brazil, A. grandis was the most frequent species associated with early blight in potato (Rodrigues et al. 2010). In some areas in Brazil with large acreages of tomato (Solanum lycopersicum L.) crops, A. tomatophila (Simmons) can be found causing early blight on potatoes (Cardoso et al., unpublished data). The exact distribution of the Alternaria species associated with potato early blight remains unclear.

The symptoms and life cycle of the causal agents of early blight in potato are similar, regardless of the species. The pathogens cause necrotic lesions on leaflets, stems, fruits or tubers and can survive in crop residues, mainly due to the presence of chlamydospores and in other hosts (Patterson, 1991; Rotem, 1994; Rodrigues et al., 2010). The host range is primarily comprised of Solanaceae species, but some members of the Asteraceae and Polygonaceae were also susceptible (Cardoso unpublished data).

In Brazil,A. solani has not been detected in potato fields lately.One plausible hypothesis to explain these facts is the displacement of A. solani by a more aggressive species, in this case, A. grandis. Nevertheless, no epidemiological studies of early blight caused by these species were conducted so far to address this issue.

Even before the reports of other species causing early blight, differences in aggressiveness according to the host of origin of the isolate were inferred in populations identified as A. solani (Bonde, 1929; Henning & Alexander, 1959; Fancelli, 1991; Castro et al., 2000; Martínez et al., 2004).More recently, with the description of the new species, it was found that A. tomatophila was reportedly more virulent than A. solani when inoculated on tomato leaves, petioles, and stems (Frazer and Zitter, 2003), and its specific metabolite profile differs from that of A. solani (Andersen et al., 2008). In turn, A. grandiswas more aggressive than A. solani when inoculated on potato leaves under controlled conditions (Cardoso, 2010). No study was designed to quantify differences in aggressiveness between A. solani and A. grandis in potato under field conditions.

Fungicide application has been the main and most effective measure adopted by growers to control early blight in potatoes, but in view of the recent findings of new species in potato crops in Brazil, there are no reliable data that provide proper support to any adjustments in control strategies, mainly timing fungicide applications. In the past decade growers have reported reduction in the efficacy of fungicides traditionally used to control the disease(Fairchild et al., 2013). The objective of this study was to quantify differences in aggressiveness of A. solani and A. grandis to potato in the field under distinct disease pressures created by two inoculum concentration and two fungicide application schedules.

4.2 MATERIAL AND METHODS

4.2.1 General aspects

This study was conducted in the municipality of Coimbra, Minas Gerais State, 20º 51'24" S and 42º 48'10" W, at 720m above the sea level. Relative humidity and temperature were recorded in automatic data logger (Hobo Pro RH / Temp) installed at 1.5m height, above the canopy of the potato plots, in the experimental field, and precipitation data were registered with Meteorological station Irriplus E1000.

Two experiments were conducted. Experiment 1 ran from 31 October 2012 to 20 January 2012 (exp1) and Experiment 2 from 14 August 2013 to 3 November 2013 (exp2). Both experiments were planted with ‘Ágata’, a cultivar susceptible to early blight (Duarte, 2012). Cultural practices including fertilization (NPK 4-14-8 formulation was used at planting and ammonium sulfate in hilling) were performed according to soil analysis and recommendations for the crop (Filgueira 2000, Ribeiro et al, 1999). Tubers were planted at distances of 0.8m between rows and 0.3 m in a row. The experimental plot measured 3.5 m (length) x 2.4 m (width) and contained 40 plants. The plots were spaced 2.5 m apart. Plots were sprinkler-irrigated throughout the experiment by applying on average 10mm/irrigation event.

Plots were artificially inoculated at 50 days after planting (DAP) with two concentrations of spore suspensions: $5x10^2$ (C1) or 10^4 conidia/mL (C2) of the type isolates of A. solani, collected in Washington USA (Simmons, 2000) or an isolate of A. grandis collected in Bueno Brandão, Minas Gerais state (Rodrigues et al., 2010). The inoculation was performed with a backpack sprayer with a conical tip nozzle, using a pressure-regulating valve to obtain a 30 lb/inch² working pressure. Furthermore, plots were sprayed weekly (P1) or at every three days (P2) with chlorothalonil (Daconil BR) at the rate of 1.3 Kg of a.i. ha^{-1}. The fungicide applications started seven days after inoculation (57 DAP) and extended until plants died.

The mixture of dimethomorph + chlorothalonil (239.7 + 1198.5 g a.i./ha) fungicides was sprayed every five days after emergence to prevent early and late blight infections before the inoculation date. Chlorothalonil sprays were suspended 15 days prior to inoculation.

The experiments were performed in a randomized complete block design, with a factorial treatment design comprised of 2 Alternaria species x 2 inoculum concentrations x 2 fungicide programs. Five blocks were used and 40 experimental units were laid out in the field. Five additional plots were used to monitor the effect of natural inoculation. These plots were not inoculated but were weekly sprayed with chlorothalonil at the same rate as previously described.

4.2.2 Inoculation and assessment of early blight severity

Alternaria spp. were grown in V8 $CaCO_3$ agar (175 mL of V8 juice, 3 g $CaCO_3$, 20 g agar, 1L) the 25 ±2 ^oC, photoperiod of 12 h. For inoculum production, sporulation was induced in 5-day-old colonies. Superficial mycelium was removed with 10mL of sterile distilled water (SDW) and a clean paintbrush. The suspension was discarded. The Petri plates were kept uncovered at 22 ±2 ^oC, for 8h under near-UV light and for 16h in the dark to induce sporulation. After 24h, conidia were removed with 10mL of SDW a clean paintbrush and the suspension concentration was adjusted for 10^4 conidia/mL. Independent spore supensions were prepared for each Alternaria species.

Early blight severity was assessed in 8 plants in the two central rows of the plots (4 in each row) using a descriptive key (Duarte et al. 2013). Severity assessments were made at every three days, starting with the first appearance of symptoms up to plants began to senesce.

4.2.3 Data analysis

Severity data were plotted to obtain disease progress curves. To estimate the disease progress rate (r) for each treatment, the monomolecular, logistic, exponential and Gompertz models were fit to the data. Adjustments were compared and the best model was selected based on the highest determination coefficient (R^2) between observed and predicted values of severity, absence of trend in the residual plots and the lower value of the residual mean square. The severity at the end of the epidemic (Y_{max}) was recorded. The duration of the epidemic in the experiment was considered from the onset of the first symptoms in any treatment up to the point in time when severity reached 100% in any treatment. The area under the disease progress curves (AUDPC) for each treatment was also calculated as previously described (Shaner and Finney, 1977). The Ymax, and AUDPC were compared by 95% confidence interval for each treatment. Estimates of r were compared by the 95% confidence interval of the difference of the estimates (Campbell and Madden, 1990).

4.3 RESULTS

4.3.1 Weather conditions during the experiments

In experiment 1, the temperature during the epidemic ranged from 15.1°C to 32.9°C with an average of 23.8°C (Figure 1C). The average relative humidity was 68.5%. Rainfall was low during the experiment and the cumulative value was 51 mm (Figure 1C).

In experiment 2, the temperature during the epidemic ranged from 7.0°C to 39.7°C with average minimum temperature of 14.0°C and average maximum temperature of 32.3°C and an average temperature of 23.2°C (Fig. 1 D). The average relative humidity was 46.1%. Rainfall was low and little during most part of the experiment, the cumulative precipitation was 3.7 mm (Figure 1C).

4.3.2 Disease progress

In experiments 1 and 2, early blight epidemics started at 7 days after inoculation (DAI), which corresponded to 57 DAP, and the disease was evaluated up to 31 DAI (Figure 1A and B). Large increments in severity values were recorded in the middle of the epidemic, in experiment 1, for plots sprayed weekly with chlorothalonil (P1) inoculated with A. grandis at 10^4 conidia/mL (C2). In experiment 2, all treatmentsreached similar severity values (Figure 1B). In both experiments, severity values until 25 DAI were lower than 10% in plots inoculated with the lower concentration of A. solani and A. grandis. The highest severity values for A. solani were recorded in C2P1 plots which in turn were similar to those recorded in plots inoculated with A. grandis at the low inoculum concentration (C1P1 and C1P2) (Figure 1A and B).

In experiment 1, the logistic model best fit the data for both A. grandis and A. solani. There were no treatment differences regarding r in plots inoculated withA.grandis. The disease progress rate in plots inoculated with A. solani at C1 was slower than in plots at C2 regardless of the spray program (Table 1). The r estimate for A. grandis in C2P1 or P2 plots was higher than those inoculated with A. solani at the same treatment combinations (Table 1).The same result was obtained when comparing the A. grandis C1P2 with A. solani C2P1.

In experiment 1, the Y_{max} values varied in plots inoculated with A. grandis at different concentrations and spray programs. Higher Y_{max} values were recorded in plots inoculated with C2. The Y_{max} did not differ when the treatments inoculated with A. solani were compared. The Y_{max} values in plots inoculated with A. grandis were higher than those in plots inoculated with A. solani in both concentrations under different fungicide spray intervals (Table 1). The average Y_{max} value in C1P2 plots inoculated with A. grandis was higher than that in C2P1 plots inoculated with A. solani.

In experiment 2, the model that best fit the disease progress was the exponential model. The progress rate in different treatmentsinoculated withA.grandisdid not differamong themselves. The progress rate in C2 plots (r = 0.18, r = 0.18, for P1 and P2, respectively) inoculated with A. solani was higher than in C1 plots (r = 0.12, r = 0.10, for P1 and P2, respectively), in both spray programs. The r estimate for A. grandis in C1P1 or P2 plots was higher than those inoculated with A. solani at the same treatment combinations (Table 1).

In experiment 2, the Y_{max} values varied in plots inoculated with A. grandis at different concentrations and spray programs.Higher Y_{max} values were recorded in plots inoculated with C2 and in P1 (Table 1). The Y_{max} values forA. solani in C2P2 plots were higher than in C1P2 plots and lower than in C2P1 (Table 1).The Y_{max} values in plots inoculated with A. grandis were higher than those in plots inoculated with A. solani in both concentrations under different fungicide spray intervals (Table 1). The average Y_{max} value in C1P2 plots inoculated with A. grandis was higher than that in C2P1 plots inoculated with A. solani.

In the five additional plots used to monitor the effect of natural inoculationthere no symptoms of the disease.

4.3.3 Area under the disease progress curve (AUDPC) of early blight caused by A. solani and A. grandis and under different fungicide application programs

In experiment 1, the AUDPC values for plots inoculated withA. grandis did not differ between the spray programs, regardless of inoculum concentration. However, in P1 plots, the AUDPC for C2 was higher than C1. The same happen for P2 plots (Table 1). In plots inoculated with A. solani the AUDPC in C2P1 plots was higher than in C1P1 plots. Higher values of AUDPC were obtained in P1 (Table 1). The AUDPC values for A. grandis in C1P1 or P2 plots were higher than those for A. solani. The same happen for C2P1 or P2 plots (Table 1). The AUDPC values in C1P2 plots did not differ from of C2P1 plots inoculated with A. solani. The AUDPC value in C1P2 plots inoculated with A. grandis was higher than that in C2P1 plots inoculated with A. solani.

In the experiment 2, AUDPC in C1 plots inoculated with A. grandis was similar in the two spray programs. Highest AUDPC values were recorded for A. grandis in C2P1 plots (Table 1). AUDPC in C1 plots inoculated with A. solani was similar in the two spray programs. There was a significant difference for A. solani in P2 plots when C1 and C2 were compared. Higher AUDPC values were calculated for the C2P1 plots.

The AUDPC values in plots inoculated with A. grandis were higher than those in plots inoculated with A. solani in both concentrations under different fungicide spray intervals (Table 1). The AUDPC values C1P2 plots with A. grandis did not differ from C2P1 plots inoculated with A. solani.

4.4 DISCUSSION

Until the beginning of the 21st century only A. solani was known as the causing agent of early blight in potato. In Brazil, the first report of the involvement A.grandis causing potato early blight was in 2010 (Rodrigues et al., 2010). Morphological and molecular comparisons of representative strains enabled to sortthe two species: A. solani and A. grandis (Rodrigues et al., 2010; Cardoso, unpublished data).We have no information concerning research on aggressiveness of these fungi. This is the first comparative study of early blight in order to understand the existing pathogen species-host relationships. The general results reported here support the hypothesis that there are differences in aggressiveness between A. grandis and A. solani.

Sufficient heterogeneity in pathogenic characters (aggressiveness and substrate preferences) havebeen observed in A. solani populations (Henning & Alexander, 1959; Weir et al. 1998; Martinez et al. 2004; Weber and Halterman 2012). In relation the new species described, that cause early blight, recent study concluded that A. tomatophila was able to infect potato leaf fragments, but with sufficiently lesser aggressiveness than for tomato and A. solani otherwise (Gannibal et al, 2014). In Brazil, A. tomatophila was less aggressive in potato than in tomatounder the same planting conditions (Cardoso, unpublished data).Thus, the most aggressive species on potato are A. solani and A. grandis.For A. grandis, a recentstudy showed that isolates of this speciescause greater lesion area on potato than A. solani (Cardoso, 2010).The environment, the traits of the pathogen, and the traits of the host can affect disease intensity (Grönberg et al., 2012). In the present study, environmental and host factors were common for A. grandis and A. solani, therefore the variations in disease level were assumed to be largely pathogen-dependent.

The detection of differences in aggressiveness of A. grandis and A. solani was supported when comparing the disease progress rates of epidemics caused by each species. Epidemics caused by A. grandis achieved higher progress rates that those for A. solani in experiment 1, when plots were inoculated with high inoculum concentration. Even at the lower inoculum concentrations and under more intensive fungicide spray program the progress rate in plots inoculated with A. grandis were higher than in plots inoculated with A. solani.In experiment 2, the weather conditions were more limiting to the progress of early blight, with greater temperature fluctuation and low relative humidity recorded during the experiment. However even under low inoculum concentration, for both species,A. grandis epidemics had higher progress rates that A. solani.

Early blight epidemic caused by A. grandis was very severe and some plots inoculated with higher inoculum concentration reached 100% severity.Overall, the epidemiological variables maximum severity (Y_{max}) and AUDPC showed similar results regarding the aggressiveness of Alternaria species.The lower value obtained for Y_{max} for A. grandis (38%), when inoculated at low concentration was higherthan the Y_{max} obtained when A. solani was inoculated at a higher concentration. AUDPC values were higher for A. grandis in all treatments except in experiment 2 when in low concentration it did not differ from A. solani in the higher concentration. Collectively, all epidemiological variables support the existence of differences in aggressiveness between A. grandis and A. solani.

Differences in epidemic of the early blight obtained for different concentrations of inoculum can be explained by the compensationhypothesis proposed by Rotem (1978). The highest concentration of inoculum of pathogens compensated low relative humidity recorded in both experiments allowing enhanced disease progression compared to lower concentration.Epidemics of early blight can occur at any season (Batista et al., 2006). However, the severity of the disease is greater under conditions of frequent rainfall and temperatures between 25 and 30ºC (Andrade, 1997; Salustiano, 2000). Regardless of inoculum concentrations, even under low humidity, early blight epidemic caused by A. grandis had higher severitythan A. solani. Evaluating the effect of different periods of leaf wetness (PLW) in the infection frequency (IF) of A. grandis and A. solani on potato, Cardoso (2010) showed that when the PLW was 2 h, the average value of IF of A. grandis was higher than that found for A. solani. Vloutoglou & Kalogerakis (2000) found that there was no development of A. solani on tomato in the absence of leaf wetness or when the PLW was less than 4 h. Thus, if the conditions for infection are limiting, especially PLW, it is believed that the spores of A. grandis germinate and infect more quickly than other species; which would contribute to the higher prevalence of this species in the area. Therefore, early blight can occur even in the driest seasons.

Differential responses in terms of pathogen aggressiveness may be less detectable under optimal environmental conditions (Pariaud et al., 2009). Previous studies under greenhouse showed that development of disease caused by different Alternaria species are favored by the same temperature range and PLW (Cardoso, 2010). For this reason, the current study was designed in order to detect differences between species under limiting conditions.

Differences in aggressiveness detected between species is an important step towards the understanding of the dynamics of the disease in potato, particularly to explain the putative displacement of A. solani in Brazil. Additionally, if the main species causing early blight in potato is A. grandis and if A. solani is not present in the country then breeding programs aimed at developing resistant varieties need to be adjusted. From an epidemiological standpoint, the pathogenic variability has direct implications for disease management. The more aggressive isolates of a pathogen also means higher use of fungicides (Kato et al. 1997). Several growers have claimed that even with the intensive application of fungicides already registered, control of early blight is not performing efficiently. Thus it is necessary to have reliable data to support adjustments in control strategies, mainly in timing fungicide application.

In summary, there is difference in aggressiveness between the species that can cause early blight in potatoes. A. grandis was more aggressive than A. solani even under unfavorable conditions such as low concentration of inoculum and in drier weather. Studies should be conducted to improve management strategies of the disease in the field now considering the high prevalence of A. grandis in potato crops in Brazil

REFERENCES

Andersen B, Dongo A, Pryor BM, 2008. Secondary metabolite profiling of Alternariadauci, A. porri, A. solani, and A. tomatophila.Mycological Research**112**, 241-250.

Andrade DFAA, 1997. Previsão e controle químico da pinta preta (Alternariasolani Sorauer), sob dois sistemas de condução do tomateiro (Lycopersiconesculentum Mill.). Viçosa, Brasil: Universidade Federal de Viçosa, dissertação de Mestrado.

Batista DC, Lima MA, Haddad F, Maffia LA, Mizubuti, ESG, 2006. Validation of decision support systems for tomato early blight and potato late blight, under Brazilian conditions.Crop Protection**25**, 664-670.

Bonde R, 1929. Physiological strains of Alternaria solani. Phytopathology**19**, 533-548.

Cardoso CR, 2010. Agressividade de Alternariatomatophila, A. grandis e A. solani em batateira e tomateiro. Viçosa, Brasil: Universidade Federal de Viçosa, dissertação de mestrado.

Castro MEA, Zambolim L, Chaves GM, Cruz, CD, Matsuoka K, 2000. Variabilidade patogênica de Alternariasolani, agente causal da pinta-preta do tomateiro. Summa Phytopathologica**8**, 24-28.

Duarte HSS, 2012. Requeima e pinta preta em batateira: resistência e epidemiologia comparativa. Viçosa, Brasil: Universidade Federal de Viçosa, tese de doutorado.

Duarte HSS, Zambolim L, Capucho AS, Nogueira Junior AF, Rosado AWC, Cardoso CR, Paul PA, Mizubuti ESG, 2013. Development and validation of a diagrammatic scale for assessment of potato early blight.Europa Journal of Plant Pathology**137**, 249-257.

Fairchild KL, Miles TD, Wharton PS, 2013. Assessing fungicide resistance in populations of Alternaria in Idaho potato fields.Crop Protection**49**, 31-39.

Fancelli MI, 1991. Comparação patogênica, cultural, serológica e eletroforética entre isolados de Alternariasolani do tomate e da batata e variabilidade patogênica de A. solani f. sp. lycopersici N. F. Piracicaba, São Paulo, Escola Superior de Agricultura Luiz de Queiroz, tese de doutorado.

Filgueira FAR, 2000. Novo manual de Olericultura: Agrotecnologia moderna na produção e comercialização de hortaliças. 2. Ed. Viçosa, BR: Editora UFV, 402p.

Frazer JT, Zitter TA, 2003. Two species of Alternaria cause early blight of potato, (Solanumtuberosum) and tomato (Lycopersiconesculentum). Phytopathology (suppl. 3), 93.

Gannibal PB, Orina AS, Mironenko NV, Levitin MM, 2014. Differentiation of the closely related species, Alternariasolani and A. tomatophila, by molecular and morphological features and aggressiveness.European Journal of Plant Pathology**139**, 609-623.

Grönberg L, Andersson B, Yuen J, 2012. Can weed hosts increase aggressiveness of Phytophthorainfestans on potato? Phytopathology**102**, 429-433.

Guenthner JF, Wiese MV, Pavlista AD, Sieczka JB, Wyman J, 1999. Assessment of pesticide use in the US potato industry.American Journal of Potato Research**76**, 25-29.

Henning RG, Alexander LJ, 1959. Evidence of existence of physiologic races of Alternariasolani.Plant Disease Reporter**43**, 298-308.

Jones JB, Jones JP, Stall RE., Zitter, TA, 1991. Compendium of Tomato Diseases. St. Paul. APS Press.

Kato M, Mizubuti ESG, Goodwin SB, Fry WE, 1997. Sensitivity to protectant fungicides and pathogenic fitness of clonal lineages of Phytophthorainfestans in the United States.Phytopathology**87**, 973-978.

Kumar V, Haldar S, Pandey KK, Singh RP, Singh AK, Singh PC, 2008. Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of Alternariasolani in India. World Journal Microbiol Biotechnol**24**,1003–1009.

Martínez SP, Snowdon R, Pons-Kuhnemann J, 2004. Variability of Cuban and international populations of Alternariasolani from different hosts and localities: AFLP genetic analysis. European Journal Plant Pathology**110**, 399-409.

McDonald BA, Linde C, 2002. The population genetics of plant pathogens and breeding strategies for durable resistance.Euphytica**124**, 163-180.

Neergaard P, 1945. Danish Species of Alternaria and Stemphylium. London: Oxford Univ. Press. 560 p.

Pariaud B, Ravigne V, Halkett F, Goyeau H, Carlier J, Lannou C, 2009. Aggressiveness and its role in the adaptation of plant pathogens.Plant Pathology**58**, 409–24.

Patterson CL, 1991. Importance of chlamydospores as primary inoculum of Alternariasolani, incitant of collar rot and early blight on tomato.Plant disease**75**, 274-278.

Reifschneider FJB, Furumoto O, Filgueira FAR, 1984. Illustrated key for the evaluation of early blight of potatoes. Plant Protection Bulletin**32**, 91-94.

Ribeiro AC, Guimarães PTG, Alvarez VH, 1999. Recomendações para o uso de corretivos e fertilizantes em Minas Gerais – 5ª Aproximação. Viçosa: CFSEMG, 359 p.

Rodrigues TTMS, 2009. Morphological, molecular characterization, and inference about recombination, for species of Alternaria related to early blight of potato and tomato. Viçosa, Brasil: Universidade Federal de Viçosa, tese de doutorado.

Rodrigues TTMS, Berbee ML, Simmons EG, Cardoso CR, Reis A, Maffia LA, Mizubuti ESG, 2010. First report of Alternariatomatophila and A. grandis causing early blight on tomato and potato in Brazil.New Disease Reports**22**, 28.

Rotem, J. 1978. Climatic and weather influences on epidemic. In: HORSFALL, J. G. & COWLING, E.B. eds. Plant disease an advanced treatise. New York, Academic Press. 397 p.

Rotem J, 1994. The genus Alternaria.Biology, epidemiology, and pathogenicity.APS Press, St. Paul. 326.

Salustiano ME, 2000. Progresso da pinta-preta do tomateiro em cultivares de tomate em diferentes épocas de plantio. Viçosa, Brasil: Universidade Federal de Viçosa, tese de doutorado.

Shaner G, Finney RE, 1977.The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat.Phytopathology**67**,1051-1056.

Shuman JL, Christ BJ, 2005. Integrating a host-resistance factor into the Fast system to forecast early blight of potato.American Journal of Potato Research**82**, 9-19.

Simmonds NW, 1971. The potential of potatoes in the tropics.Tropical Agriculture, 48, 291-299.

Simmons EG, 2000.Alternariathemes and variations (244-286) species on Solanaceae.Mycotaxon**75**,1-115.

Suassuna ND, Maffia LA, Mizubuti ESG, 2004.Aggressiveness and host specificity of Brazilian isolates of Phytophthorainfestans. Plant Pathology**53**, 405-413.

Van Der Walls JE, Korsten L, Slippers B, 2004. Genetic diversity among Alternariasolani isolates from potatoes in South Africa. Plant Disease**88**, 959-964.

Vloutoglou I, Kalogerakis SN, 2000. Effectes of inoculum concentration, wetness duration and plant age on development of early blight (Alternariasolani) and on shedding of leaves in tomato plants. Plant Pathology **49**, 339-345.

Weber B, Halterman DA, 2012. Analysis of genetic and pathogenic variation of Alternariasolani from a potato production region.European Journal of Plant Pathology**134**, 847–858.

Weir TL, Huff DR, Christ BJ, Romaine CP, 1998. RAPD-PCR analysis of genetic variation among isolates of Alternariasolani and Alternariaalternata from potato and tomato. Mycologia**90**, 813-821.

TABLE

Table 1.The severity at the end of the epidemic (Y_{max}), area under disease progress curve (AUDPC) and disease progress rate (r) quantified for early blight epidemic caused by Alternaria solani and A. grandis when inoculated to potato and confidence interval (CI) for each variable (in parenthesis) for experiments 1 and 2.For both experiments, concentration 1=$5x10^2$ conidia/ml, concentration 2= 10^4 conidia/ml, spray 1= weekly application of chlorotalonil and spray 2= application at every three days.

	EXPERIMENT2012					
Species	**Concentration1**					
	Spray1			**Spray2**		
	Y_{max}	**AUDPC**	**rlog**[a]	**Y_{max}**	**AUDPC**	**rlog**
Alternariagrandis	38.80 (33.96 - 43.64) [C]	188.72 (173.17 - 204.27)	0.28	35.40 (29.74 - 41.06)	158.54 (137.11 - 179.99)	0.28
Alternariasolani	9.8 (4.84 – 14.77)	61.31 (58.52 - 64.10)	0.16	7.80 (4.18 - 14.77)	49.14 (42.23 – 56.04)	0.16
	Concentration 2					
	Spray 1			**Spray 2**		
	Y_{max}	**AUDPC**	**rlog**	**Y_{max}**	**AUDPC**	**rlog**
Alternariagrandis	79.60 (64.66 - 94.54)	496.98 (323.93 - 670.04)	0.29	65.0 (49.32 - 80.68)	389.39 (269.55 - 509.22)	0.27
Alternariasolani	26.80 (24.11 - 29.49)	160.62 (153.16 - 167.98)	0.20	15.0 (9.11 - 20.88)	78.75 (72.46 - 85.03)	0.20

Table 1 Cont.

EXPERIMENT2013						
Species	**Concentration1**					
	Spray1			**Spray2**		
	Y_{max}	**AUDPC**	**Rexp**[b]	**Y_{max}**	**AUDPC**	**rexp**
Alternariagrandis	31.20 (24.67 - 37.73)	164.31 (143.47 – 185.15)	0.20	32.20 (24.53 - 39.87)	153.35 (132.36–174.33)	0.23
Alternariasolani	12.3 (9.09 - 15.51)	89.49 (68.83 - 110.15)	0.12	6.666 (3.74 - 9.46)	57.44 (40.46–74.42)	0.10
	Concentration 2					
	Spray 1			**Spray 2**		
	Y_{max}	**AUDPC**	**rexp**	**Y_{max}**	**AUDPC**	**rexp**
Alternaria grandis	89.80 (85.38 - 94.22)	447.48 (419.78–475.18)	0.20	49.40 (43.95 - 54.85)	257.53 (230.03–285.03)	0.20
Alternariasolani	26.20 (24.16 - 28.24)	142.25 (126.70 - 157.81)	0.18	16.10 (11.49 - 20.71)	91.59 (82.75–100.43)	0.18

adisease progress rate logistic model; b disease progress rate exponential model; c95 % confidence interval

Table 2- Confidence interval at 95% probability of the b2 estimate, for comparisons of progress rate of early blight epidemics caused by Alternaria grandis (Ag)and A. solani (As)on potato. Plots were inoculated with $5x10^2$ (C1) or 10^4 conidia/mL (C2) and subjected to either weekly application of chlorothalonil (P1) or at every three days (P2).

Comparisons	**Experiment 1**	**Experiment 2**
	Rates (logistic)	**Rates (exponential)**
AgC1P1/ AgC1P2	(-0.0327 - 0.0607)	(-0.0140 - 0.0686)
AgC1P1/ AgC2P1	(-0.0471 - 0.0763)	(-0.0256 - 0.0316)
AgC1P2/ AgC2P2	(-0.0217 - 0.0887)	(-0.0012 - 0.0688)
AgC2P1/ AgC2P2	(-0.0342 - 0.1024)	(-0.0087 - 0.0277)
AsC1P1/ AsC1P2	(-0.0384 - 0.0384)	(-0.0118 - 0.0508)
AsC1P1/ AsC2P1	(0.0039 - 0.0806)*	(0.0396 - 0.0932)*
AsC1P2/ AsC2P2	(0.0368 - 0.0922)*	(0.0396 - 0.1063)*
AsC2P1/ AsC2P2	(-0.0277 - 0.0277)	(-0.0163 - 0.0423)
Alternaria grandis X Alternaria solani		
AgC1P1/ AsC1P1	(-0.0131 - 0.0815)	(0.0445 - 0.1143)*
AgC1P2/ AsC1P2	(-0.0339 - 0.0859)	(0.0880 - 0.1645)*
AgC2P1/ AsC2P1	(0.0344 - 0.1478)*	(-0.0017 - 0.0337)
AgC2P2/ AsC2P2	(0.0099 - 0.1041)*	(-0.0101 - 0.0491)
AsC2P1/ AgC1P1	(0.0388 - 0.1142)*	(-0.0187 - 0.0447)

* The interval did not include zero, rates of progress of early blight are statistically different at 5% probability (we reject H0: A = 0).

FIGURE

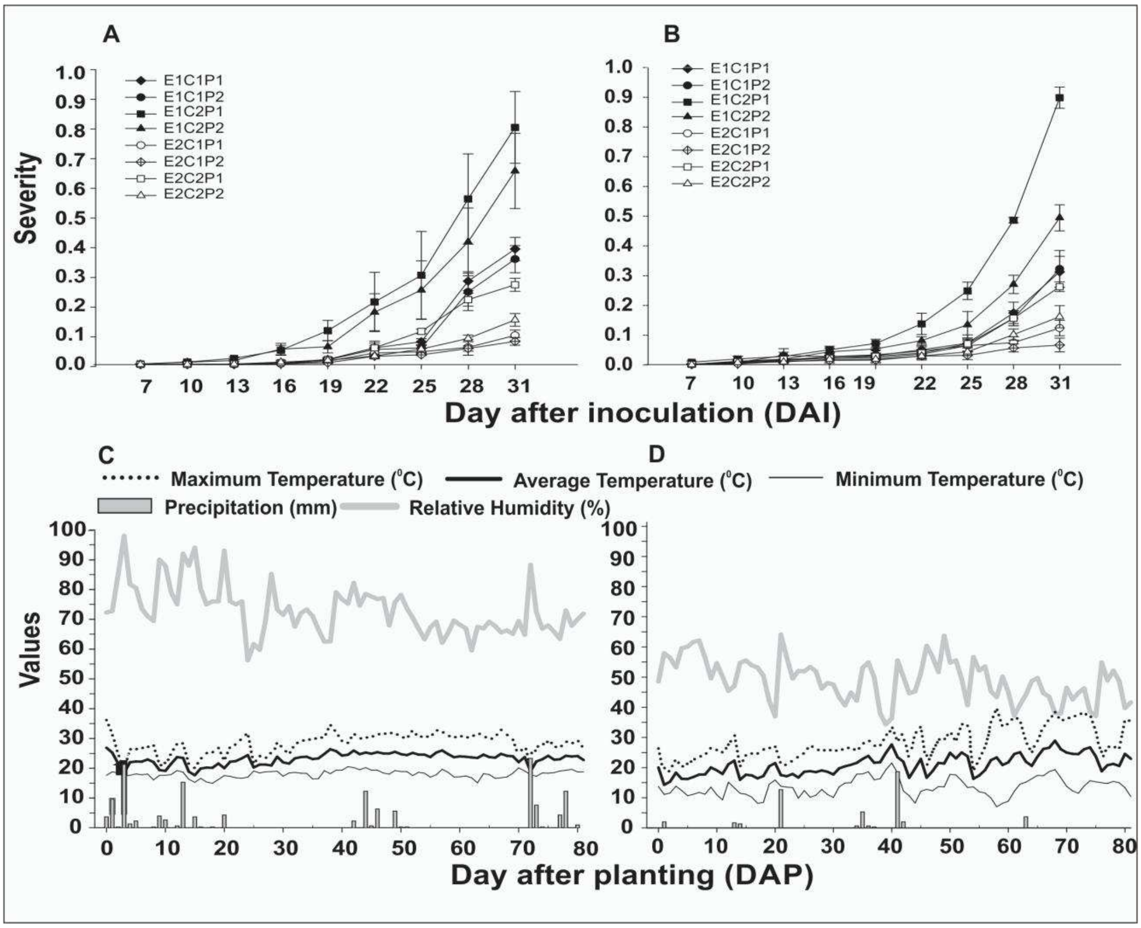


Figure 1 – Early blight progress curves on potato in experiment 1 (A) and 2 (B). For both experiments: E1: Alternaria grandis; E2: A. solani; C1: concentration of 5x10^2 conidia/mL; C2: concentration of 10^4 conidia/mL; P1: weekly application of chlorothalonil and P2: application of chlorothalonil every three days. Vertical bars represent the standard deviation of the mean. Weather conditions recorded during the experiment 1 (C) and experiment 2 (D)

CONCLUSÃO GERAL

1. Sequências de DNA das regiões desidrogenase gliceraldeído-3-fosfato, calmodulina, actina, membrana Plasma ATPase permitiram distinguir as três espécies mais prevalentes em batateira e tomateiro: A. solani, A. grandis e A. tomatophila.
2. Análise de highresolutionmelting (HRM) baseada na RNA polimerase II subunidade beta e os genes de proteínas de manutenção mini-cromossomos foi uma ferramenta útil e rápida na identificação e diferenciação de Alternaria spp. associadas a pinta preta.
3. Ageratum conyzoides, Erigeron bonariensis, S. capsicoides, S. lycocarpum, S. viarum e Rumex acetosa foram suscetíveis a Alternaria spp.
4. Desenvolvimento da pinta preta em pimentão e pimenta variou de acordo com a espécie do patógeno.
5. As espécies do patógeno em seus hospedeiros supostamente de origem são mais agressivas.
6. A cultura hospedeira predominante em cada região afetou a intensidade da epidemia.
7. Em geral, A. grandis tem maior agressividade que A. solani, quando ambas as espécies são inoculadas em batateira.