**COSMA RAISSA BEZERRA DO NASCIMENTO**

# ETIOLOGICAL CHARACTERIZATION OF SMALL-SPORED *Alternaria* AND EPIDEMIOLOGY OF POTATO BROWN SPOT IN BRAZIL

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

Adviser: Eduardo Seiti Gomide Mizubuti

Co-adviser: Thaís Carolina da S. Dal'Sasso

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Assent:

Cosma Raissa Bezerra do Nascimento

Cosma Raissa Bezerra do Nascimento
Author

Eduardo Seiti Gomide Mizubuti
Adviser

*With longing, I dedicate this dissertation to my mother, Maria Liêda do Nascimento, in memory, for having encouraged me to seek new paths and face challenges with my head held high. Her love and dedication will forever prevail in our family.*

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# ABSTRACT

NASCIMENTO, Cosma Raissa Bezerra do, M.Sc., Universidade Federal de Viçosa, February, 2023. **Etiological characterization of small-spored *Alternaria* and epidemiology of potato brown spot in Brazil**. Adviser: Eduardo Seiti Gomide Mizubuti. Co-adviser: Thaís Carolina da Silva Dal'Sasso.

This dissertation investigates potato brown spot, a disease that can significantly impact potato quality and yield in various parts of the world. The study focuses on small-spored *Alternaria* species recovered from potato leaf lesions collected in the major Brazilian potato-producing states. The isolates were characterized using morphological and molecular approaches and tested for pathogenicity. More than one small-spored *Alternaria* species can cause brown spot in potato, including *A. arborescens* and a new *Alternaria* sp., that was identified for the first time as a causal agent of the disease. Furthermore, the study examines the effects of temperature on the basic epidemiological/ecological variables associated with small-spored *Alternaria* species. The results demonstrate that temperature affects mycelial growth, sporulation, and germination of small-spored *Alternaria* isolates from the main potato-producing regions. The highest values for most phenotypic traits evaluated were in the range of 25 and 30 °C, and growth patterns varied among small-spored species. The severity of brown spot may vary according to the distribution of the small-spored *Alternaria* species, which highlights the importance of understanding the ecological characteristics of the pathogen for disease management. Overall, this dissertation provides valuable insights into the factors influencing the occurrence and severity of potato brown spot and identifies potential strategies for its control.

Keywords: *Solanum tuberosum*. Identification. Pathogenicity. Epidemiological components.

# RESUMO

NASCIMENTO, Cosma Raissa Bezerra do, M.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Caracterização etiológica de *Alternaria* de esporos pequenos e epidemiologia da mancha marrom em batata no Brasil.** Orientador: Eduardo Seiti Gomide Mizubuti. Coorientadora: Thaís Carolina da Silva Dal'Sasso.

Esta dissertação investiga a mancha marrom da batata, uma doença que pode afetar significativamente a qualidade e o rendimento da batata em várias partes do mundo. O estudo se concentra em espécies de *Alternaria* de pequenos esporos recuperadas de lesões foliares de batata nos principais estados brasileiros produtores de batata. Os isolados foram caracterizados usando abordagens morfológicas e moleculares e testados quanto à patogenicidade. Mais de uma espécie de *Alternaria* de esporos pequenos pode causar mancha marrom na batata, incluindo *A. arborescens* e uma nova espécie de *Alternaria* sp., identificadas pela primeira vez como agentes causais da doença. Além disso, o estudo examina os efeitos da temperatura nas variáveis epidemiológicas/ecológicas básicas associadas a espécies de *Alternaria* de esporos pequenos. Os resultados demonstram que a temperatura afeta o crescimento micelial, a esporulação e a germinação de isolados de *Alternaria* de esporos pequenos das principais regiões produtoras de batata. Os maiores valores para a maioria das características fenotípicas avaliadas foram na faixa de 25 e 30 °C, e os padrões de crescimento variaram entre as espécies de esporos pequenos. A severidade da mancha marrom pode variar de acordo com a distribuição das espécies de *Alternaria* de esporos pequenos, o que destaca a importância de compreender as características ecológicas do patógeno para o manejo da doença. No geral, esta dissertação fornece informações valiosas sobre os fatores que influenciam a ocorrência e a severidade da mancha marrom da batata e identifica estratégias potenciais para seu controle.

Palavras-chave: *Solanum tuberosum*. Identificação. Patogenicidade. Componentes epidemiológicos.

# SUMMARY

## General introduction

Species of the genus *Alternaria* stand out worldwide as major potato foliar fungal pathogens (Landschoot et al. 2017a). Two important diseases caused by *Alternaria* spp. have had major impacts on potato production and had sparked the attention of growers, extension agents, and potato pathologists worldwide: early blight, caused by large-spored species of *Alternaria*, and brown spot, caused by small-spored species of *Alternaria* (Vandecasteele et al. 2018). The main distinctions between these diseases are manifested in the specificities of the infections, including the evolution of symptoms and their progression (Soleimani and Kirk 2011; Tymon at al. 2016a). Nevertheless, brown spot is substantially less studied than early blight and in Brazil there is almost no information about this disease.

Brown spot epidemics are polycyclic and high disease progress rates can be recorded if environmental conditions are favorable (Meng et al. 2015a). The main economic impact of brown-spot is assumed to result from the infection on mature leaf tissue when the physiological demands for tuber filling are at a critical stage and this reflects in high yield loss (Taheri 2019; Jones and Perez 2022). In addition to reducing tuber quality, losses can range from 70 to 80% in net tuber weight (Soleimani and Kirk 2012).

In the last decade, different species of small-spored *Alternaria* have been associated with brown spot, such as *A. alternata*, *A. tenuissima*, *A. dumosa*, *A. interrupta*, *A. arborescens*, *A. longipes*, *A. infectoria*, *A. telliensis*, and *A. arbusti*. The disease has been reported to occur in different countries, such as Iran (Taheri 2019), Russia (Orina et al. 2011; Kokaeva et al. 2018), Pakistan (Shoaib et al. 2014), China (Zheng et al. 2015), United States (Tymon et al. 2016b), Belgium (Landschoot et al. 2017b; Vandecasteele et al. 2018), Algeria (Bessadat et al. 2020), India (Lingwal et al. 2022), and South Korea (Choi et al. 2022). In Brazil, except for the first report of brown spot on potatoes in 1994 (Boiteux and Reifschneider 1994), no detailed study on the occurrence (surveys), etiology, epidemiology, or disease control was found, despite the growing number of complaints and concerns by potato growers countrywide (Nascimento & Mizubuti, unpublished data).

The accurate identification and evaluation of pathogenicity of small-spored *Alternaria* species is crucial to expand the knowledge about the brown spot. Small-spored *Alternaria* spp. appear to have a versatile lifestyle and, consequently, a versatile ecological niche since many species can be saprophytes associated with different substrates, including seeds, plants, agricultural products, animals, soil. These species are also present in the air, causing respiratory diseases. (Woudenberg et al. 2013). Other species such as *A. longipes*, *A. mali*, *A. gaisen*, and *A. arborescens* are plant pathogens, but in many cases the same pathogenic species is also reported as saprophyte (Woudenberg et al. 2015). Furthermore, large-spored and small-spored *Alternaria* species can co-occur in the same lesion and symptoms under field conditions can be very similar (Agrios 2005), raising objections to the ability of small-spored species to infect potato leaves by themselves. Rigorous species identification and pathogenicity tests need to be conducted in order to ascertain a species as an etiological agent of brown spot.

Different species of small-spored *Alternaria* can respond differentially to the environmental variables potentially leading to variation of the pathogen populations by location. Furthermore, climate change has increased the occurrence of many pathogens and influenced the impact in areas where their incidence was previously low (Escuredo et al. 2019). Studies evaluating sporulation, germination, and mycelial growth rates and their relationships with ecological factors were carried out to characterize the differences between *Alternaria* species (Zhu and Xiao 2015; Kumar Bais et al. 2019; Wang et al. 2021; Sandoval-Contreras et al. 2021; Parvin et al. 2021; Camiletti et al. 2022). Therefore, it is necessary to test whether the small-spored *Alternaria* species associated with potato brown spot in Brazil differ from each other in terms of ecological requirements.

In the first chapter, an extensive study was conducted with the objective of identifying small-spored *Alternaria* spp. associated with brown spot in different geographic areas in Brazil. In the second chapter, the effect of temperature on basic epidemiological variables was assessed to test for any differential response of the pathogen species to this ecological factor.

## Brief literature review

### Overview of Potato brown spot

Potato (*Solanum tuberosum* L.) is the main non-cereal food crop in the world and an important staple food widely consumed by the population (Embrapa 2016; Zaheer and Akhtar 2016; Hong et al. 2017). In 2019, the world potato production was estimated to be around 370 million tons and China is the largest producer, followed by India and Russia (FAO 2021). In Brazil, 3.8 million tons of potatoes were produced in 116,420.00 ha in 2021, reaching an average yield of 33.09 tons/ha (IBGE - Instituto Brasileiro de Geografia e Estatística 2021). These numbers make potato as the most widely cultivated vegetable crop in terms of acreages.

Cultivation of potatoes is challenging as yield is threatened by several plant pathogens that cause high losses (Dias and Iamauti 2005; Filgueira 2008; Zhang et al. 2020). The species of the genus *Alternaria* are the most important fungal foliar pathogens of potatoes (Landschoot et al. 2017a). Morphologically, *Alternaria* spp. can be divided into large-spored and small-spored species, causing early blight and potato brown spot, respectively (Simmons 2007). The brown spot has gained prominence in recent years as a result of climate change caused by global warming, which contributes to the expansion of the pathogen into new hosts and also widening its distribution in potato growing regions throughout the world (Kirk and Wharton 2012; Dube 2014; Ahmed 2017). Losses resulting from the disease can reach, on average, around 20%, but may reach up to 80% in severe cases (Soleimani and Kirk 2012).

Brown spot epidemics can develop under a wide range of weather conditions. Temperatures between 20 and 30 °C, continuous high humidity, or alternating wet and dry periods favor sporulation, spore germination, and infection of plant tissues (Kirk and Wharton 2012; Soleimani and Kirk 2012). However, brown spot severity depends mainly on the frequency of wetting of the foliage by rain, fog, dew, or irrigation, the nutritional status of the foliage, and on the susceptibility of the host (Stevenson et al. 2001; Vandecasteele et al. 2018). The increase of host susceptibility to brown spot is associated with tissue age, a characteristic component of many pathosystems involving *Alternaria* species. Together with other *Alternaria* spp., the small-spored species are considered disease-causing agents of aging plants (Rotem 1994). The preference for old plants may be related to the infection strategies of small-spored *Alternaria* species, to the favorable environmental conditions, as well as to the availability of nutritional resources in the aged tissue. For *A. alternata*, field evaluations have shown that epidemics develop at high progress rates as the season progresses and leaves grow older (Leiminger et al. 2015; Lingwal et al. 2022). The survival of *Alternaria* species is guaranteed by the saprophytic characteristic and the ability to remain viable in the soil as conidia, chlamydospores, and mycelium or in decomposing plant tissues (Rotem 1994; Kokaeva et al. 2018). Furthermore, the pathogen is asexually propagated and causes polycyclic diseases. Conidia are dispersed by wind and rain splash, serving as both the primary and secondary inocula for new disease cycles (Ding et al. 2019a). The disease begins with spores landing on the leaves of susceptible plants and, after germination, the germ tube penetrates the tissues directly through the epidermis, or through openings such as stomata, or wounds (Kirk and Wharton 2012).

The symptoms of brown spot are characterized by small, irregular, circular lesions on the leaves, dark brown in color, approximately 10 mm in diameter. The lesions can group and form large necrotic lesions with or without a yellow halo, which coalesce and significantly damage the leaf blade. Dark-colored superficial elongated lesions can also form on the stems and petioles (Stevenson et al. 2001; Kirk and Wharton 2012; Leiminger et al. 2015; Lingwal et al. 2022). In young leaves, the symptoms can be confounded with those of early blight, which at early stages, are also characterized by small circular lesions. However, brown spot lesions do not have concentric rings (van der Waals et al. 2011; Soleimani and Kirk 2012; Zhang et al. 2020).

## Etiology

*Alternaria alternata* (Fr.) Keissl is an ubiquitous small-spored species that can be saprophyte or pathogen (Woudenberg et al. 2015). This is the predominantly reported species causing brown spot in potato crops in different countries (Droby et al. 1984; Thomma 2003; Tymon et al. 2016a). *Alternaria alternata* was not considered a potato pathogen until 1984, when it was isolated from lesions and Koch's postulates were fulfilled on intact and detached potato leaves (Droby et al. 1984). In recent years, in addition to *A. alternata*, different small-spored species such as *A. tenuissima*, *A. dumosa*, *A. interrupta*, *A. arborescens*, *A. longipes*, *A. infectoria*, *A. telliensis* and *A. arbusti* have been reported to cause brown spot in potato in different countries, such as Iran (Taheri 2019), Russia (Orina et al. 2011; Kokaeva et al. 2018), Pakistan (Shoaib et al. 2014), China (Zheng et al. 2015), United States (Tymon et al. 2016b), Belgium (Landschoot et al. 2017b; Vandecasteele et al. 2018), Algeria (Bessadat et al. 2020), India (Lingwal et al. 2022), and South Korea (Choi et al. 2022). However, using whole genome sequencing, it was shown that *A. alternata*, *A. tenuissima*, *A. dumosa*, and *A. interrupta* do not correspond to distinct phylogenetic species, therefore, they should be synonymized under *A. alternata* (Woudenberg et al. 2015).

*Alternaria alternata* was first reported causing brown spot on potato in Brazil in 1994 (Boiteux and Reifschneider 1994). The pathogen was isolated from potato leaves and Koch's postulates were fulfilled. However, since then, no studies have been conducted to assess the role of *A*. *alternata* or other small-spored species in the potato crop in the country. Curiously, even with the increasing number of complaints by growers of a "different kind of early blight', no additional studies were conducted.

Brown spot can be difficult to diagnose in the field, as large-spored and small-spored *Alternaria* species have many similar biological and epidemiological characteristics. *Alternaria* pathogens can coexist in the same infected leaf, in addition to being able to cause similar symptoms in the early stages of infection (Leiminger et al. 2015; Bessadat et al. 2017; Ding et al. 2019a, 2019b). The possibility of both species being isolated from the same lesions generates disagreements regarding the ability of the small-spored species to infect potato leaves (Stammler et al. 2014; Tymon et al. 2016a; Bessadat et al. 2017). Rotem (1994) reported *A. alternata* as a weak pathogen, occurring in up to 25% of lesions initiated by *A. solani*. Other studies consider *A*. *alternata* as a secondary invader that saprophytically grows on lesions and, therefore, is often isolated from leaf spots (Stammler et al. 2014); as an endophytic fungus (Vandecasteele et al. 2018); or completely unable to infect and cause necrotic lesions on potato leaflets (Turkensteen et al. 2010; Spoelder et al. 2014).

The pathogenicity of small-spored *Alternaria* species has been demonstrated and the severity of epidemics caused by the pathogen has increased in different parts of the world, besides standing out as the predominant species in many areas (Droby et al. 1984; Boiteux and Reifschneider 1994; van der Waals et al. 2011; Shtienberg 2014; Lawrence et al. 2014; Zheng et al. 2015; Leiminger et al. 2015; Tymon et al. 2016a; Vandecasteele et al. 2018; Ding et al. 2019b; Taheri 2019; Lingwal et al. 2022; Choi et al. 2022). The emergence of these new epidemics may be the result of the capacity of these species to gain pathogenicity through the horizontal flow of genes or chromosomes, which also helps explain the origin of different virulence profiles among these species reported in potato, since these mechanisms can increase the adaptation of the pathogen to the environment (Hatta et al. 2002; Thomma 2003; Akagi et al. 2009; Vandecasteele et al. 2018).

The taxonomic complexity of the genus *Alternaria* is another factor that contributes to instability in the identification of species related to brown spot (Lawrence et al. 2016). Conventionally, *Alternaria* species are identified based on conidial morphology, including shape, size, coloration, septation, ornamentation, and patterns of secondary sporulation, and to a less extent, by their association with hosts and metabolic and biochemical profiles (Ellis 1971; Brun et al. 2013; Lawrence et al. 2016). For small-spored species, identification is typically based on conidial size and branching patterns (Landschoot et al. 2017a). Given the high morphological variability, this genus has undergone considerable taxonomic flux and its classification can be divided into five main stages since its proposition in 1816 (Nees von Esenbeck 1816) until the last revisions carried out between 2003 and 2015 (Lawrence et al. 2016). The numerous descriptions and revisions based on morphology resulted in an increasing number of new species (Saccardo 1886; Wiltshire 1933, 1938; Neergaard 1945; Simmons and Roberts 1993), with emphasis on the studies by Simmons (Simmons 1967, 1971, 1989, 1992, 2007), who completly reassessed the taxa related to *Alternaria* and described 275 morphospecies (Simmons 2007).

The overlapping of morphological characteristics between species/sections of *Alternaria* has led to the use of molecular phylogenetic taxonomy tools which have an important role in the delimitation of species by enabling a stable and predictable recognition of taxa (Lawrence et al. 2016; Zhang et al. 2020). Phylogenetic hypotheses are produced from the sequence of genomic regions, such as the internal transcribed spacer (ITS), *Alternaria* major allergen gene (*Alt a 1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), endopolygalacturonase (*EndoPG*), RNA polymerase second largest subunit (*Rpb2*), translation elongation factor 1-alpha (*Tef1*), an anonymous gene region (OPA10-2) and calmodulin (Pryor and Gilbertson 2000; Pryor and Bigelow 2003; Hong et al. 2005; Andrew et al. 2009; Lawrence et al. 2013, 2014; Armitage et al. 2015; Woudenberg et al. 2013, 2015; Ozkilinc et al. 2018).

Based on molecular data, the genus *Alternaria* has recently been restructured and divided into 27 monophyletic subgeneric taxonomic groups called *sections* (sect. *Alternantherae*, *Alternaria*, *Brassicicola*, *Chalastospora*, *Cheiranthus*, *Crivellia*, *Dianthicola*, *Embellisia*, *Embellisioides*, *Euphorbiicola*, *Eureka*, *Gypsophilae*, *Infectoriae*, *Japonicae*, *Nimbya*, *Panax*, *Phragmosporae*, *Porri*, *Pseudoalternaria*, *Pseudoulocladium*, *Radicina*, *Soda*, *Sonchi*, *Teretispora*, *Ulocladioides*, *Ulocladium*, and *Undifilum*), each typified by a representative species (Woudenberg et al. 2013; Lawrence et al. 2016). The *Alternaria* section contains most of the small-spored *Alternaria* species and is separated into 11 phylogenetic species: *A. alternata*, *A. burnsii, A. tomato, A. jacinthicola, A. iridiaustralis, A. eichhorniae, A. betae-kenyensis,* *A. gaisen, A. alstroemeriae, A. longipes*, and *A. gossypina*. Additionally, there is the *A. arborescens* species complex (AASC), comprising four morphospecies, *A. arborescens*; *A. cerealis*; *A. geophila*; and *A. senecionicola*, but the first set of genetic data did not allow complete separation of the members of AASC (Woudenberg et al. 2015). Later, Landschoot et al. (2017b) using the histone h3 gene, demonstrated that the isolates belonging to the AASC could be subdivided into groups A and B. Therefore, these taxa and sections will continue to change over time as new studies of *Alternaria* are conducted (Lawrence et al. 2016).

## Epidemiology

Despite the high support of molecular methods, the pathogenicity of *A. alternata*-like individuals found in potato fields is still under debate (Vandecasteele et al. 2018). Data supporting the ability of *A. alternata* to infect plants are available, but there are also many reports on the variation in virulence of different isolates, along with their host specificity or the lack of it. Thus, the pathogenicity of *A*. *alternata* still remains to be elucidated (Kokaeva et al. 2018). Small-spored *Alternaria* species exhibit high genetic (Andrew et al. 2009; Dini-Andreote et al. 2009; Lengi et al. 2014; Woudenberg et al. 2015; Zhang et al. 2020), morphological (Simmons 2007; Tymon et al. 2016b; Ozkilinc et al. 2018) and pathogenic variability (Roberts et al. 2000; Slavov et al. 2004; Tymon et al. 2016a; Bessadat et al. 2017; Vandecasteele et al. 2018). Understanding the distribution of these species, the pathogenic nature and the mechanisms by which such variation arises within a population is of paramount importance to understanding the success of these pathogens (Strandberg 1992; Chaerani and Voorrips 2006).

Studies of variability in populations of small-spored plant pathogenic *Alternaria* spp. are mostly conducted towards the understanding of host adaptation and response to fungicides (Brown 1998). However, studies involving the biology of the pathogen and the epidemiological components of the disease are important for the adoption of effective management strategies (Brown 1998). As a first step, the epidemiological variables most likely to influence disease development must be parametrized. The latent period (LP), incubation period (IP), lesion area, lesion expansion rate, and infection frequency (IF) are variables worth analyzing (Carlisle et al. 2002; Suassuna et al. 2004; Chacon et al. 2007; Cardoso 2010). The LP and IP correspond, respectively, to the time interval between inoculation and spore production and symptom appearance (Griffiths and Jones 1987). The IF is the probability of a spore deposited on the surface of the host to produce an injury in the absence of competitive interactions (Pariaud et al. 2009).

In addition to the relative differences in the epidemiological components associated with the virulence of *Alternaria* spp., they exhibit different responses regarding environmental variables, which may be related to preference for hosts (Cardoso 2010) or variation in small-spored species due to differences in local populations of the pathogen. Several studies addressing the epidemiological components were carried out to characterize the resistance of cultivars to early blight (Paula and Oliveira 2003; Dita Rodriguez et al. 2006; Cardoso 2010). However, the epidemiological components of brown spot have been poorly studied, and no work has been conducted with Brazilian small-spored *Alternaria* isolates associated with the potato crop. Additionally, it is necessary to analyze whether the small-spored *Alternaria* species associated with potato brown spot differ from each other in terms of ecological requirements.

# Chapter 1: Etiological studies of brown spot of potatoes caused by small-spored *Alternaria* species in Brazil.

## Abstract

Potato brown spot has the potential to significantly reduce potato quality and yield in different parts of the world. Small-spored *Alternaria* isolates were recovered from potato leaf lesions collected from major Brazilian potato-producing states during 2020 to 2021. The isolates were characterized using morphological and molecular approaches and tested for pathogenicity on detached-leaf and whole-plants. Multilocus sequence analyses were performed with six loci, Alternaria major allergen gene (*Alt a 1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), internal transcribed spacer (ITS), RNA polymerase second largest subunit (*Rpb2*), translation elongation factor1 - alpha (*Tef1*) and an anonymous gene region (OPA10-2). Pathogenicity tests demonstrated that inoculations of 57 of the 59 small-spored isolates resulted in brown necrotic lesions on detached potato leaves. However, no lesions were observed in assays using whole-plants. Variations were found in terms of virulence estimated by infection frequency, incubation period, and latent period. In total, three small-spored *Alternaria* species, *A. alternata*, *A. arborescens*, and a new species *Alternaria* sp. were identified based on morphological characters in combination with multigene analysis of the DNA sequences. Our results indicated that more than one small-spored *Alternaria* species can be the causal agent of brown spot in Brazil and this is the first report of *A. arborescens* causing brown spot on potato in the country.

## Introduction

Although species of the genus *Alternaria* are pathogenic to numerous crops, they have been featured as notorious pathogens of solanaceous plants such as potatoes. The economically important diseases on potatoes caused by *Alternaria* are early blight, caused by the large-spored species, such as *A. solani*, *A. grandis*, and *A. linariae* (formerly, *A*. *tomatophila*), and brown spot, caused by the small-spored species (Rodrigues et al. 2010; Vandecasteele et al. 2018). Brown spot has gained attention due to potential potato yield losses, which can reach up to 70% (Stevenson et al. 2001; Ahmed 2017).

Brown spot is characterized by small, dark-brown lesions, circular to irregular in shape, that can coalesce and cause significant foliar damage (Soleimani and Kirk 2012; Zhang et al. 2020). The etiological agent most commonly associated with this disease is *A. alternata*, a well-known small-spored species (Thomma 2003; Landschoot et al. 2017b). *Alternaria alternata* can infect more than 100 plant species and cause different diseases in economically important crops such as broccoli, tomato, potato, cauliflower, tobacco, pepper, citrus, pear, strawberry, apple, etc. (Meena et al. 2017; Wang et al. 2020). Nevertheless, there is mounting evidence, mainly from molecular analysis, that several small-spored *Alternaria* spp. are causing brown spot, including *A. arborescens*, *A. arbusti*, *A. dumosa*, *A. infectoria*, *A. interrupta*, *A. longipes*, *A. telliensis* and *A. tenuissima* (Taheri et al. 2009; Ardestani et al. 2010; Orina et al. 2011; Shoaib et al. 2014; Zheng et al. 2015; Tymon et al. 2016b; Landschoot et al. 2017b; Vandecasteele et al. 2018).

Despite the growing number of reports of small-spored *Alternaria* spp. causing potato brown spot epidemics, the disease has been generally overlooked and its spatio-temporal dynamics is poorly understood (Ding et al. 2019a). In Brazil, *A. alternata* is the only small-spored *Alternaria* species reported in association with the potato crop (Boiteux and Reifschneider 1994). In a recent systematic review, there is no information on the etiological and pathogenic characterization of the causal agent of potato brown spot in Brazil (Nascimento & Mizubuti, unpublished data). The precise identification of small-spored *Alternaria* species, the causal agents of brown spot, is essential to generate information on aspects related to the biology of the pathogen(s) and the epidemiology of brown spot (Chaerani and Voorrips 2006; Meng et al. 2015b). Considering that brown spot occurs in wide climatic conditions (Soleimani and Kirk

2012; Meng et al. 2015b) the different species of the pathogen can respond differentially to the ecological variables and also in relation to the genetic background of the host.

The similarity of the reproductive structures that characterize *Alternaria* species and the overlap of morphological traits, make the delimitation of the causal agents of brown spot a complex task (Rotem 1994; Simmons 2007; Andrew et al. 2009). Thus, the low resolution of these taxonomic attributes inappropriately leads to the grouping and classification of phylogenetically distinct individuals into single species of small-spored *Alternaria* (Tymon et al. 2016b)*.* Due to the difficulties presented in the analysis of morphological characters, molecular systematics based on data from conserved DNA sequences, has been useful to the characterization and taxonomy of *Alternaria*. Partial sequences of appropriate genomic regions are detached from the plasticity portrayed by morphological characters, allowing for more reliable analyses and grouping of individuals into taxonomic units (Armitage et al. 2015; Tymon et al. 2016b; Woudenberg et al. 2015; Florea and Puia 2020).

In this perspective, phylogenetic analyses with nine gene regions were used to discriminate 168 small-spored *Alternaria* isolates within the *Alternaria* section: The 18S nrDNA (SSU), 28S nrDNA (LSU), internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), RNA polymerase second largest subunit (*Rpb2*), translation elongation factor 1-alpha (*Tef1*), Alternaria major allergen gene (Alt a 1), endopolygalacturonase (*EndoPG*), and anonymous gene region (OPA10-2) (Woudenberg et al. 2015). The reconstructed phylogeny allowed the identification of 11 distinct phylogenetic species and a species complex inside the *Alternaria* section, providing a clear and stable species classification of this section.

Many small-spored species are saprophytic and are commonly found associated with different substrates. This lifestyle makes isolation of pathogenic small-spored *Alternaria* from brown spot lesions a difficult task (Woudenberg et al. 2013; Tymon et al. 2016a). The similarity of the life cycle of large-spored and small-spored species of *Alternaria* (Agrios 2005), their co-occurrence in the same host plant, and the similar symptoms of brown spot and early blight also contribute to raise objections to the ability of the small-spored species to infect potato leaves. Many studies indicate that small-spored species are made of invasive, endophytic, saprophytic or secondary organisms (Kirk and Wharton 2012; Stammler et al. 2014; Bessadat et al. 2017; Vandecasteele et al. 2018). It remains, therefore, to be proven whether small-spored

*Alternaria* spp. are indeed pathogenic and capable of causing epidemics when occurring as a single species, not interacting with large-spored *Alternaria* species.

Quantitative differences in the virulence of individuals of a given species are also important to be studied, mainly those related to eventual host preferences. Virulence is defined as the amount of disease produced in a host-pathogen interaction in a given period of time (Casadevall and Pirofski 2001). Usually, virulence is estimated through epidemiological components of the disease, such as latent period, incubation period, area under the disease progress curve, lesion area, rate of lesion expansion, and frequency of infection (Carlisle et al. 2002; Suassuna et al. 2004; Chacon et al. 2007; Pariaud et al. 2009; Cardoso 2010). In the case of large-spored pathogenic species of *Alternaria* spp., relative differences in epidemiological components that result in host preference or adaptation have been demonstrated (Andrivon 1993; Cardoso 2010). It is possible that such differences may also exist in small-spored *Alternaria* species, justifying further investigation.

The objective of this work was to identify and characterize the small-spored *Alternaria* isolates obtained from naturally infected potato leaves in commercial fields in Brazil and to assess their pathogenicity. A collection of 253 isolates was formed during two years (2020 - 2021) by sampling different fields in the main potato producing regions. Partial sequences of six genetic regions, the ITS region; *Gapdh*; *Rpb2*; *Tef1*; *Alt a 1*; and OPA10-2 were evaluated using single and multilocus analyses. In addition, we investigated the pathogenicity of these small-spored species and measured epidemiological components that may be associated with virulence.

## Material and Methods

### Sampling and isolates

Potato leaves with typical symptoms of brown spot were randomly sampled in commercial fields located in Minas Gerais, Paraná, São Paulo, Rio Grande do Sul, Santa Catarina, Goiás, and Bahia, main potato-producing states in Brazil. Samples were collected during the 2020 and 2021 growing seasons. Three leaf samples from each area were collected and placed in exsiccated paper sheets to preserve the material and to be transported to the laboratory for processing.

From each leaf, one to three lesions were chosen to attempt isolation. Fungal cultures were obtained using direct isolation of the pathogen, which consisted of direct transfer of conidia present in infected leaf tissues to Petri dishes containing potato dextrose agar (PDA) medium. Colonies with morphological characteristics similar to those described for *Alternaria* (Simmons, 2007) and producing small spores were selected for monoconidial isolation. The monoconidial isolation process consists of obtaining a suspension of conidia, which was subsequently inoculated on a water-agar (WA) plate, followed by the transfer of a single conidium to a Petri dish containing PDA medium. Pure colonies were grown at 25 °C under a 12 h photoperiod for 7 days until storage. The small-spored *Alternaria* isolates were preserved by the Castellani method (Castellani 1967) and on silica gel (Grivell and Jackson 1969). All isolates were added to the fungal culture collection of the Laboratory of Population Biology of Plant Pathogens, at Universidade Federal de Viçosa. In total, 253 small-spored *Alternaria* isolates were obtained and of these, 59 isolates were selected based on the collected region and used in the experiments (**Figure 1)**.

### Plant material

Potato plants, variety ‘Agata’, were used in all experiments. 'Agata' is the most planted cultivar in Brazil and is susceptible to several diseases, including early blight (Silva et al. 2014; Peeten et al. 2011). Nevertheless, susceptibility of 'Agata' to small-spored *Alternaria* isolates has not been evaluated. Potato seed-tubers were planted in 5L-plastic pots filled with a mixture of soil, sand, and commercial substrate MecPlant, in the proportion of 1:0.5:1, respectively. The plants were kept in a greenhouse set at 25 °C (± 3 °C) during the day and approximately 20 °C at night, with at least 12 h of natural light. All cultural practices were carried out following the recommendations for the crop, but fungicide sprays using active ingredients effective against *Alternaria* spp. were not used. Six weeks after emergence, the plants were selected for the whole-plant or detached-leaf pathogenicity tests.

### Inoculum preparation

The isolates were subcultured to oat-agar medium (30 g of oat and 20 g of agar, per 1L) and placed to grow in a BOD incubator at 25 ± 2 °C with a 12 h photoperiod. After seven days, 10 mL of distilled water was added to each colony and the aerial mycelium was scraped. Subsequently, the plates were kept open under 40

W black light (Sylvania®) at 23 ± 2 ºC for 48 h and 12 h photoperiod, to allow dehydration and induce sporulation. The inoculum suspension was obtained by adding 10 ml of gelatin (1%) (Sigma-Aldrich, St Louis, MO, USA) to the sporulating colonies. The concentration of the spore suspension was adjusted to $10^5$ conidia/mL by counting the number of conidia in the Neubauer chamber.

### Detached-leaf pathogenicity test

Potato leaves were selected and placed on plates lined with moistened paper towels to form a high-humidity environment. The petioles were wrapped with a cotton ball soaked in water to maintain hydration and turgor of the plant tissues. The leaflets of a detached leaf were delimited at different points for inoculation with a drop of 10 µL of conidia suspension. The plates were kept at 25 ± 2 °C, with a photoperiod of 12 h. In addition to the 59 small-spored *Alternaria* isolates collected from different locations, three other small-spored isolates were included in the analyses: *A. alternata* (UFVAa-628), *A. alternata* (CBS 916.96) and *A. arborescens* (CBS 102605) (https://wi.knaw.nl/page/Collection). The positive control leaves were inoculated with *A. grandis* (UFVAg-638), while the negative control was inoculated with gelatin. The presence or absence of lesions was evaluated 24 h after inoculation and was performed daily up to seven days after inoculation. Symptomatic leaves were examined under a stereomicroscope (10X), and conidia were transferred to a PDA medium in a Petri dish to confirm Koch's postulates.

### Brown spot virulence components

The incubation period (IP), latent period (LP), and infection frequency (IF) of brown spot were estimated for all 62 small-spored isolates used in the detached-leaf test. The IP was determined as the time (days) that elapsed between inoculation and the onset of symptoms. The IP was evaluated daily and considered finished on the seventh day or when 100% of the leaves showed brown spot lesions. The LP was determined as the time (days) elapsed from inoculation to sporulation. The infection frequency (%) was quantified at the end of the assessment time and it was defined as the proportion of the number of lesions by the total number of inoculated sites.

## Pathogenicity test in whole-plants

Sixteen isolates (UFVAssp-02, UFVAssp-04, UFVAssp-10, UFVAssp-22, UFVAssp-26, UFVAssp-51, UFVAssp-66, UFVAssp-75, UFVAssp-97, UFVAssp-112, UFVAssp-225, UFVAssp-239, UFVAssp-302, UFVAssp-310, UFVAssp-323, UFVAssp-332) were selected for the whole plant pathogenicity assay based on the IF values. Isolates that had IF values greater than 70% in at least one experiment were used to validate the results of the detached-leaf assays. The plants used in this experiment were grown as described above. Leaves were delimited at different inoculation points. A 10 µL-drop of spore suspension (1 x $10^5$ conidia/mL) was placed in marked areas of the leaves. Positive and negative controls were the same as in the previous experiment.

The plants were kept in a greenhouse at approximately 21 to 25°C under a 14 h natural light photoperiod. The presence or absence of lesions was initially evaluated 24 h after inoculation and daily assessments were conducted up to 21 days post-inoculation. Symptomatic leaves were also examined and conidia were transferred to PDA in a Petri dish to complete Koch's postulates.

## DNA extraction

The pathogenic isolates were cultured in a liquid medium (10 g sucrose, 2 g L-asparagine, 2 g yeast extract, 1 g $KH_2PO_4$, 0.1 g $MgSO_4.7H_2O$, 0.44 mg of $ZnSO_4.7H_2O$, 0.48 mg $FeCl_3.6H_2O$, and 0.36 mg of $MnCl_2.H_2O$). The fungal cultures were incubated for seven days at 25 ± 2 °C with continuous agitation under a photoperiod of 12 h. The mycelium was then washed with distilled water, placed on filter paper for drying, and macerated in the TissueLyser® for 2 min at a frequency of 30 Hz until obtaining a fine powder. Genomic DNA extraction was performed using the Wizard® kit. Genomic DNA Purification (Promega Corporation), following the manufacturer's instructions. DNA was quantified by spectrophotometry (NanoDrop 2000 Thermo Scientific), and DNA integrity assessment was performed on agarose gels (1%) using molecular weight lambda phage.

## Polymerase Chain Reaction (PCR)

The isolates used in the pathogenicity test in whole-plants were included in the phylogenetic analyses. Six genetic regions were amplified following published protocols (Woudenberg et al. 2013, 2015), with some modifications. The ITS region was amplified with primers ITS1 and ITS4 (White et al. 1990), the *Alt a 1* region with Alt-for and Alt-rev (Hong et al. 2005), the *Gapdh* region with Gpd1 and Gpd2 (Berbee et al. 1999), the OPA10-2 region with OPA 10-2R and OPA 10-2L (Andrew et al. 2009), the *rpb2* region with rpb2-5f2 and frpb2–7cr (Sung et al. 2007; Liu et al. 1999) and the *Tef1* gene with EF1-728F and EF1-986R (Carbone and Kohn 1999), conditions for amplification are shown in **Table 1**. PCR reactions were performed with the GoTaq® G2 DNA Polymerase Kit, in a thermocycler (BIO-RAD. T100™ Thermal Cycler) with a total volume of 12,5 µL.

**Table 1:** Pairs of primers used to amplify and sequence genetic regions used in molecular phylogenetic analysis to identify small-spored *Alternaria* isolates associated with brown spot in potato.

| Genetic region* | Primer | Sequence | Condition** | Reference |
|---|---|---|---|---|
| ITS | ITS1 | TCCGTAGGTGAACCTGCGG | 5 min 94 ° C, 35 × (45 s 94 ° C, 45 s 52 ° C, 90 s 72 ° C), 6 min 72 ° C. | White et al. 1990 |
|  | ITS4 | TCCTCCGCTTATTGATATGC |  | White et al. 1990 |
| *Alt a 1* | Alt-rev | ATGCAGTTCACCACCATCGC | 5 min 94 ° C, 40 × (30 s 94 ° C, 30 s 55 ° C, 60 s 72 ° C), 7 min 72 ° C. | Hong et al. 2005 |
|  | Alt-for | ACGAGGGTGAYGTAGGCGTC |  | Hong et al. 2005 |
| *Gapdh* | gpd1 | CAACGGCTTCGGTCGCATTG | 4 min 94 ° C, 35 × (30 s 94 ° C, 45 s 60 ° C, 60 s 72 ° C), 5 min 72 ° C. | Berbee et al. 1999 |
|  | gpd2 | GCCAAGCAGTTGGTTGTGC |  | Berbee et al. 1999 |
| OPA10-2 | OPA 10-2R | GATTCGCAGCAGGGAAACTA | 5 min 94 °, 35 x (30 s 94 ° C, 30 s 62 ° C e 45 s 72 ° C) , 7 min 72 ° C. | Andrew et al. 2009 |
|  | OPA 10-2L | TCGCAGTAAGACACATTCTACG |  | Andrew et al. 2009 |
| *Rpb2* | *rpb2-5f2* | GGGGWGAYCAGAAGAAGGC | 5 min 94 ° C, ( 5x ( 45 s a 94 ° C, 45 s a 60 ° C, 2 min a 72 ° C), 30 x ( 54 ° C), 7 min 72 ° C. | Sung et al. 2007 |
|  | *frpb2*–7cr | CCCATRGCTTGYTTRCCCAT |  | Liu et al. 1999 |
| *Tef1* | *ef1-728f* | CATCGAGAAGTTCGAGAAGG | 5 min 94 ° C, 40 × (30 s 94 ° C, 30 s 52 ° C, 45 s 72 ° C), 7 min 72 ° C. | Carbone and Kohn 1999 |
|  | *ef1-986r* | TACTTGAAGGAACCCTTACC |  | Carbone and Kohn 1999 |

* ITS = Internal transcribed spacer, *Alt a 1* = Alternaria major allergen gene, *Gapdh* = glyceraldehyde-3-phosphate dehydrogenase, OPA10-2 = anonymous noncoding region, *Rpb2* = RNA polymerase second largest subunit, *Tef1* = translation elongation factor 1-alpha. ** PCR steps of denaturation, annealing, and extension.

After amplification, PCR products were purified using the ExoSAP-IT™ kit following the protocol: 2 µL of enzyme for each 5 µL of PCR product; incubation at

37°C for 10 min, followed by enzyme inactivation at 80°C for 10 min. The purified samples were sequenced in both directions.

## Phylogenetic analysis

The sequences of each region were aligned with reference sequences obtained from GenBank (**Table S2**) using the MUSCLE tool implemented in the MEGA11 Software (Tamura et al. 2021). Phylogenetic analysis was performed by Bayesian Inference (BI) method for individual data partitions and then for the concatenated dataset of the six genomic regions.

Bayesian inferences were performed with MrBayes v3.2.7a on the Cipres Science Gateway (CIPRES Cluster). The nucleotide substitution model was chosen among several options based on the Bayesian Information Criterion (BIC) by the jModelTest v2.1.6 package in CIPRES. The HKY+G model was chosen for *Alt a 1* and *Gapdh* partitions, and the K80+G model for ITS, OPA10-2, *Rpb2*, and *Tef1*. Two simultaneous runs with one cold chain and three heated chains were conducted. The total number of generations was five million, and the temperature was set as 0.20. Trees were sampled at every 500 generations for each analysis and 25 percent of the initially generated trees were discarded to obtain the consensus tree. The diagnosis of chain convergence was carried out according to two criteria. i- The standard deviation of the divided frequencies at the end of each run must be kept below 1.5%, as indicated in the MrBayes output. ii- The effective sample size (ESS) for each parameter above 200, when evaluated with the Tracer v1.7.2 software (Rambaut et al. 2018). Subsequently, a Bayesian 50 % majority rule consensus tree was obtained based on posterior probability (PP) equivalent to the bipartition frequencies obtained in the two independent runs. The phylogenetic trees of the BI analysis for each genetic region and the multigene phylogeny were visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited in CorelDraw®. Branch support values were added based on Bayesian probabilities. *Alternaria grandis* (CBS 116695) was used as an outgroup (**Table S2**). The sequences generated in the study will be deposited in the GenBank database.

## Morphological characterization

The morphological characterization of the isolates was carried out to verify the stability of the characteristics traditionally used to separate large-spored from small-spored *Alternaria* species (Simmons 2007). Five isolates identified phylogenetically as *A. alternata* (UFVAssp-04, UFVAssp-97) *A. arborescens* (UFVAssp-239) and *Alternaria* sp. (UFVAssp-26, UFVAssp-302) were selected and characterized in potato carrot agar (PCA) and V8 medium (Simmons 2007). The detailed examination of the morphological structures of the isolates was carried out in slide microculture, which consisted of subculture of the actively growing colony onto blocks of PCA, and V8 media already fixed on the slide (Aylmore and Todd 1984). The slides were placed in the center of Petri dishes and maintained under a cycle of 8 h of fluorescent light and 16 h of darkness at 25 ± 2°C. The cultures were analyzed after 5 days. Photographs were taken with an Olympus BX53 microscope equipped with a digital camera, Olympus Q-Color5™. Measurements of morphological traits such as chain ornamentation, conidia and conidiophores size, and number of septa were examined (n = 30) using the Olympus cellSens Dimension 1.9 software system. Cultural characteristics of the isolates in V8 and PCA medium were also evaluated, such as colony growth and color. Morphological separations were based on descriptions of *Alternaria* spp. (Simmons 2007).

## Data analysis

The IP, LP, and IF data were submitted to analysis of variance. The means and standard errors for each of  the small-spored *Alternaria* isolates were estimated. The assumptions for analysis of variance were evaluated according to the normality of the errors and standard residual graphs. The morphological characteristics were also submitted for analysis of variance, followed by the elaboration of descriptive analyses, such as mean, minimum, and maximum values. The frequency distribution of morphological data was used to assess the overlapping of replicates measured among isolates. All analyses were carried out using R version 4.2.2 (R Development Core Team, 2021).

## Results

**Isolate collection.** A total of 253 monosporic isolates were obtained from potato leaf samples collected from commercial potato plantations between 2020 and 2021 with brown spot symptoms. The state of Minas Gerais (n=141) had the highest number of municipalities collected, followed by Paraná (n=33), Rio Grande do Sul (n=32), Goiás (n=19), São Paulo (n=18), Bahia (n=7) and Santa Catarina (n=3) (**Figure 1)**. Brown spot is distributed across the main potato producing states in Brazil. In most areas, isolates of small-spored individuals were observed together with large-spored ones in the same necrotic lesions or in different lesions formed in a leaf.

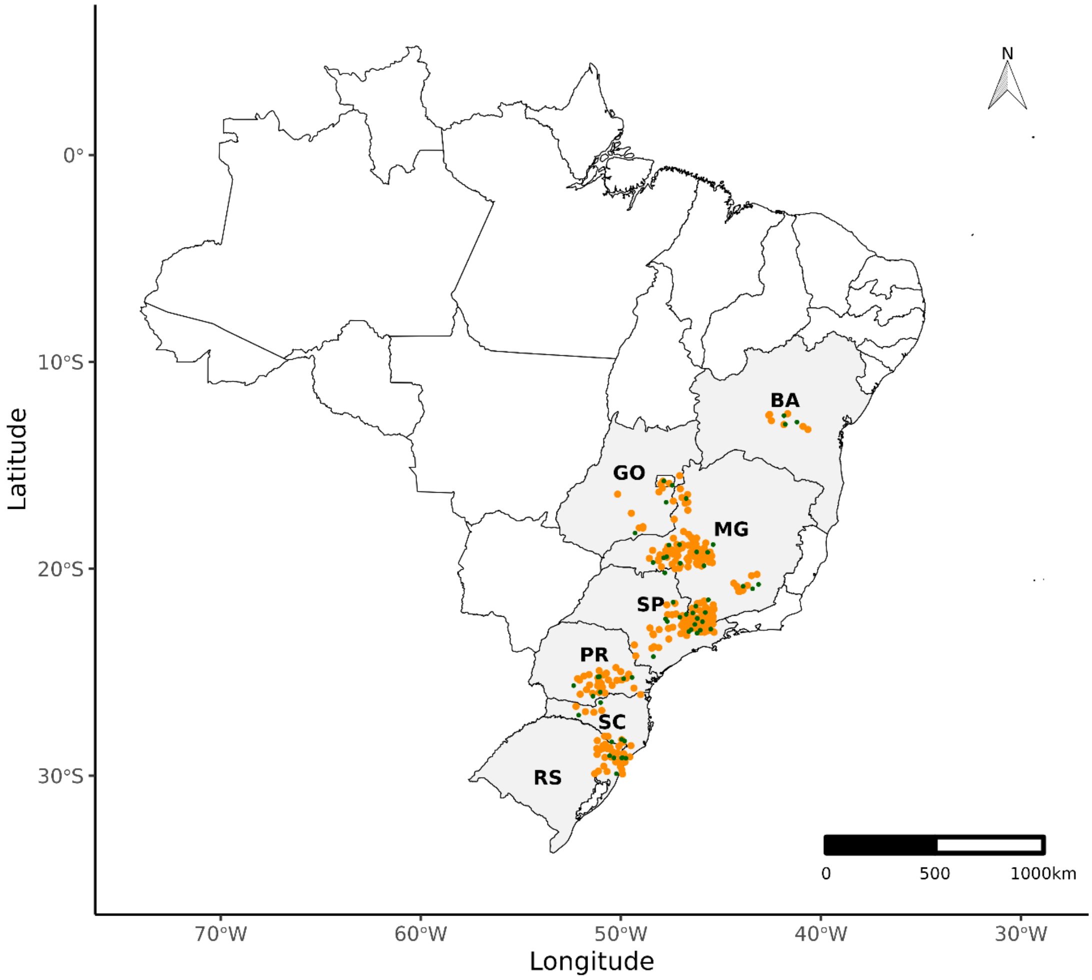

**Figure 1 -** Brazilian map showing small-spored *Alternaria* isolates that were sampled from potato leaves with brown spot symptoms in the main potato-producing states. The orange dots represent the total number of isolates sampled (n = 253). The green dots represent the isolates that were considered pathogenic according to the pathogenicity tests (n = 59). BA = Bahia, GO = Goiás, MG = Minas Gerais, SP = São Paulo, PR = Paraná, SC = Santa Catarina, and RS = Rio Grande do Sul states.

**Detached-leaf pathogenicity test.** Small-spored *Alternaria* isolates were considered pathogenic when typical necrotic lesions were formed on the inoculated detached leaves. Of the 62 isolates inoculated, 59 were pathogenic. Three isolates did not cause symptoms in any of the replicates. Regarding the isolates collected in the potato producing regions, 57 of the 59 tested, were pathogenic. Symptoms observed on detached leaves expanded from the inoculation site and developed as light to dark brown necrotic ovoid lesions, circular, with and without a yellow halo, similar to lesions seen on samples of infected leaves collected under field conditions (**Figure 2**). The severity of symptoms differed according to the isolate. The control isolate *A. grandis* (UFVAg-638) was consistently more virulent based on the number of injuries caused, showing lesions in all inoculation sites, while the *A. arborescens* (CBS 102605) isolates exhibited a moderate level of virulence, the *A. alternata* (CBS 916.96) isolate did not cause any lesions in potato leaves. Symptoms were not observed in gelatin-treated negative control leaves. Re-isolation of fungi was performed to verify their pathogenic nature.

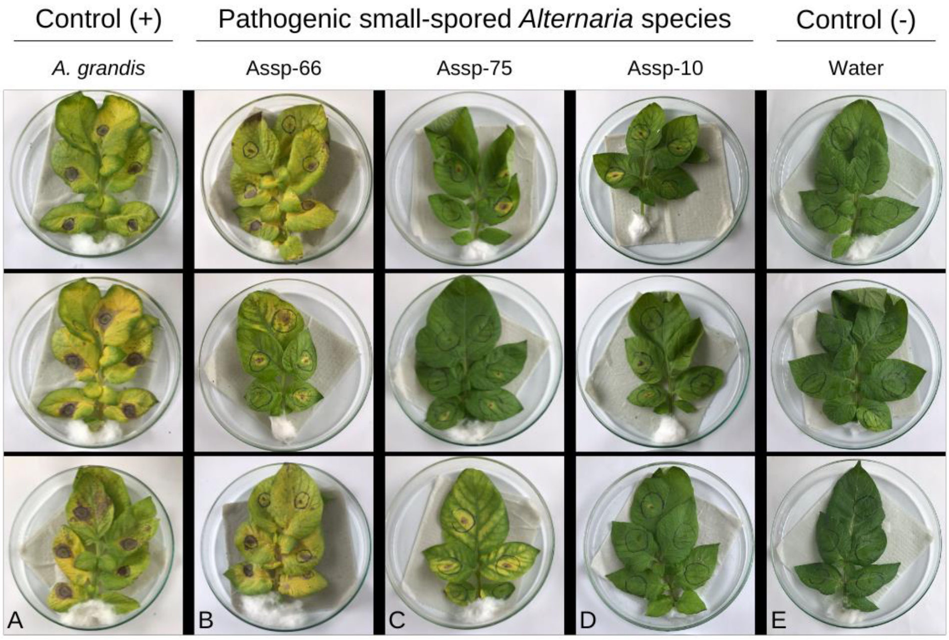

**Figure 2 -** Symptoms induced by representative isolates of small-spored *Alternaria* and *A*. *grandis* inoculated onto detached 'Agata' potato leaves. Virulence was determined based on the number of lesions observed at the inoculation sites, with the most virulent isolates displaying the most pronounced symptoms, including a higher lesion count. Control (+): positive control representing leaves inoculated with *A*. *grandis*. Control (-): negative control representing leaves sprayed with water. Images were captured 7 days following the inoculation

**Infection frequency (IF).** Based on Hartley's test ($F_{max} = 1.31$), the two IF experiments had similar variances and data were pooled for analysis (**Table S3**). The IF values differed among isolates ($p < 0.001$). For *A. grandis* (UFVAg-638) the IF was 100% and this was higher than small-spored *Alternaria* isolates (**Figure 3**). For small-spored isolates, the highest IF values were recorded for UFVAssp-225, UFVAssp-97, and UFVAssp-239: 77.5%, 68.5%, and 65.6%, respectively. The UFVAssp-142 and UFVAssp-182 isolates did not cause symptoms, therefore IF was not estimated.

**The incubation period (IP).** The IP values varied among isolates in experiment 1 ($p < 0.01$) (**Table S3**). The IP for UFVAssp-217 isolate, 5.5 days, was the longest. The IP for most isolates ranged from 2 to 3 dai (**Figure 4**). In experiment 2, there were no significant differences among isolates ($p = 0.41$).

**Latent period (LP).** The LP differed significantly among isolates ($P < 0.001$) (**Table S3**). Of the 59 isolates, seven did not complete the LP, as well as *A. grandis* (UFVAg-638) and *A. arborescens* (CBS 102605) (**Figure 5**). The *A. alternata* (UFVAg-628) isolate had the longest LP among the small-spored isolates (7.5 dai). Sporulation for 30 isolates was observed at asymptomatic inoculated sites; i.e. sporulation without necrotic lesions.

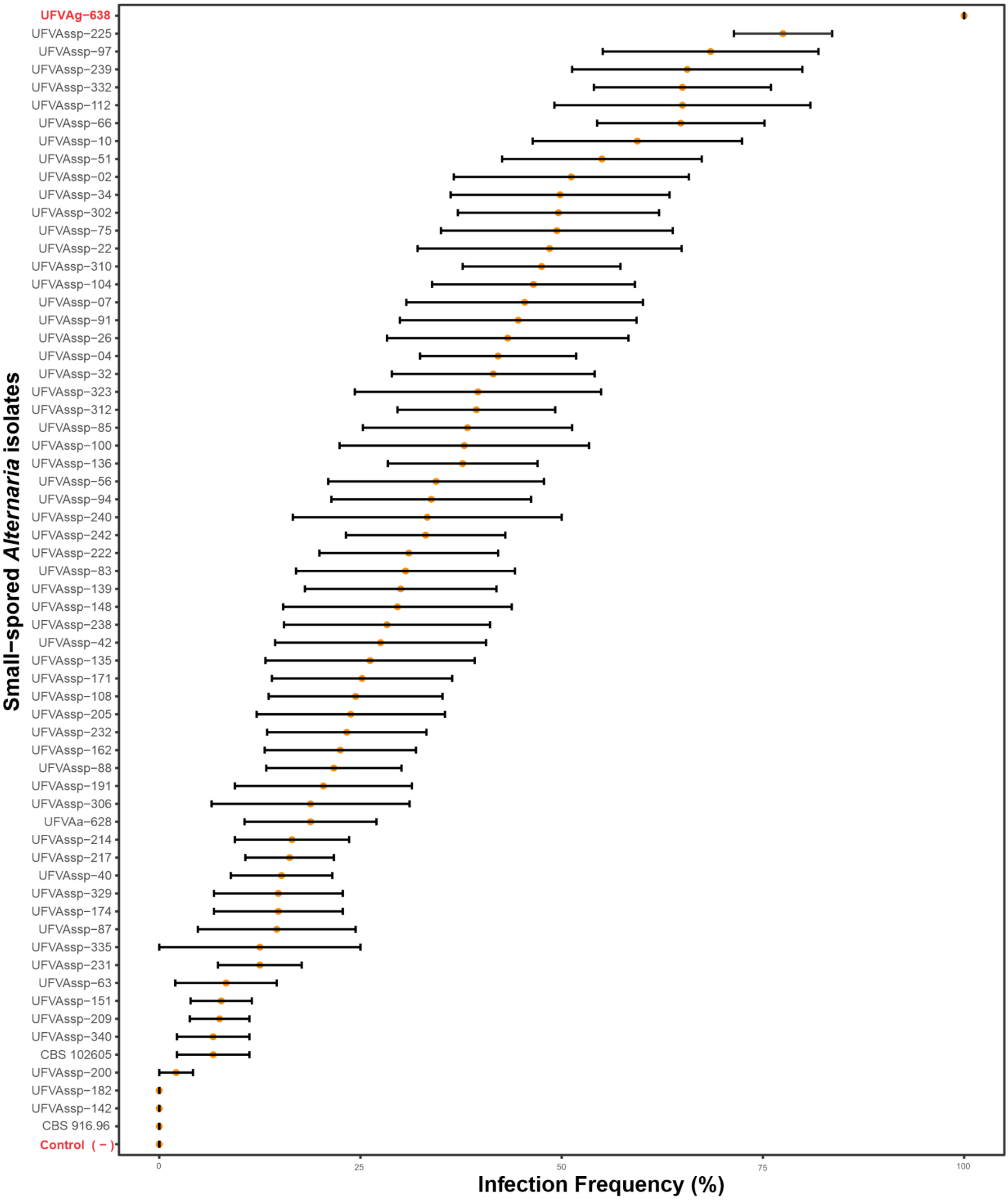

**Figure 3** - Infection frequency for small-spored *Alternaria* isolates inoculated on detached potato 'Agata' leaves. Values for the two experiments were pooled and averages represent eight data points. The IF averages are represented by orange dots. Vertical bars represent the standard error of the mean ( ± ). CBS 916.96 is isolate *A. alternata* (CBS 916.96) and CBS 102605 is isolate *A. arborescens* (CBS 102605). Text in red are the positive and negative controls: leaves inoculated with *A. grandis* and leaves sprayed with water, respectively.

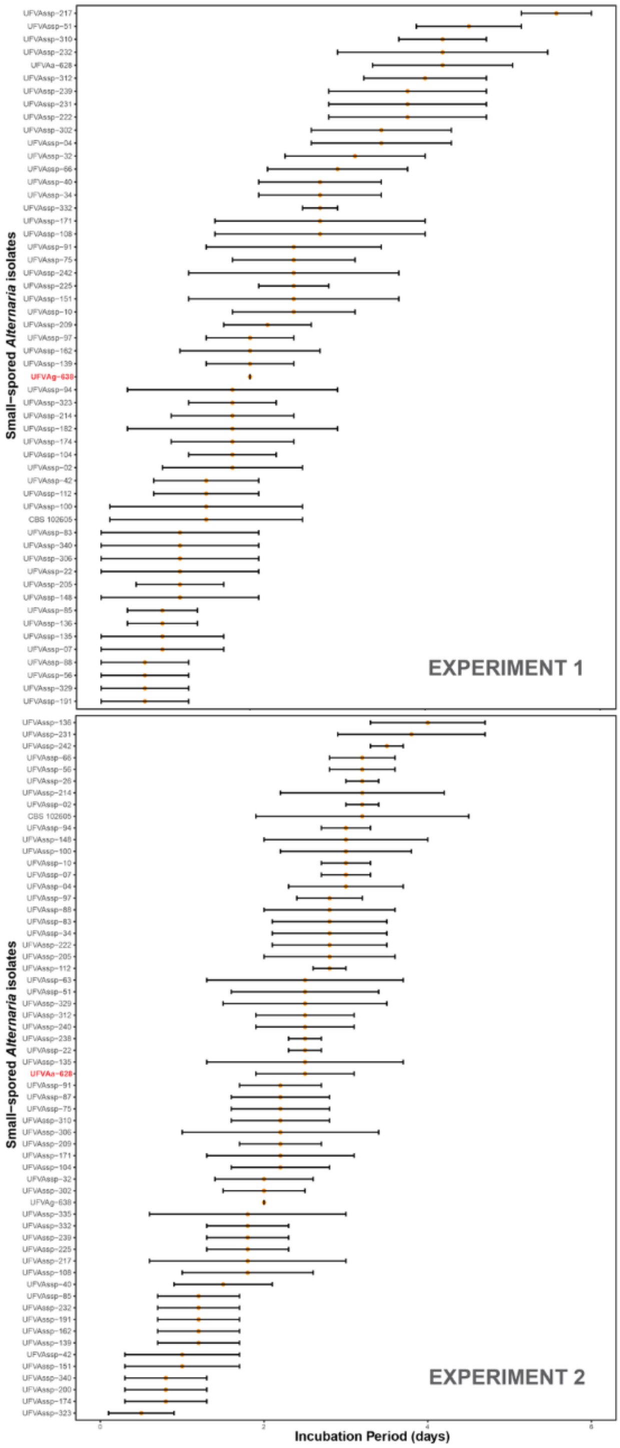

**Figure 4 -** Incubation period in two separate experiments in which small-spored *Alternaria* isolates were inoculated on detached potato 'Agata' leaves. Values for the two experiments are presented and averages represent four data points. The IP averages are represented by orange dots. Vertical bars represent the standard error of the mean ( ± ). CBS 916.96 is isolate *A. alternata* (CBS 916.96) and CBS 102605 is isolate *A. arborescens* (CBS 102605). In red are the control treatments: positive control representing leaves inoculated with *A. grandis* and Control (-) negative control representing leaves inoculated with water.

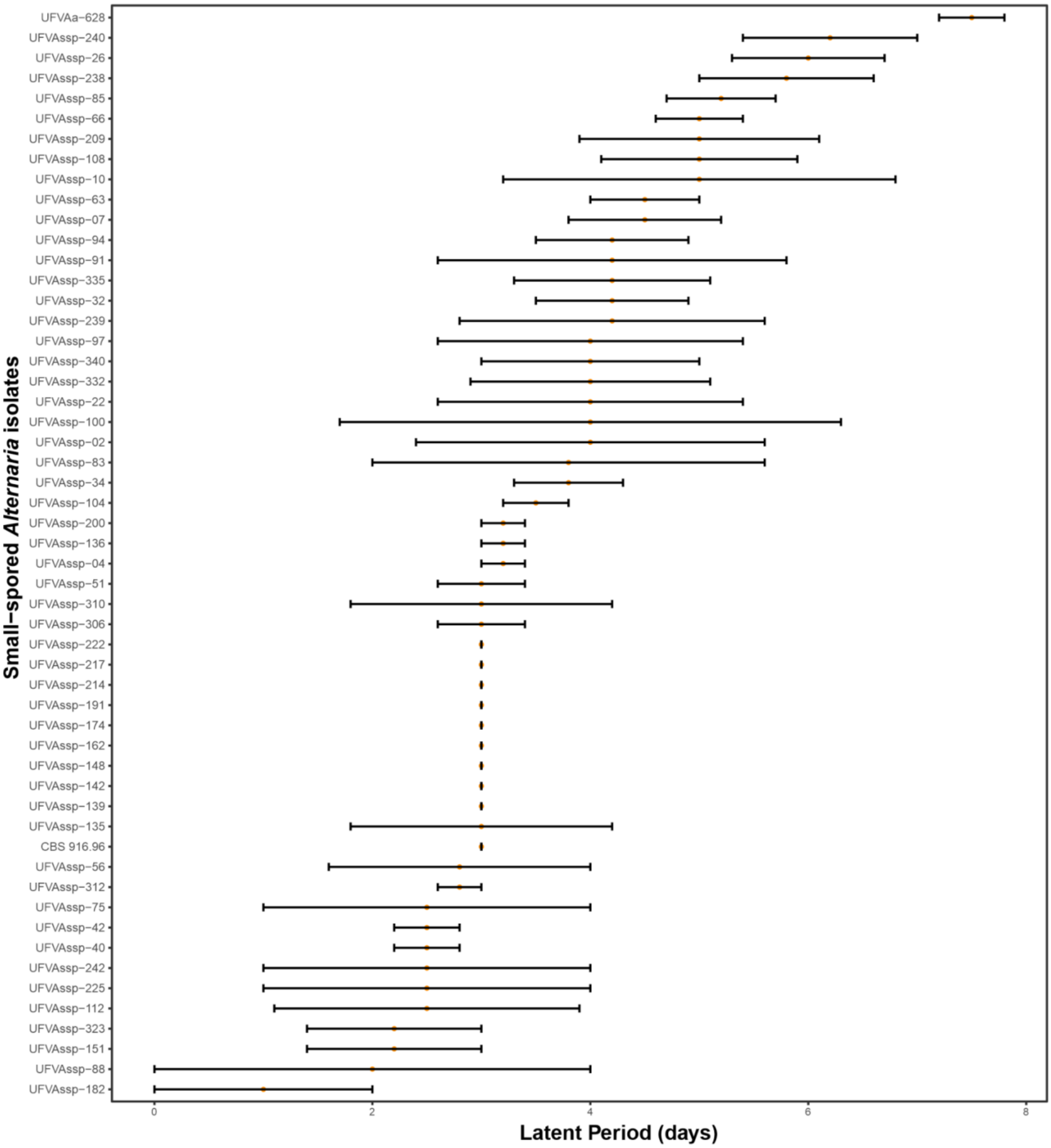

**Figure 5 -** Latent period for small-spored *Alternaria* isolates inoculated on detached potato 'Agata' leaves. The LP averages represent four data points and are presented by orange dots. Vertical bars represent the standard error of the mean ( ± ). CBS 916.96 is isolate *A. alternata* (CBS 916.96) and CBS 102605 is isolate *A. arborescens* (CBS 102605). In red are the control treatments: positive control representing leaves inoculated with *A. grandis* and Control (-) negative control representing leaves inoculated with water.

**Pathogenicity test in whole plants.** Sixteen highly virulent isolates under detached leaf test were selected and used in pathogenicity test in whole plants grown under greenhouse conditions. No symptoms were observed in whole plants.

**Phylogenetic analysis.** All sixteen isolates that were pathogenic in the detached leaf test were grouped in section *Alternaria* (Woudenberg 2015). PCR reactions of eight isolates for the OPA10-2 gene failed, these isolates were included as missing data in the multigene phylogeny. The aligned sequences from *Alt a 1* (474 aligned characters), *Gapdh* (588 aligned characters), ITS (565 aligned characters), *Rpb2* (861 aligned characters), *Tef1* (344 aligned characters) and OPA10-2 (634 aligned characters) resulted for a total of 115, 74, 36, 137, 55, and 94 single nucleotide polymorphisms, respectively. The phylogenetic analysis using ITS sequences did not provide enough resolution to reliably sort the species (**Figure S3**). Therefore, multigene analysis was performed for the six gene regions and separately for the *Alt a 1*, *Rpb2* and *Tef1* regions.

The alignments for the six concatenated genes contained 3466 characters, including alignment gaps. The Bayesian phylogeny of the six genes distinguished two main clades: *A. alternata* and *A. arborescens species complex (AASC)* (**Figure 6**).

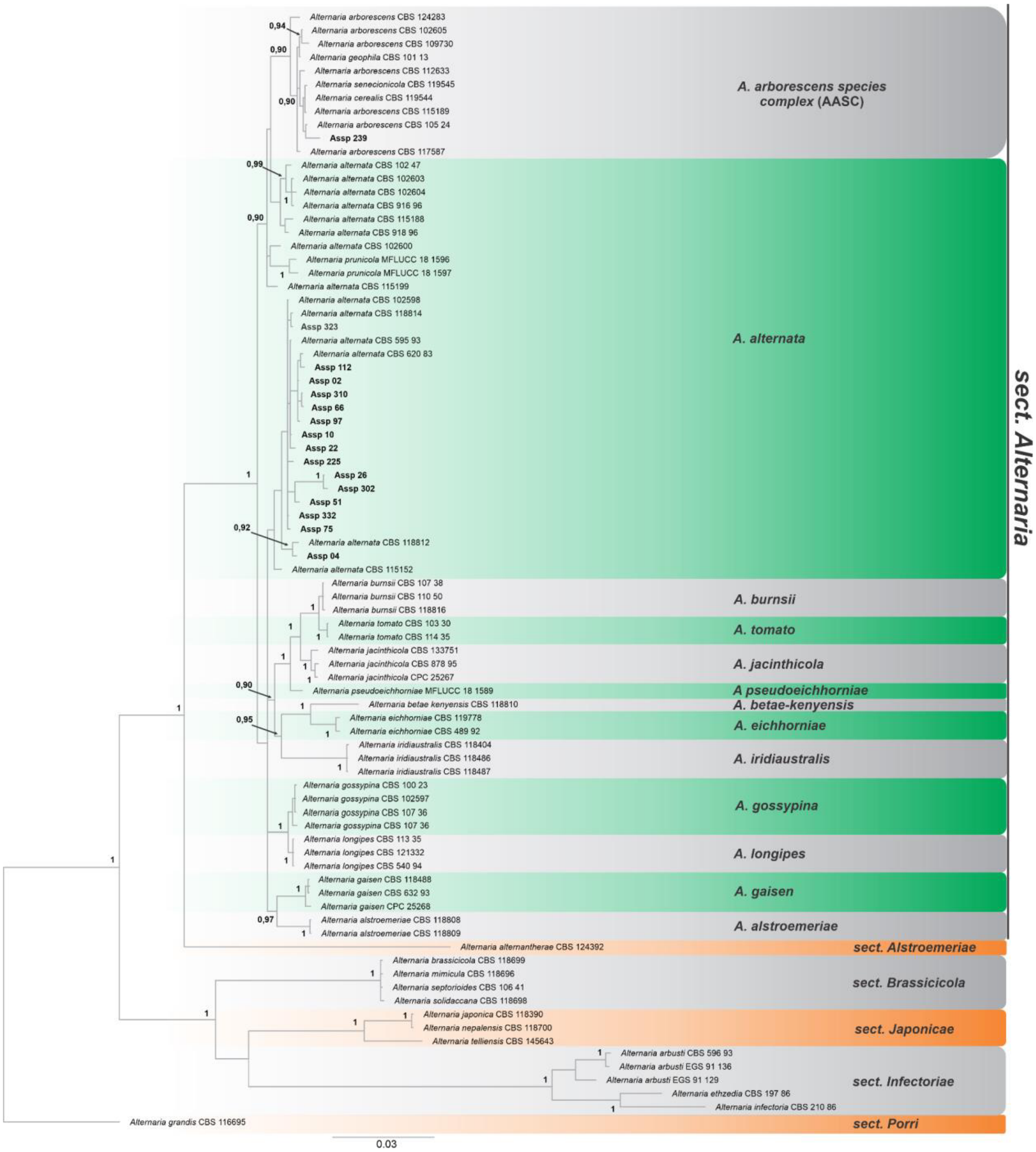

**Figure 6** - Bayesian 50 % majority rule consensus tree based on the *Alt a 1*, *Gapdh*, ITS, *Rpb2*, *Tef1* and OPA10-2 of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.90 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site. The isolates used in the analyses are indicated by their code, printed in **bold**.

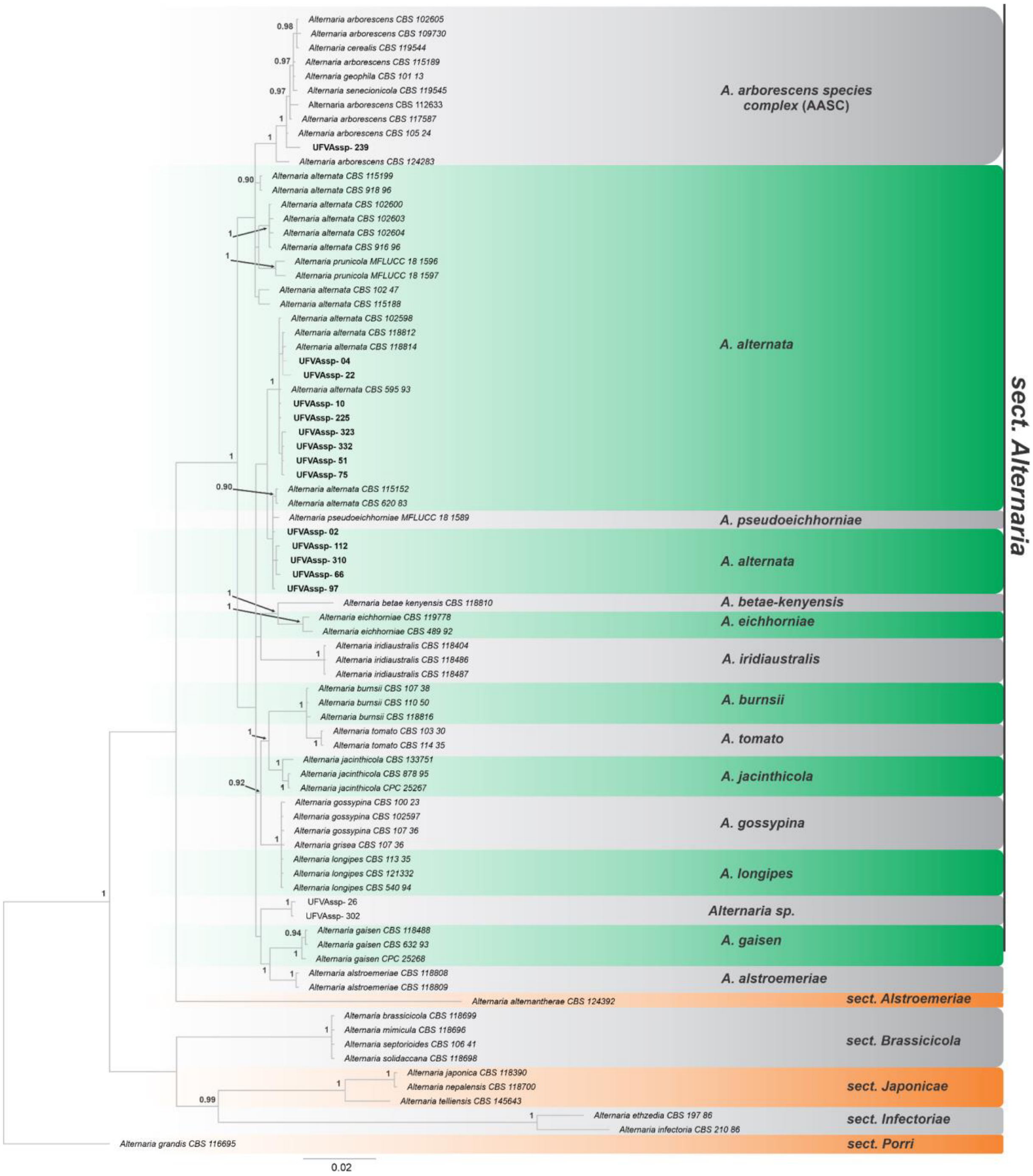

**Figure 7** - Bayesian 50 % majority rule consensus tree based on the *Alt a 1*, *Rpb2* and *Tef1* of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.90 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site. The isolates used in the analyses are indicated by their code, printed in **bold**.

Fifteen isolates of the *A. alternata* clade were separated in three distinct groups based on Bayesian posterior probabilities (PP). Furthermore, the analysis of the concatenated dataset separated the *A. alternata* clade from other taxa belonging to the sect. *Alternaria* (**Figure 6**).

For the alignment with the three concatenated genomic regions (1679 aligned characters), the phylogenetic analysis could adequately resolve clades within the section *Alternaria*, despite having grouped *A. pseudoeichhorniae* with *A. alternata* (**Figure 7**). Thirteen of the 16 isolates were grouped into the *A. alternata* clade (PP = 1), and these were divided into three subclades. The first subclade includes reference-isolates *A. alternata* (CBS 118812) and *A. alternata* (CBS 118814) and isolates UFVAssp-04 and UFVAssp-22. The second was composed of six isolates grouped with the reference *A. alternata* (CBS 595.93), while the third subclade was formed by five isolates that were grouped with two CBS reference strains together with *A. pseudoeichhorniae*. The UFVAssp-26 and UFVAssp-302 isolates always grouped together in a separate clade (**Figure S1, S2,S4, S5**). The UFVAssp-239 isolate, as in the previous analysis, grouped with the *A. arborescens* clade (PP = 1). The two phylogenetic analyses, however, did not result in clear clades for most of the isolates. The subclades formed by these isolates are inconsistent with the individual phylogenetic analyses (**Figure S1 - S5**). The partial sequences of *Rpb2*, *Alt a 1* and OPA10-2 were more informative and allowed better grouping of most isolates. The *Gapdh* and *Tef1* genes failed to consistently separate species within the *Alternaria* sect (Woudenberg 2015). The ITS gene was less successful in separating species in this study. Although the single-gene phylogenies are not fully congruent, the *Rpb2* and *Alt a 1* genes can provide significant statistical support in identifying small-spored *Alternaria* species.

**Analysis of morphological characteristics.** There was little variation among the small-spored *Alternaria* isolates regarding the main morphological characteristics; i.e. conidia and conidiophore size and number of septa (**Figure S6**). Measurements of morphological characters in synthetic media are described in **Table 2**. There were differences among the mean values of all measured variables among isolates ($p < 0.05$). However, the interaction between the isolates and the culture medium was not significant for conidial width ($p = 0.104$), number of transverse septa ($p = 0.062$), or conidiophore width ($p = 0.363$).

**Table 2:** Measurement of different morphological structures of small-spored *Alternaria* isolates in V8 and PCA medium.

| Isolate | Specie | Medium V8 | | | | | |
|---|---|---|---|---|---|---|---|
| | | Conidium size | | Septa | | Conidiophore size | |
| | | Length (μm) | Width (μm) | Transverse | Longitudinal | Length (μm) | Width (μm) |
| CBS 916.96[a] | *A. alternata* | 29.3 ± 6.4 (18.9 - 44.3) | 11.3 ± 2.5 (5.6 - 14.5) | 3.4 ± 0.8 (2 - 5) | 3.4 ± 1.2 (0 - 4) | 45.5 ± 20.2 (15.1 - 88.9) | 4.1 ± 0.6 (3.0 - 5.2) |
| UFVAssp-04 | *A. alternata* | 22.8 ± 3.6 (17.2 - 31.6) | 9.5 ± 1.8 (6.4 - 13.3) | 3.3 ± 0.6 (2 - 5) | 3.3 ± 0.7 (0 - 2) | 38.8 ± 15.6 (11.2 - 85.6) | 4.2 ± 0.5 (3.0 - 5.0) |
| UFVAssp-239 | *A. arborescens* | 25.3 ± 6.1 (15.0 - 43.2) | 9.5 ± 1.9 (6.4 - 14.6) | 3.4 ± 0.9 (1 - 5) | 3.4 ± 0.9 (0 - 3) | 83.3 ± 49.2 (16.9 - 227.1) | 4.5 ± 0.6 (3.2 - 5.7) |
| UFVAssp-26 | *Alternaria* sp. | 24.6 ± 7.5 (13.6 - 45.1) | 9.6 ± 1.3 (7.7 - 12.5) | 3.4 ± 1.1 (1 - 6) | 3.4 ± 0.6 (0 - 2) | 97.5 ± 46.7 (11.7 - 211.6) | 4.1 ± 0.5 (3.2 - 4.9) |
| UFVAssp-302 | *Alternaria* sp. | 17.5 ± 3.5 (11.6 - 24.2) | 8.8 ± 1.7 (5.6 - 11.8) | 2.0 ± 0.9 (1 - 4) | 2.0 ± 0.6 (0 - 2) | 129.2 ± 41.8 (69.3 - 235.4) | 4.3 ± 0.5 (3.2 - 5.5) |
| UFVAssp-97 | A. alternata | 21.9 ± 3.1 (15.8 - 29.1) | 9.2 ± 1.9 (5.4 - 13.2) | 3.1 ± 0.6 (2 - 4) | 3.1 ± 0.8 (0 - 3) | 75.5 ± 45.1 (15.4 - 143.3) | 4.1 ± 0.7 (2.8 - 6.2) |

| Isolate | Specie | Medium PCA | | | | | |
|---|---|---|---|---|---|---|---|
| | | Conidium size | | Septa | | Conidiophore size | |
| | | Length (μm) | Width (μm) | Transverse | Longitudinal | Length (μm) | Width (μm) |
| CBS 916.96[a] | *A. alternata* | 27.5 ± 4.7 (20.9 - 40.2) | 10.1 ± 2.1 (6.5 - 14.6) | 3.4 ± 0.9 (2 - 6) | 3.4 ± 1.0 (0 - 3) | 42.5 ± 19.1 (18.6 - 78.5) | 4.1 ± 0.4 (3.3 5.1) |
| UFVAssp-04 | *A. alternata* | 24.8 ± 4.4 (17.4 - 37.7) | 8.5 ± 1.3 (5.9 - 11.4) | 3.8 ± 0.6 (3 - 5) | 3.8 ± 0.8 (0 - 3) | 53.9 ± 18.4 (17.9 - 88.8) | 4.3 ± 0.7 (3.2 - 6.2) |
| UFVAssp-239 | *A. arborescens* | 27.4 ± 7.4 (13.4 - 40.4) | 8.9 ± 1.5 (5.9 - 12.2) | 3.8 ± 1.1 (2 - 6) | 3.8 ± 0.4 (0 - 2) | 97.2 ± 62.0 (34.3 - 279.4) | 4.5 ± 0.5 (3.6 - 5.7) |
| UFVAssp-26 | *Alternaria* sp. | 20.2 ± 4.9 (13.8 - 35.3) | 9.5 ± 1.9 (3.4 - 12.8) | 2.8 ± 1.2 (1 - 5) | 2.8 ± 0.8 (0 - 3) | 142.2 ± 37.3 (64.9 - 211.5) | 4.3 ± 0.5 (3.4 - 5.7) |
| UFVAssp-302 | *Alternaria* sp. | 27.3 ± 6.9 (15.7 - 42.1) | 9.1 ± 2.1 (6.5 - 15.1) | 3.7 ± 1.2 (1 - 6) | 3.7 ± 0.5 (0 - 2) | 104.4 ± 44.6 (43.0 - 190.1) | 4.2 ± 0.6 (3.3 - 5.4) |
| UFVAssp-97 | A. alternata | 25.5 ± 5.4 (18.2 - 37.4) | 9.7 ± 2.5 (4.6 - 14.5) | 3.2 ± 1.1 (0 - 5) | 3.2 ± 0.6 (0 - 2) | 77.0 ± 32.3 (21.4 - 134.2) | 3.8 ± 0.5 (2.9 -5.0) |

[a] reference *A. alternata* from Simmons (2007).

The dimensions of the conidia of *A*. *alternata* isolates UFVAssp-04 and UFVAssp-97 varied from 15.8 to 37.4 x 4.6 to 14.5 μm (length × width). The conspicuous beak presented by some replicates was considered when measuring the length of the conidium. The number of transverse septa ranged from 0 to 5, while the longitudinal septa ranged from 0 to 3 (**Table 2**). The conidia measuring 11.2 to 143.3 x 2.8 to 6.2 μm (length × width) were mostly formed in branched and rarely unbranched chains, supported by short primal conidiophores. The conidia dimensions of isolates UFVAssp-26 and UFVAssp-302 varied from 11.6 to 45.1 x 3.4 to 12.5 μm (length × width). The number of transverse septa and longitudinal septa varied from 1 to 6 and 0 to 3, respectively. The conidia were produced in branched chains, and the dimensions of the long primary conidiophores varied from 11.7 to 235 x 3.2 to 5.7 μm (length × width). The conidia of *A*. *arborescens* UFVAssp-239 measured 13.4 to 43.2 x 5.9 to 14.6 μm (length × width). The number of transverse septa ranged from 0 to 5, while occasionally longitudinal septa were present (**Table 2**). Conidia were produced in chains with a higher degree of branching and supported by long primary conidiophores measuring 16.9 to 279.4 µm x 3.2 to 5.7 µm (length × width).

The frequency distributions of the PCA culture medium show greater overlap compared to V8 for each of the morphological character assessed (**Figure 8A, 8B, 8C**). The length of the conidia was frequently observed in the range of 20 to 30 µm, higher than the frequency of the width of the conidia of 7 to 10 µm, characterizing the shape of the conidia as ellipsoid or ovoid. For all isolates, longitudinal septa were less frequent compared to transverse ones.

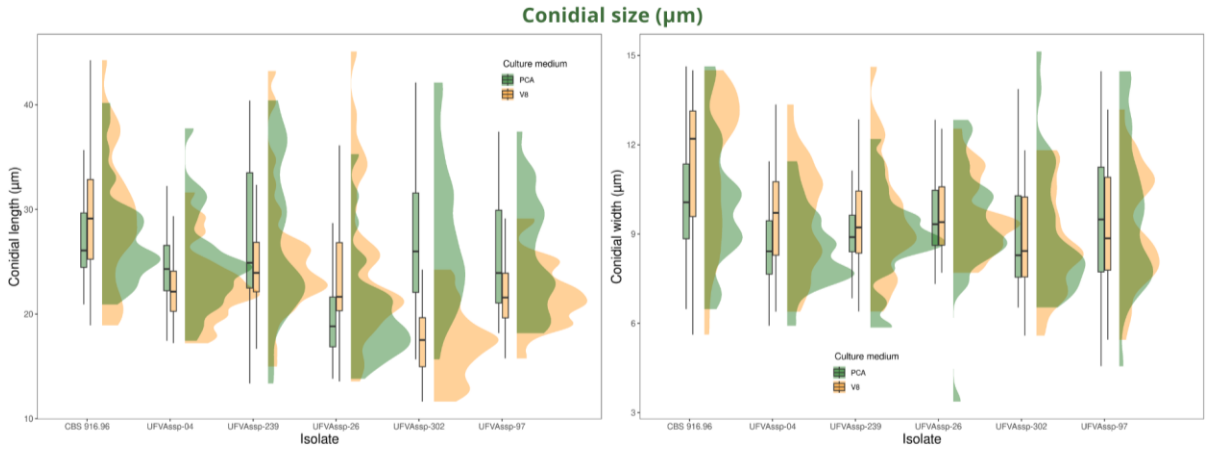

**Figure 8A** - Frequency distribution of the conidial size measured for the identification of small-spored *Alternaria* isolates (n = 30) grown in PCA or V8 medium.

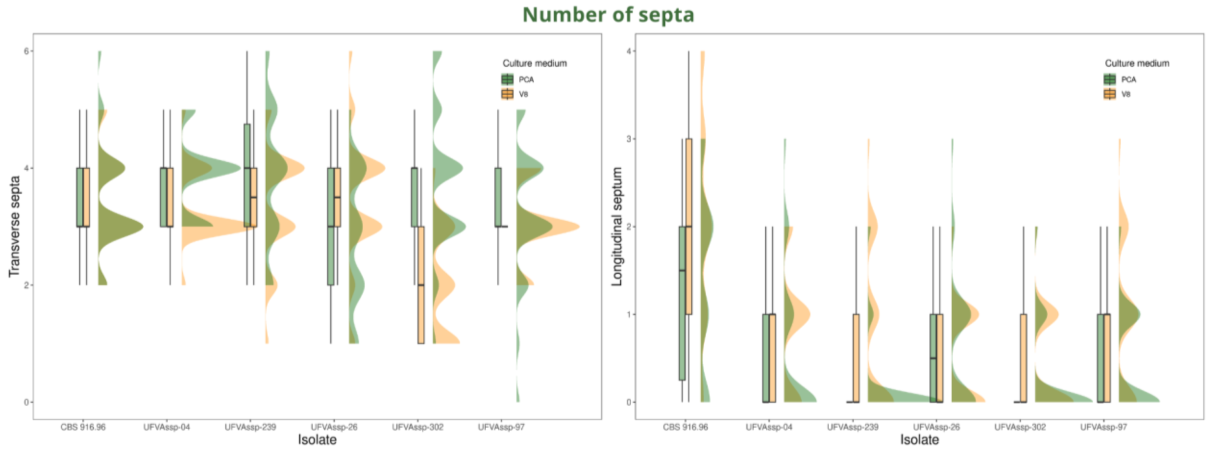

**Figure 8B** - Frequency distribution of the number of septa measured for the identification of small-spored *Alternaria* isolates (n = 30) grown in PCA or V8 medium.

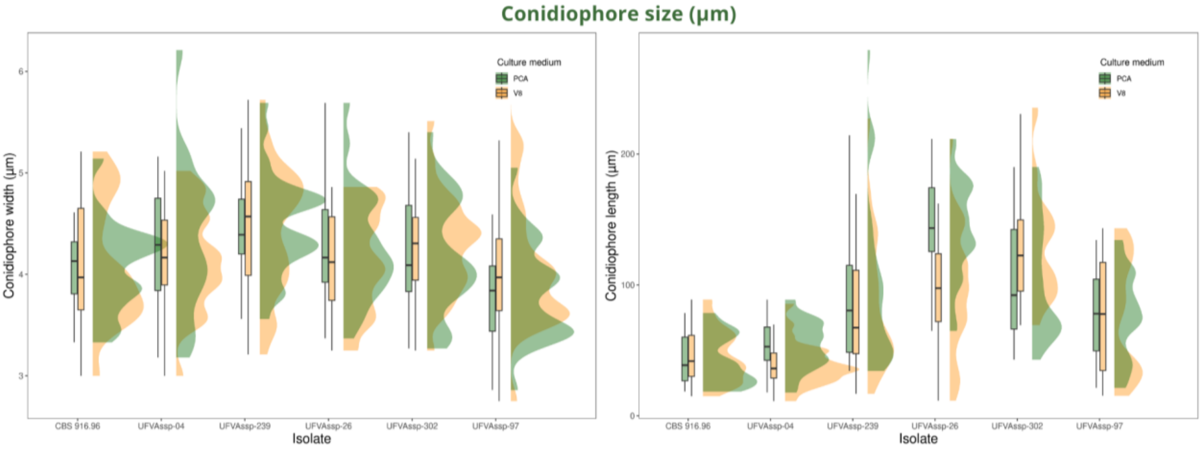

**Figure 8C** - Frequency distribution of the conidiophore size measured for the identification of small-spored *Alternaria* isolates (n = 30) grown in PCA or V8 medium.

The cultural characteristics of the colonies are presented in **Table 3**. Differences in growth on PCA or V8 media in relation to colony diameter were significant ($p \leq 0.05$). In PCA, the highest growth was observed for isolate UFVAssp-04, average diameter of 72.8 mm (69.1 - 75 mm), while the lowest growth was observed for UFVAssp-26 and UFVAssp-302, with average diameter of the colonies of 54.2 mm (53 - 58.8 mm) and 55 mm (52.7 - 56.5 mm), respectively. In V8, the isolate UFVAssp-239 stood out with the largest colony diameter 73.3 mm (71.1 - 74.7 mm), while UFVAssp-97 had the lowest growth 68.3 mm (64.1 - 71.7 mm).

Colony morphology varied on both media. All colonies produced aerial mycelia with color changes as they grew on the plate (sectors) (**Figure S6**).

**Table 3:** Measurement of mycelial growth of small spored *Alternaria* in V8 and PCA medium.

| **Isolate** | | **Mycelial growth (mm)** | |
|---|---|---|---|
| | | **Medium PCA** | **Medium V8** |
| UFVAssp-04 | *A. alternata* | 72.8 ± 2.6 (69.1 - 75.0) | 72.0 ± 1.7 (70.0 - 73.9) |
| UFVAssp-26 | *Alternata* sp. | 54.2 ± 1.3 (53.0 - 55.8) | 69.0 ± 1.8 (67.9 - 71.7) |
| UFVAssp-97 | *A. alternata* | 72.6 ± 1.0 (71.2 - 73.5) | 68.3 ± 3.2 (64.1 - 71.7) |
| UFVAssp-239 | *A. arborescens* | 62.3 ± 4.1 (58.1 - 66.4) | 73.3 ± 1.6 (71.1 - 74.7) |
| UFVAssp-302 | *Alternata* sp. | 55.0 ± 1.6 (52.7 - 56.5) | 70.2 ± 1.9 (67.8 - 72.4) |
| CBS 916.96[a] | *A. alternata* | 70.0 ± 0.4 (69.6 - 70.3) | 61.0 ± 1.3 (59.6 - 62.2) |

[a] reference *A. alternata* from Simmons (2007).

The coloration of the aerial mycelium was visually evaluated without scale, and three shades were assigned: shades of gray (light gray/dark gray), shades of olive green (light/dark), and a whitish tone.

In PCA, the colonies formed compact mycelia with 2–3 concentric sectors according to the hue. Isolate UFVAssp-04 showed less abundant aerial mycelium with an irregular whitish border, followed by a light olive green sector and dark olive green center, unlike isolate UFVAssp-97, which had a thin, well-defined whitish border. Isolate UFVAssp-97 showed a change in hue that was less pronounced, with a light olive green color dominant in the colony. The isolates UFVAssp-26 and UFVAssp-303 showed similarity in the color of the colony, the edge presented a thin white layer with little distinction, followed by an extensive light gray sector, and in the center, a light olive green. Isolate UFVAssp-239 had a very thin and clearly defined whitish border, followed by a light olive green sector and a dark olive green center.

In V8, isolates form dense, cottony aerial mycelia with 2-3 concentric sectors of different shades. Isolate UFVAssp-04 had a regular and well-defined whitish border, followed by another dark gray sector. Isolate UFVAssp-97, had a less accentuated light gray border that extended to the center, with a smaller dark gray sector. The isolate UFVAssp-26 had the most whitish and cottony margin in relation to the other isolates, followed by a light gray sector and another dark gray sector located in the center of the colony. UFVAssp-302 showed a whitish border followed by a light gray color extending to the center of the colony. The UFVAssp-239 isolate showed a very thin and clearly defined whitish border, followed by a dark gray color towards the center.

## Discussion

This is the first study carried out with small-spored *Alternaria* isolates obtained from potato leaves with symptoms of brown spot collected in the main Brazilian potato-producing states. Based on molecular phylogenetic analyses of a subset of 16 unidentified, pathogenic to potato, small-spored isolates resulted in 13 isolates identified to the species level as *A*. *alternata*. One isolate of *A*. *arborescens* (UFVAssp-239) and two isolates of *Alternaria* sp. (UFVAssp-26 and UFVAssp-302) that grouped closely to *A. gaisen* and *A. alstroemeriae* were also identified and were pathogenic to detached leaves of potato. Thus, 57 of the 59 isolates of small-spored *Alternaria* were pathogenic. Koch's postulates were completed when the assay was conducted using detached leaves.

Variation in pathogenicity of small-spored *Alternaria* isolates can be due to the inoculation method, whether on whole-plants, detached-leaf or leaf disks. Stammler et al. (2014) and Leiminger et al. (2015) demonstrated that *A*. *alternata* was unable to infect potato leaves in whole-plants, however, pathogenicity was ascertained using inoculation in whole plants and symptoms appeared between 15 and 20 days after inoculation (Droby et al. 1984; Boiteux and Richinaider 1994; van der Waals et al. 2011; Taheri et al. 2019; Bessadat et al. 2020; Lingwal et al. 2022; Choi et al. 2022). In detached-leaf trials, *A. alternata* was unable to cause lesions while *A. solani* promptly infected the host (Spoelder et al. 2014). On the other hand, several reports demonstrated the ability of *A. alternata*, *A. tenuissima* and *A. arborescens* to infect detached leaves, with symptoms appearing three days after inoculation (Droby et al. 1984, Tymon et al. 2016a; Zheng et al. 2015; Ding et al. 2019a; Lingwal et al. 2022). Tymon et al. (2016a) evaluated the effect of wounding at the inoculation site in leaves still attached to whole plants and also on detached leaves. The species examined were *A. arborescens* and *A. arbusti* and wounding was required for symptoms to occur. The virulence of *A. alternata* and *A. arborescens* were tested on leaf disc and were clearly capable of causing lesions, although no apparent disease progression was observed (Vandercaslee 2018).

Unlike other studies with small-spored *Alternaria* on potatoes, the isolates in the current work caused infections without the need of wounding the plant tissue (Tymon et al. 2016). The fact that the pathogenicity was demonstrated without tissue injury contributes to strengthen the evidence that the small-spored isolates were indeed the etiological agent of potato brown spot. Nevertheless, no symptoms developed when whole-plants were inoculated. Pathogenicity only in the detached-leaf test can be explained by the fact that the removal of these leaves induced senescence of tissues, favoring colonization. It is well-known that *Alternaria* species are prevalent in senescent or old tissues (Barnes 1979; Thomma 2003). Despite the fact that physiologically mature potato plants were used for the pathogenicity assay, the right conditions for infection may not have been achieved for infection to occur.

The quantification of the different epidemiological components on a detached leaf using several isolates increases the reliability of the results. After an extensive review, no studies with epidemiological components of potato brown spot caused by small-spored *Alternaria* were found. For large-spored *Alternaria* species causing early blight there are several studies about epidemiological components, mainly to quantify levels of resistance (Pelletier 1989; Dita Rodriguez et al. 2006; Cardoso 2010; Abuley et al. 2018). The variation in IF, IP, and LP seem to point to differential aggressiveness among isolates and adaptation variation of these isolates within populations of small-spored *Alternaria* in Brazil. Although small in magnitude, the variation in IF among the small-spored isolates was significant. The IF was significantly higher for *A. grandis* (UFVAg-638) (100% infection), confirming its high adaptation to potatoes. The incubation period values observed in our work were similar to those reported in other studies. Symptoms were observed 2 to 3 days after inoculation in detached leaf assays (Droby et al. 1984; Singwal et al. 2022). The ability of the pathogen to rapidly colonize tissues of hosts gives it a greater advantage in adverse environmental conditions (Suassuna et al. 2004). Brazilian populations of small-spored *Alternaria* also rapidly colonized potato tissues, possibly giving them a greater epidemiological advantage under changing environmental conditions.

Latent period is one of the most important epidemiological components due to its relationship with the number of generations of the pathogen (Suassuna et al. 2004). There was variation among the isolates regarding LP. For some isolates the LP was shorter than the IP, suggesting that there is also variation in the way these isolates colonize the host. The LP was significantly high for more virulent isolates, including *A. grandis* (UFVAg-638), but no spores were formed on leaves. Interestingly, for some small-spored *Alternaria* isolates spores were formed in inoculated areas that did not develop necrotic lesions. This phenomenon has been recorded in *A. alternata* pathosystems reinforcing that the pathogen can colonize the host epiphytically or even internally as endophyte, establishing an asymptomatic symbiosis with the plants (Lawrence et al. 2016; DeMers 2022).

Quantitative differences in epidemiological components may be related to the genetic structure of the small-spored *Alternaria* populations and contribute to assessing the low specificity of potatoes to these small-spored *Alternaria* species in Brazil. In China, studies have shown the simultaneous presence in potato production areas of populations of *A. solani* and *A. alternata* with different genetic structures (Meng et al. 2015b, 2018), and there may be an exchange of genetic material between large-spored and small-spored *Alternaria* species. In addition, variations inherent to the presence of selective host toxins (HST) must be considered, which are vital in the pathogenesis process and contribute to the virulence of *A. alternata* isolates (Hatta et al. 2002; Meena and Samal 2019). The genes responsible for HST biosynthesis in *A.*

*alternata* are located in accessory chromosomes that can be transmitted by horizontal transfer between different lineages, favoring the emergence of small-spored *Alternaria* with new specificities and variations in virulence profiles (Akagi et al. 2009; Hou et al. 2016; Wang et al. 2019). Horizontal transfer of virulence genes was demonstrated for *A. arborescens*. It was postulated that this may be involved in the evolutionary diversification of this species in gaining pathogenicity to potato and acquiring host-selective toxin genes ( Akagi et al. 2009; Hu et al. al. 2012; Tymon et al 2016b).

Based on morphological and multigenic phylogenetic analyses, three small-spored *Alternaria* species that cause brown spot in Brazil could be identified: *A. alternata*, *A. arborescens* and a potential new species of *Alternaria*. However, at this point no new species proposition will be put forth, but rather, the individuals will be referred to as *Alternaria* sp. since complementary analyses are needed to properly conclude about the identification of the isolates

The *Alt a 1*, *Gapdh*, ITS, *Rpb2*, *Tef1*, OPA10-2, and *EndoPG* genomic regions have been widely used for taxonomic studies of small-spored *Alternaria* spp. (Tymon et al. 2016; Landschoot et al. 2017; Ding et al. 2019; Singwal et al. 2022). Six loci, *Alt a 1*, *Gapdh*, ITS, *Rpb2*, *Tef1*, and OPA10-2 were used to distinguish species in sect. *Alternaria* (Woudenberg et al. 2015). In addition to isolates of the *Alternaria* section, representative isolates of small-spored *Alternaria* pathogenic to potato from other sections were included in analysis, such as *A. arbusti* in sect. *Infectoria* (Tymon et al. 2016b) and *A. telliensis* in sect. *Japonicae* (Bessadat et al. 2020). Even though there are no reports of species infecting potatoes in the sect. *Brassicicola* sequences of isolates of this section were added because it harbors small-spored species.

Single-gene phylogenies based on partial sequences of ITS and *Gapdh* had a reduced number of polymorphisms which resulted in a low discriminative power to delineate small-spored *Alternaria* isolates within the sect. *Alternaria*. The individual topologies of these two genes could not distinguish species of *A. arborescens* - AASC from *A. alternata* as previously reported elsewhere (Woudenberg et al., 2015 , Zheng et al., 2015, Landschoot et al. 2017a; Ding et al. 2019a). These regions are frequently used in the classification and identification of fungi (Woudenberg et al. 2013, Lawrence et al. 2013), but for *Alternaria* species they have proven more effective in distinguishing only between small-spored and large-spored individuals (Landschoot et al. 2017a; Ding et al. 2019a). Partial sequences of OPA10-2 and *Tef1* were not informative enough for the sorting of the isolates pathogenic to potato. The anonymous region

OPA10-2 that encodes a protein with unknown function had a reasonable number of polymorphisms and is used in taxonomical studies, however, amplification was successful for only half of the isolates studied. There was low polymorphism in the *Tef1* region and few subclusters within the small-spored *Alternaria* cluster were detected in the analysis. Limited resolution of this gene has been reported and it is considered as not powerful enough to differentiate *Alternaria* sections (Lawrence et al. 2013). Despite having lower resolution than other genes, *Tef1* was used because it was able to separate the *A*. *alternata* species complex from other species within the *Alternaria* section (Woudenberg et al. 2015). In the present work, this gene was able to separate *A*. *alternata* from *A. arborescens* species complex (AASC)

The partial sequences of the genes *Alt a 1* and *Rpb2* resulted in the highest percentages of informative characters and the reconstructed phylogenetic trees presented the highest Bayesian posterior probabilities. *Alt a 1* is an allergen produced by *Alternaria* species and is associated with human asthma (Hong et al. 2005). This gene evolved rapidly but is highly preserved among *Alternaria* species, so it is a good genomic region to be used in the taxonomy and classification of *A. alternata* (Hong et al. 2005; Teifoori et al. 2019). In general, because these two regions had a greater number of informative sites, a greater number of subclusters is usually obtained in phylogenetic analysis and allow to separate *A*. *alternata* from other species within the *Alternaria* section.

After analyzing each gene individually, a concatenated dataset with six genes, *Alt a 1*; *Gapdh*; ITS; *Rpb2*; *Tef1*; and OPA10-2 was constructed and the phylogenetic analysis was run and compared with another concatenated dataset that contained the *Alt a 1*, *Rpb2*, *Tef1* genes that had resulted in highest resolution for the separation of species within the sect. *Alternaria*. The topology of the tree constructed for the six-gene concatenated dataset showed a lower resolution in separating species compared to the three-gene tree. However, there are some similar subclusters between the two topologies that are supported by high PP values.

The consensus tree of six concatenated genes revealed that small-spored *Alternaria* isolates collected from potatoes were grouped into the *A. alternata* group, and one isolate grouped in a clade of the *A. arborescens* species complex (AASC). For the analysis of the three-gene concatenated dataset, the isolates were spread into three branches and 13 isolates (UFVAssp-02, UFVAssp-04, UFVAssp-10, UFVAssp-22, UFVAssp-51, UFVAssp-66, UFVAssp-75, UFVAssp-97, UFVAssp-112, UFVAssp-

225, UFVAssp-310, UFVAssp-323, UFVAssp-332) grouped with *A. alternata* reference isolates; one isolate (UFVAssp-239) grouped in the AASC, as in the previous analysis, and two isolates (UFVAssp-26 and UFVAssp-302 ) grouped into a separate clade. The multigene phylogeny with the three-gene dataset showed better resolution and better-supported branches. However, the inconsistencies observed in the phylogenetic analysis of single genes, such as *Gapdh*, ITS, *Tef1* and OPA10-2, together with the analysis of the six-gene concatenated dataset are not sufficient to distinguish the species within the sect. *Alternaria* using this dataset. Both the phylogenetic analysis of the individual *Alta 1* and Rpb2 regions and the three-gene dataset indicated a strong phylogenetic reconstruction and can be used for molecular characterization of unknown strains.

Morphological analysis revealed few, but important differences among the small-spored *Alternaria* that were pathogenic to potato. In the past, the taxonomy of the genus *Alternaria* was based mainly on morphological characteristics such as conidia color, number of septa, conidia size and wall structure; beak size and type; size of conidiophores; cultural characteristics and sporulation arrangements (Simmons and Roberts 1993; Simmons 2007; Woudenberg et al. 2015). Here, the isolates grew on PCA and V8 media and were identified based on the morphological characters described by Simmons (2007): evaluation of the macrostructure such as color and growth of the colony, and of the microstructures, such as size and shape of the conidiophores and conidia, septation number, and chain ornamentation.

Overlapping of characters among taxa were common. However, morphological variations associated with conidiophore size and chain branching degree provided support for the separation of the two phylogenetic lineages that we identified in sect. *Alternaria*. The species identified as *A. alternata* had short conidiophores and produced single or branched chains, while the *A. arborescens* isolate had long conidiophores and a larger cluster of conidia in branched chains with an arborescent appearance. Morphological observations, in particular the sporulation pattern, allowed the differentiation of *A. alternata* and *A. arborescens* isolates in other studies (Simmons and Roberts 1993 ; Simmons 1995; Simmons 2007; Woudenerg et al. 2013; Landschoot et al. 2017a).

Morphological analyses to distinguish *Alternaria* spp. has been questioned because the characteristics used are plastic and may be affected by environmental variation (Andrew et al. 2009; Tymon et al. 2016a). However, in the present study, the morphological analysis provided important support to choose the results of the three-gene phylogeny and the identification of the small-spored *Alternaria* species pathogenic to potato.

In summary, this study contributed to the identification of small-spored *Alternaria* individuals as etiological agents of the brown spot of potato using a combination of morphological, pathological and molecular analyses. In addition to *A. alternata*, *A. arborescens* and a putatively new species are causing brown spot in potato fields in Brazil. *A*. *alternata* and *A*. *arborescens* have also been identified causing brown spot in potato fields in Europe. Although morphology is able to distinguish between distinct taxa of small-spored *Alternaria*, our results suggest that DNA sequences are important to elucidate the speciation processes underlying the *Alternaria* associated with potato plants.

## Supplementary material

**TABLE S1:** Location and number of small-spored *Alternaria* isolates collected from diseased potato leaves in Brazil.

| Isolate - code[1] | Location[2] | Date of Collection |
|---|---|---|
| UFVAssp-002* | Chapada Diamantina - BA | June 2020 |
| UFVAssp-004** | Chapada Diamantina - BA | June 2020 |
| UFVAssp-007 | Ponta Grossa - PR | October 2020 |
| UFVAssp-010* | Patrocínio - MG | February 2020 |
| UFVAssp-022* | Patrocínio - MG | February 2020 |
| UFVAssp-026** | Perdizes - MG | February 2020 |
| UFVAssp-032 | Uberaba - MG | February 2020 |
| UFVAssp-034 | Uberaba - MG | February 2020 |
| UFVAssp-040 | Sacramento - MG | February 2020 |
| UFVAssp-042 | Perdizes - MG | February 2020 |
| UFVAssp-051* | São Gotardo - MG | February 2020 |
| UFVAssp-056 | Rio Paranaíba - MG | February 2020 |
| UFVAssp-063 | Rio Paranaíba - MG | February 2020 |
| UFVAssp-066* | São Francisco de Paula - RS | January 2020 |
| UFVAssp-075* | Guarapuava - PR | January 2020 |
| UFVAssp-083 | Guarapuava - PR | January 2020 |
| UFVAssp-085 | Contenda - MG | February 2020 |
| UFVAssp-087 | Andradas - MG | April 2020 |
| UFVAssp-088 | Andradas - MG | April 2020 |
| UFVAssp-091 | São Francisco de Paula - RS | May 2020 |
| UFVAssp-094 | Bom Jesus - RS | May 2020 |
| UFVAssp-097** | Morrinhos - GO | May 2020 |
| UFVAssp-100 | Buri - SP | June 2020 |
| UFVAssp-104 | Cristalina - GO | July 2020 |
| UFVAssp-108 | Munhoz - MG | April 2020 |
| UFVAssp-112* | Guarapuava - PR | July 2020 |
| UFVAssp-135 | Itaverava - MG | November 2020 |
| UFVAssp-136 | Conselheiro Lafaiete - MG | November 2020 |

| | | |
|---|---|---|
| UFVAssp-139 | Ouro Branco - MG | November 2020 |
| UFVAssp-142 | Senador Amaral - MG | November 2020 |
| UFVAssp-148 | Senador Amaral - MG | January 2020 |
| UFVAssp-151 | Camanducaia - MG | January 2020 |
| UFVAssp-162 | Camanducaia - MG | January 2020 |
| UFVAssp-171 | Munhoz - MG | February 2020 |
| UFVAssp-174 | Bueno Brandão - MG | February 2020 |
| UFVAssp-182 | Bom Repouso - MG | February 2020 |
| UFVAssp-191 | Ipuiúna - MG | March 2020 |
| UFVAssp-200 | Espírito Santo do Dourado - MG | March 2020 |
| UFVAssp-205 | Bom Jesus - RS | December 2020 |
| UFVAssp-209 | Bom Jesus - RS | December 2020 |
| UFVAssp-214 | São José dos Ausentes - RS | December 2020 |
| UFVAssp-217 | São Francisco de Paula - RS | December 2020 |
| UFVAssp-222 | Senador Amaral - MG | November 2020 |
| UFVAssp-225* | Irati - PR | December 2020 |
| UFVAssp-231 | Bueno Brandão - MG | December 2020 |
| UFVAssp-232 | São José dos Ausentes - RS | November 2020 |
| UFVAssp-238 | Palmas - PR | February 2021 |
| UFVAssp-239** | Água Doce - SC | January 2021 |
| UFVAssp-240 | Água Doce - SC | January 2021 |
| UFVAssp-242 | Divinolândia - SP | January 2021 |
| UFVAssp-302** | Mucugê - BA | March 2021 |
| UFVAssp-306 | Guarapuava - PR | March 2021 |
| UFVAssp-310* | Leme - SP | March 2021 |
| UFVAssp-312 | Holambra - SP | March 2021 |
| UFVAssp-323* | Holambra - SP | March 2021 |
| UFVAssp-329 | Rio Paranaíba - MG | March 2021 |
| UFVAssp-332* | Cristalina - GO | July 2021 |
| UFVAssp-335 | Cristalina - GO | July 2021 |
| UFVAssp-340 | Cristalina - GO | July 2021 |
| UFVAa-628 | Rio Paranaíba - MG | March 2017 |

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[1] UFVAssp = code of the culture collection "*Alternaria* small spored" (Assp) of the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil);

[2] Location refers to states of Bahia (BA), Goiás (GO), Minas Gerais (MG), Paraná (PR), Rio Grande do Sul (RS), Santa Catarina (SC) and São Paulo (SP);

* Isolate used in phylogenetic analysis;

** Isolate used in morphological characterization.

**TABLE S3:** Analysis of Variance (ANOVA) of potato brown spot in detached-leaf.

| **Source of variation** | **Infection frequency** | | | **Experiment 1 Incubation period** | | | **Experiment 2 Incubation period** | | | **Latent period** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **DF** | **F** | ***P*** | **DF** | **F** | ***P*** | **DF** | **F** | ***P*** | **DF** | **F** | ***P*** |
| Isolate | 63 | 3.69 | 4.38e-$^{16}$ ***** | 63 | 1.69 | 0.003***** | 63 | 1.03 | 0.41 | 63 | 3.62 | 4.3e-$^{12}$ ***** |
| Residuals | 448 | | | 192 | | | 192 | | | 192 | | |

* Significant *P* values

**TABLE S2:** GenBank accession number of *Alternaria* species sequences used in this study.

| [1]**Species name and strain number** | **Country/ Host / Substrate** | [2] **GenBank accession number** | | | | | |
|---|---|---|---|---|---|---|---|
| | | ITS | gapdh | tef1 | rpb2 | Alta-1 | OPA10-2 |
| ***Sect. Alternantherae*** | | | | | | | |
| *A.alternantherae* CBS 124392; | China, *Solanum melongena* | KC584179 | KC584096 | KC584633 | KC584374 | KP123846 | - |
| ***Sect. Alternaria*** | | | | | | | |
| *A. alstroemeriae* CBS 118808 | EUA, *Alstroemeria sp.* | KP124296 | KP124153 | KP125071 | KP124764 | KP123845 | KP124601 |
| *A. alstroemeriae* CBS 118809 | Australia, *Alstroemeria sp* | KP124297 | KP124154 | KP125072 | KP124765 | - | KP124602 |
| *A. alternata* CBS 118814 | USA, *Solanum lycopersicum* | KP124357 | KP124211 | KP125133 | KP124825 | KP123906 | KP124669 |
| *A. alternata* CBS 595.93 | Japan, *Pyrus pyrifolia* | KP124320 | KP124175 | KP125096 | KP124788 | JQ646399 | KP124627 |
| *A. alternata* CBS 118812 | USA, *Daucus carota* | KC584193 | KC584112 | KC584652 | KC584393 | KP123905 | KP124668 |
| *A. alternata* CBS 102598 | USA, *Minneola tangelo* | KP124329 | KP124184 | KP125105 | KP124797 | KP123878 | KP124638 |
| *A. alternata* CBS 102603 | Israel, *Minneola tangelo* | KP124333 | KP124188 | KP125109 | KP124801 | KP123882 | KP124642 |
| *A. alternata* CBS 102604 | Israel, *Minneola tangelo* | KP124334 | AY562410 | KP125110 | KP124802 | AY563305 | KP124643 |
| *A. alternata* CBS 918.96 | UK, *Dianthus chinensis* | AF347032 | AY278809 | KC584693 | KC584435 | AY563302 | KP124633 |
| *A. alternata* CBS 620.83 | USA, *Nicotiana tabacum* | KP124315 | KP124171 | KP125091 | KP124783 | KP123868 | KP124622 |
| *A. alternata* CBS 115152; | China, *Psychotria serpens* | KP124348 | KP124202 | KP125124 | KP124816 | KP123896 | KP124658 |
| *A. alternata* CBS 916.96 | India, *Arachis hypogaea* | AF347031 | AY278808 | KC584634 | KC584375 | AY563301 | KP124632 |
| *A. alternata* CBS 115199 | South Africa, *Minneola tangelo* | KP124351 | KP124205 | KP125127 | KP124819 | KP123899 | KP124661 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *A. alternata* CBS 102600 | USA, *Citrus reticulata* | KP124331 | KP124186 | KP125107 | KP124799 | KP123880 | KP124640 |
| *A. alternata* CBS 102.47 | USA, *Citrus sinensis* | KP124304 | KP124161 | KP125080 | KP124773 | KP123855 | KP124610 |
| *A. alternata* CBS 115188 | South Africa, *Citrus clementina* | KP124349 | KP124203 | KP125125 | KP124817 | KP123897 | KP124659 |
| A. cerealis CBS 119544 (**AASC**) | New Zealand, *Avena sativa* | KP124408 | JQ646321 | KP125186 | KP124878 | KP123955 | KP124722 |
| A. senecionicola CBS 119545 (**AASC**) | New Zealand, *Senecio skirrhodon* | KP124409 | KP124260 | KP125187 | KP124879 | KP123956 | KP124723 |
| *A. geophila* CBS 101.13 (**AASC**) | Switzerland, *peat soil* | KP124392 | KP124244 | KP125170 | KP124862 | KP123940 | KP124705 |
| *A. arborescens* CBS 102605 (**AASC**) | USA, *Solanum lycopersicum* | AF347033 | AY278810 | KC584636 | KC584377 | AY563303 | KP124712 |
| *A. arborescens* CBS 105.24 (**AASC**) | Unknown, *Solanum tuberosum* | KP124393 | KP124245 | KP125171 | KP124863 | KP123941 | KP124706 |
| *A. arborescens* CBS 124283 (**AASC**) | Russia, *Oryza* sp. | KP124416 | KP124267 | KP125194 | KP124885 | KP123963 | KP124730 |
| *A. arborescens* CBS 117587 (**AASC**) | Netherlands, *Brassica* sp. | KP124406 | KP124258 | KP125184 | KP124876 | KP123953 | KP124720 |
| *A. arborescens* CBS 115189 (**AASC**) | South Africa, *Citrus clementina* | KP124402 | KP124254 | KP125180 | KP124872 | KP123949 | KP124716 |
| *A. arborescens* CBS 112633 (**AASC**) | South Africa, *Malus domestica* | KP124400 | KP124252 | KP125178 | KP124870 | KP123947 | KP124714 |
| *A. arborescens* CBS 109730 (**AASC**) | USA, *Solanum lycopersicum* | KP124399 | KP124251 | KP125177 | KP124869 | KP123946 | KP124713 |
| *A. betae-kenyensis* CBS 118810 | Kenya, *Beta vulgaris* var. *cicla* | KP124419 | KP124270 | KP125197 | KP124888 | KP123966 | KP124733 |
| *Alternaria burnsii* CBS 118816 | India, *Rhizophora mucronata* | KP124423 | KP124273 | KP125201 | KP124892 | KP123970 | KP124737 |
| *Alternaria burnsii* CBS 107.38 | India, *Cuminum cyminum* | KP124420 | JQ646305 | KP125198 | KP124889 | KP123967 | KP124734 |
| *Alternaria burnsii* CBS 110.50 | Mozambique, *Gossypium* sp. | KP124421 | KP124271 | KP125199 | KP124890 | KP123968 | KP124735 |
| *A. eichhorniae* CBS 489.92 | India, *Eichhornia crassipes* | KC146356 | KP124276 | KP125204 | KP124895 | KP123973 | KP124740 |
| *A. eichhorniae* CBS 119778 | Indonesia, *Eichhornia crassipes* | KP124426 | KP124277 | KP125205 | KP124896 | - | KP124741 |
| *A. gaisen* CBS 118488 | Japan, *Pyrus pyrifolia* | KP124427 | KP124278 | KP125206 | KP124897 | KP123975 | KP124743 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *A. gaisen* CBS 632.93 | Japan, *Pyrus pyrifolia* | KC584197 | KC584116 | KC584658 | KC584399 | KP123974 | KP124742 |
| *A. gaisen* CPC 25268 | Portugal, unknown | KP124428 | KP124279 | KP125207 | KP124898 | KP123976 | KP124744 |
| *A. gossypina* CBS 100.23 | Unknown, *Malus domestica* | KP124429 | KP124280 | KP125208 | KP124899 | KP123977 | KP124745 |
| *A. gossypina* CBS 107.36 | Indonesia, soil | KP124431 | JQ646310 | KP125210 | KP124901 | JQ646393 | KP124747 |
| *A. gossypina* CBS 102597 | USA, *Minneola tangelo* | KP124432 | KP124281 | KP125211 | KP124902 | KP123978 | KP124748 |
| *A. iridiaustralis* CBS 118486 | Australia, *Iris* sp | KP124435 | KP124284 | KP125214 | KP124905 | KP123981 | KP124751 |
| *A. iridiaustralis* CBS 118404 | New Zealand, *Iris* sp. | KP124434 | KP124283 | KP125213 | KP124904 | KP123980 | KP124750 |
| *A. iridiaustralis* CBS 118487 | Australia, *Iris* sp. | KP124436 | KP124285 | KP125215 | KP124906 | KP123982 | KP124752 |
| *A. jacinthicola* CBS 878.95 | Mauritius, *Arachis hypogaea* | KP124437 | KP124286 | KP125216 | KP124907 | KP123983 | KP124753 |
| *A. jacinthicola* CBS 133751 | Mali, *Eichhornia crassipes* | KP124438 | KP124287 | KP125217 | KP124908 | KP123984 | KP124754 |
| *A. jacinthicola* CPC 25267 | Unknown, *Cucumis melo* | KP124439 | KP124288 | KP125218 | KP124909 | KP123985 | KP124755 |
| *A. longipes* CBS 113.35 | Unknown, *Nicotiana tabacum* | KP124440 | KP124289 | KP125219 | KP124910 | KP123986 | KP124756 |
| *A. longipes* CBS 540.94 | USA, *Nicotiana tabacum* | AY278835 | AY278811 | KC584667 | KC584409 | AY563304 | KP124758 |
| *A. longipes* CBS 121332 | USA, *Nicotiana tabacum* | KP124443 | KP124292 | - | KP124913 | KP123989 | KP124760 |
| *A. prunicola* MFLUCC 18-1596 | China, *Cherry* sp. | MH827033 | MH853647 | MH853705 | MH853720 | MH853694 | - |
| *A. prunicola* MFLUCC 18-1597 | China, *Cherry* sp. | MH827035 | MH853649 | MH853707 | MH853722 | MH853696 | - |
| *A. pseudoeichhorniae* MFLUCC 18-1589 | China, *Cherry* sp. | MH827030 | MH853644 | MH853702 | MH853717 | - | - |
| *A. tomato* CBS 114.35 | Unknown, *Solanum lycopersicum* | KP124446 | KP124295 | KP125225 | KP124916 | KP123992 | KP124763 |
| *A. tomato* CBS 103.30 | Unknown, *Solanum lycopersicum* | KP124445 | KP124294 | KP125224 | KP124915 | KP123991 | KP124762 |
| **Sect. Brassicicola** | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *A. brassicicola* CBS 118699 | USA, *Brassica oleracea* | - | KC584103 | KC584642 | KC584383 | - | - |
| *A.mimicula* CBS 118696 | USA, *Lycopersicon esculentum* | NR_135959 | - | KC584669 | KC584411 | - | - |
| *A. solidaccana* CBS118698 | Bangladesh, soil | NR_136049 | KC584141 | KC584690 | KC584432 | - | - |
| *A. septorioides* CBS106.41 | Netherlands, *Reseda odorata* | KC584216 | KC584136 | KC584685 | KC584427 | - | - |
| **Sect. Infectoriae** | | | | | | | |
| *A. arbusti* CBS 596.93 | USA, *Pyrus pyrifolia* | - | FJ214806.1 | - | - | - | - |
| *A. arbusti* EGS 91-129 | Japan, *Pyrus pyrifolia* | JQ693643 | JQ693621 | - | - | - | - |
| *A. arbusti* EGS 91-136 | USA, *Pyrus pyrifolia* | JQ693644 | JQ646365 | - | - | - | - |
| *A. ethzedia* CBS 197.86 | Switzerland, *Brassica napus* | AF392987 | AY278795 | KC584657 | KC584398 | AY563284.1 | - |
| *A. infectoria* CBS 210.86 | UK, *Triticum aestivum* | DQ323697 | AY278793 | KC584662 | KC584404 | FJ266502.1 | - |
| **Sect. Japonicae** | | | | | | | |
| *A. japonica* CBS 118390 | USA, *Brassica chinensis* | KC584201 | KC584121 | KC584663 | KC584405 | - | - |
| *A. nepalensis* CBS 118700 | Nepal, *Brassica* sp. | KC584207 | KC584126 | KC584672 | KC584414 | - | - |
| *A. telliensis* CBS 145643 | Algeria, *Solanum* sp. | MT013035 | MK904523 | MK904550 | MK904536 | MK940315.1 | |
| **Sect. Porri** | | | | | | | |
| *Alternaria grandis* CBS 116695 | USA, *Solanum tuberosum* | KJ718241 | KJ718070 | KJ718587 | KJ718416 | KJ718748 | |

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[1] CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Personal collection of P.W. Crous, Utrecht, The Netherlands; E.G.S.: Personal collection of Dr. E.G. Simmons; MFLUCC: Mae Fah Luang University Culture Collection, Thailand.

[2] Accession numbers in Genbak; - : no product

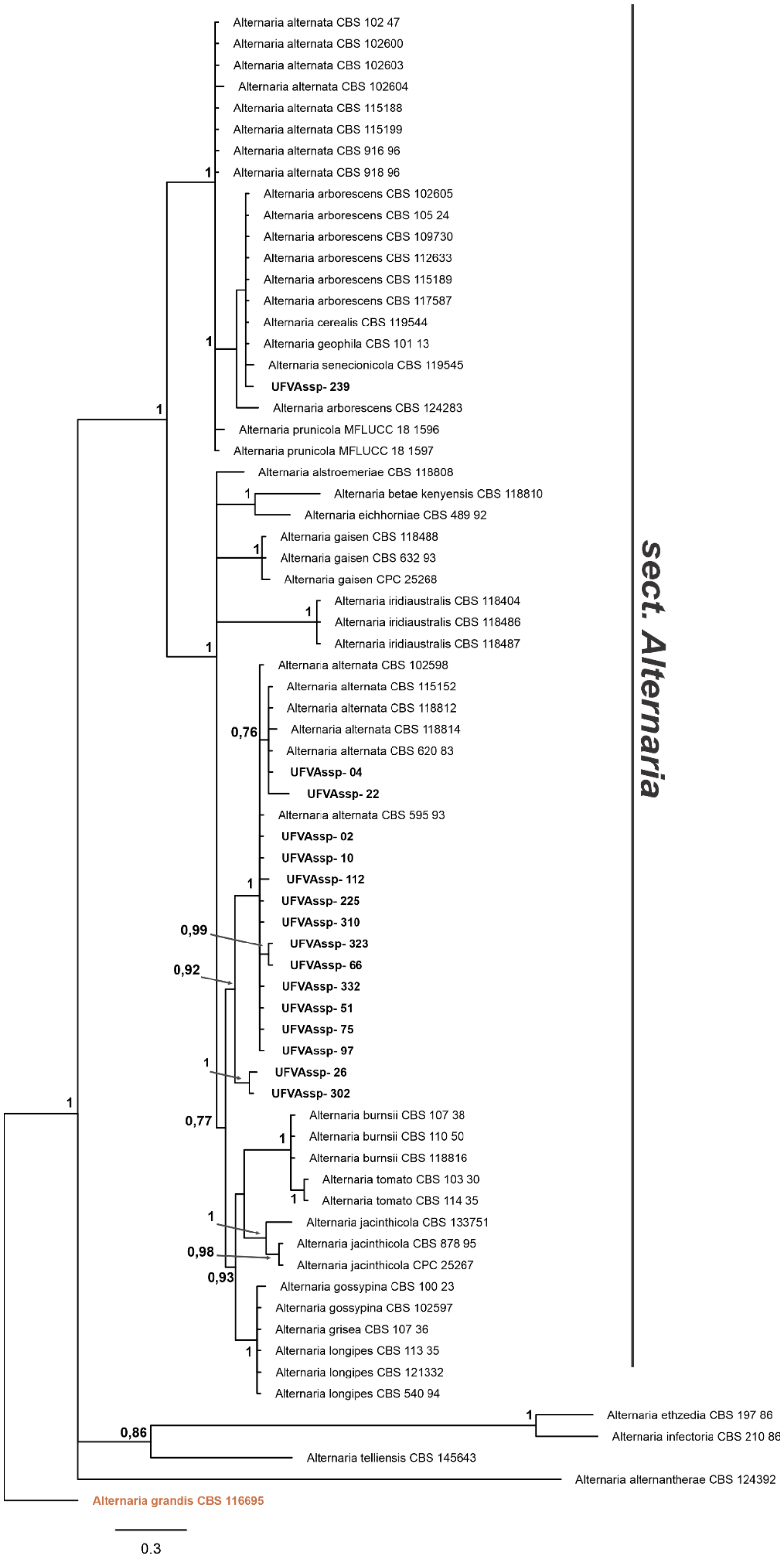

**Figure S1:** Consensus tree constructed based on the *Alt a 1* of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.75 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site.

**Figure S2:** Consensus tree constructed based on the *Gapdh* of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.75 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site.

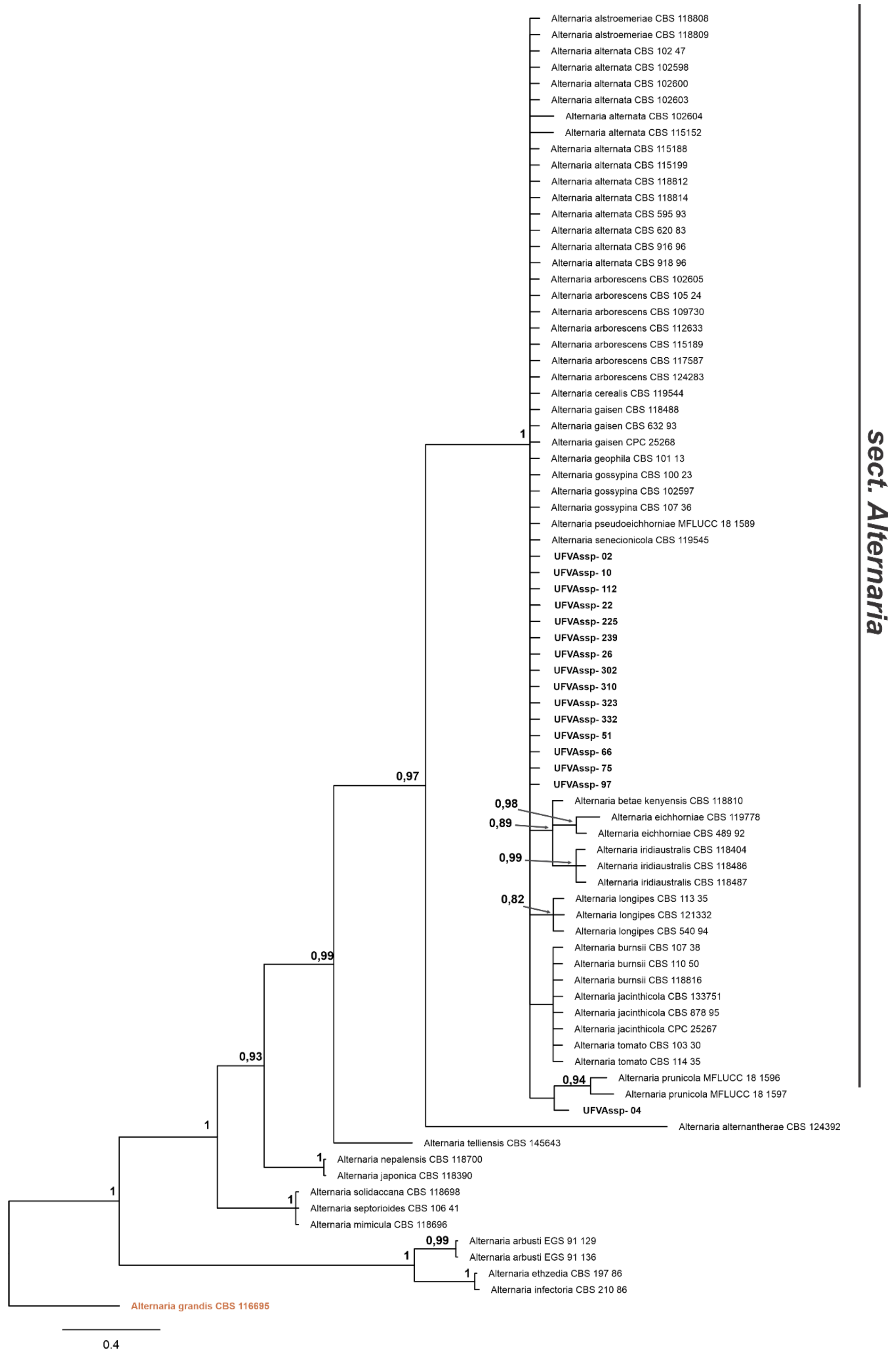

**Figure S3:** Consensus tree constructed based on the ITS of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.75 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site.

**Figure S4:** Consensus tree constructed based on the *Rpb2* of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.75 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site.

**Figure S5:** Consensus tree constructed based on the *Tef1* of the 16 small-spored *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in the tree). Bayesian posterior probabilities (%) were also calculated and values are shown after the slash at each node >0.75 (PP). The tree was rooted in *A. grandis* (CBS 116695). The scale bar represents the expected number of substitutions per site.

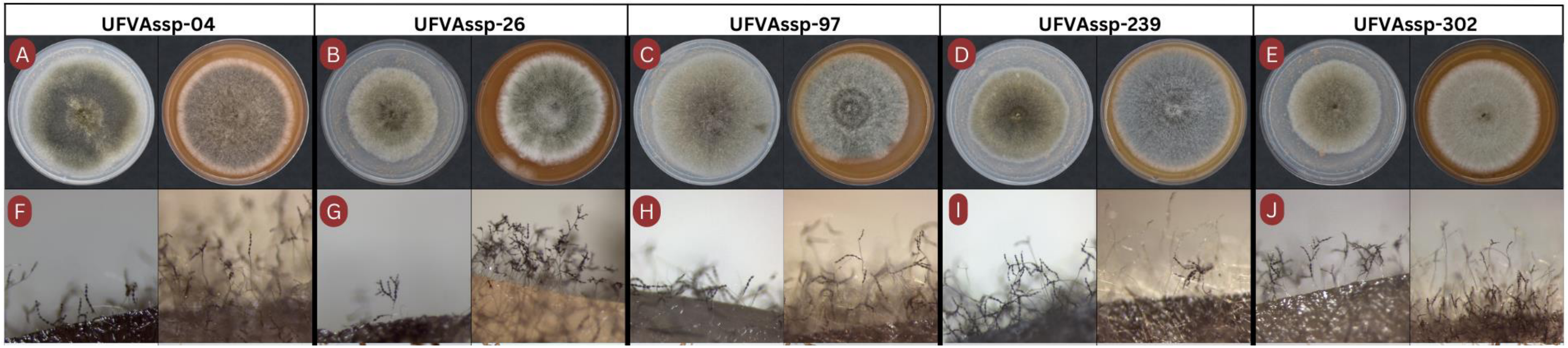

**Figure S6:** Morphological characteristics of small-spored *Alternaria* isolated from potato: *A. alternata* (UFVAssp-04, UFVAssp-97) *A. arborescens* (UFVAssp-239) and *Alternaria* sp. (UFVAssp-26, UFVAssp-302). A to E, colony morphology in PCA and V8 after 7 days under cold white fluorescent light with 8 h photoperiod and 16 h darkness; F-J spore chain branches in PCA and V8.

# Chapter 2: Mycelial growth, sporulation and germination at different temperatures of isolates of small-spored *Alternaria* pathogenic to potato

## Abstract

Potato brown spot has gained the attention of growers and consultants due to the increase in the occurrence of epidemics across the country. Allegedly, climate change is causing higher temperatures to be recorded in potato-producing regions in Brazil, contributing to an increase in the frequency and severity of brown spot epidemics in potato-producing regions. So far, there is no information on the effects of temperature on the basic epidemiological/ecological variables associated with small-spored *Alternaria* species in Brazil. Therefore, this study aimed to evaluate the influence of different temperatures on mycelial growth, sporulation, and germination of small-spored *Alternaria* isolates from the main potato-producing regions. Temperature affected both mycelial growth, sporulation and conidial germination. The highest values for most phenotypic traits evaluated were in the range of 25 and 30 °C. Growth patterns varied among small-spored species. *Alternaria alternata* grew faster than *A. arborescens* at all temperatures. This response was also observed for sporulation and conidial germination. There was no effect of the origin of the isolate on the response to temperature, for all variables. Thus, the severity of brown spot may vary according to the distribution of the small-spored *Alternaria* species.

## Introduction

Until recently, brown spot, caused by small-spored *Alternaria* species complex, was commonly known in most potato-growing regions as a mild disease, occurring only in aged tissues (Rotem 1994; Lawrence et al. 2013). However, climate change caused by global warming has contributed to an increase in records of the expansion of these small-spored taxa into new hosts and their wider distribution in potato-growing regions around the world (Kirk and Wharton 2012; Dube 2014; Ahmed 2017).

The plant-pathogen interaction is a multifaceted process, mediated by the pathogen, plant, and environment (Marcuzzo et al. 2020; Fagodiya et al. 2022). In the host, the environment can affect the vegetative and reproductive development and the genetic susceptibility of the plants. Regarding the pathogen, the environment influences survival, development rate, sporulation, direction and distance of pathogen dispersal, as well as the rate of germination and penetration of spores into plant tissues (Agrios, 2005). The most important environmental factors for the development of epidemics are humidity and temperature (Escuredo et al. 2019; Fagodiya et al. 2022).

Among the factors that make up climate, temperature is one of the main drivers for the distribution, growth, and survival of plant pathogens (Agrios, 2005). Changes in expansion areas, infection patterns, and the severity of various agricultural pests have marked the current scenario as a result of climate change caused by rising temperatures (Meng et al. 2015a; Escuredo et al. 2019). Therefore, it may be appropriate to state that temperature is the most frequently investigated variable in climate change biology research related to plant pathogens and crop disease risks (Juroszek et al. 2020).

The use of phenotypic markers such as mycelial growth rate, sporulation and germination have also been used to assess the influence of temperature on variability within populations of fungal species (Arauz and Sutton 1989; Olanya et al. 2009; Mannaa and Kim 2018; Kumar Bais et al. 2019). This environmental range allows *Alternaria* species to create specific strategies to colonize and develop in the hosts, causing problems in different crops of economic importance (Töfoli et al. 2019), such as the potato crop.

The optimum temperature for fungal growth covers a wide range of temperatures usually varying from 10 to 40 °C. Usually, the optimal growth temperature for most fungi is in the range of 25-30 °C (Magan 2007). For *A. alternata*, there are reports of optimal temperature for growth ranging from 25 to 30 °C (Pose et al. 2009). The sporulation rate of *Alternaria* isolates was differently influenced by temperature (Vloutoglou and Kalogerakis 2000; Escuredo et al. 2019) and high numbers of *Alternaria* spores were found when cultures were grown at 20 to 30 °C (Escuredo et al. 2019; Camiletti et al. 2022). Temperature is one of the most important environmental factors influencing conidial germination. Not only the number of conidia that germinate is affected, but temperature also affects the velocity of germination and the elongation of the germ tube (Vloutoglou et al. 1996). The effects of temperature on conidial germination of different *Alternaria* species have been studied in detail, *in vitro* or *in vivo* (Rotem, 1994; Vloutoglou et al. 1996; Escuredo et al. 2019; Fagodiya et al. 2022). For most *Alternaria* species, the optimal germination temperature is around 25°C (Rotem, 1994). More specifically, for *A. alternata* isolates the optimal temperature varies between 25 and 30 °C (Hatzipapas et al. 2002; Camiletti et al. 2022). Furthermore, non-germinated conidia of *Alternaria* species are considered very resistant to unfavorable environmental conditions (Rotem, 1994). When temperature is not in a favorable range, conidia can stay viable and resume germination when optimal temperatures occur.

Quantifying the variability in response to temperature in populations of plant pathogens can help understand differences in epidemiological processes such as disease progress rate and final disease severity. In Brazil, the average temperatures in potato producing regions are probably higher than those recorded in other regions in temperate climate countries during the crop season. Higher temperatures may be contributing to the increased frequency and severity of brown spot epidemics in Brazil. However, very little is known about this pathosystem and no studies have been performed to assess the basic epidemiological characteristics of potato brown spot. Therefore, the objective of this work was to evaluate the influence of different temperatures on mycelial growth, germination, and sporulation of small-spored *Alternaria* isolates collected in Minas Gerais, Paraná, São Paulo, Rio Grande do Sul, Santa Catarina, Goiás, and Bahia.

# Materials and methods

## Sampling and isolates

Fifteen small-spored Alternaria isolates, which were collected in the previous study, were employed in all the experiments. The isolates were obtained from samples of potato leaves with typical symptoms of brown spot, randomly sampled from commercial fields located in the main potato producing states of Brazil during the 2020 and 2021 growing seasons. One isolate *A. alternata* (UFVAssp-628) was collected in 2017, eight isolates were identified by molecular phylogenetic analysis as *A. alternata,* one as *A. arborescens* and one as *Alternaria* sp. new species (**Figure 1**).

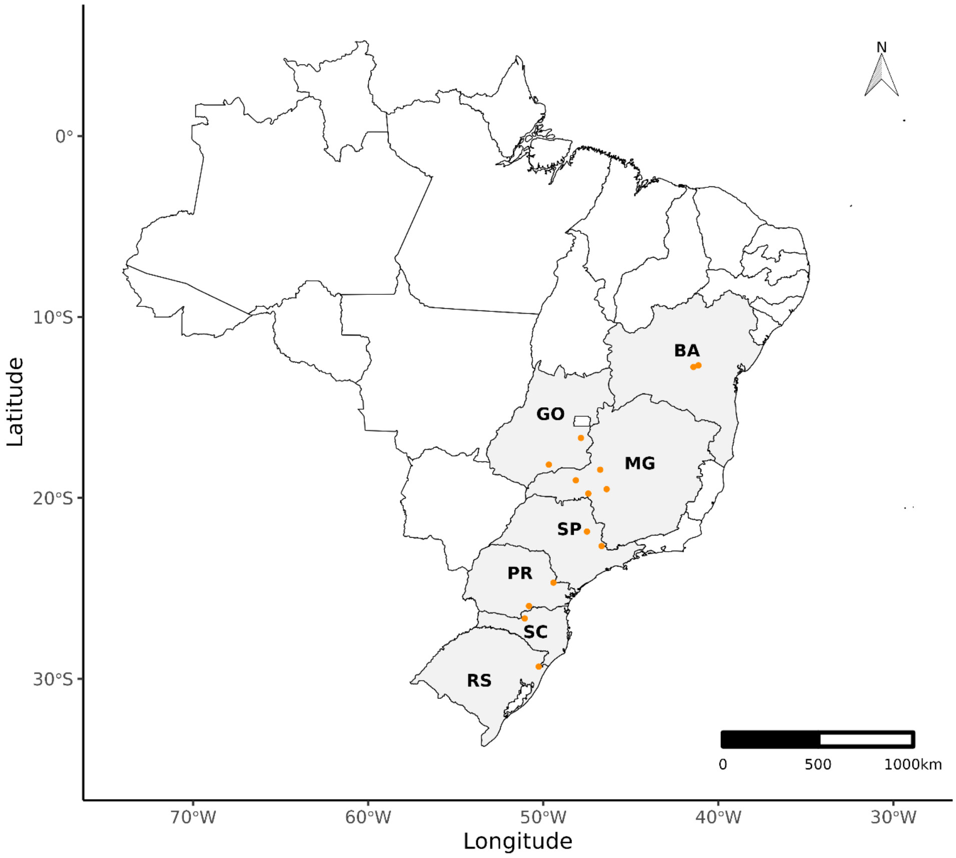

**Figure 1 -** Brazilian map showing small-spore Alternaria isolates that were sampled from potato leaves with brown spot symptoms in the main potato-producing states. The orange represent the isolates used in this study (n = 14). BA = Bahia, GO = Goiás, MG = Minas Gerais, SP = São Paulo, PR = Paraná, SC = Santa Catarina, RS = Rio Grande do Sul states.

## Mycelial growth at different temperatures

Mycelium discs (0.5 cm in diameter) from each isolate were removed from the margin of the colony at seven days of growth and transferred to the center of Petri plates containing potato dextrose agar (PDA) medium. The plates were kept in incubators at 15, 20, 25, 30, and 35 °C with 12h photoperiod. The incubators were stabilized at the respective temperatures 24 h before plates were transferred in. Colony diameter measurements using a digital caliper were performed daily until the 10th day of incubation. Colony diameter was assessed in two perpendicular directions. The experiment was performed twice.

### Sporulation at different temperatures

Four runs of the experiment were conducted to assess the effects of temperature on sporulation, two under "discontinuous incubation period" and two under “continuous” incubation period.

For the two runs under discontinuous incubation period the sporulation was assessed at the end of the period of measurements of colony diameter described above. Therefore, plates were removed from the incubators on a daily basis for one to two hours and this was considered as a discontinuation of the incubation conditions. For the two runs under continuous incubation, the plates were kept inside the incubators set at the different temperatures for seven days and sporulation was assessed at the end of the incubation period.

For both incubation conditions, mycelium discs (0.5 cm in diameter) from each isolate were removed from the margin of the colony at seven days of growth, transferred to the center of Petri plates containing PDA medium, and incubated as described above. Concomitantly, the influence of dehydration of the medium on sporulation was also evaluated under both "discontinuous" and "continuous" incubation conditions by maintaining the plates with and without sealing with two layers of PVC film. After 15 days of incubation, a total of 10 mL of sterilized distilled water was added to each Petri plate and the surface of the colony was scraped with a sterile Drigalski loop to suspend the conidia. The suspension obtained was filtered through gauze and a drop of 20 µL of lactoglycerol was added to prevent conidial germination. A 100 µL-aliquot of the conidia suspension from each isolate was placed in a clear, flat-bottomed 96-well microplate. Sterilized distilled water was used as a control. The sporulation of the isolates was estimated by assessing the concentration of conidia in suspension with a spectrophotometer based on the reading of absorbance at 600 nm (OD600). To calculate the relationship between sporulation and absorbance, the spore concentrations of some isolates at 25°C were measured in a Neubauer chamber, performing the spore count in five quadrants of the chamber for each plate (spores/cm²). The experiment was performed twice.

### Germination at different temperatures

A 10 µL-aliquot of spore suspension from each isolate was placed on sterile slides. The slides were distributed in plastic boxes (Gerbox) (11 cm length x 11 cm width x 3,5 cm height) lined with moistened paper towels and kept at 15, 20, 25, 30, and 35 °C. After 4 h, a drop of lactoglycerol was added to each slide to stop the germination process. The number of germinated conidia was counted from a total of 100 conidia arbitrarily selected per slide. A conidium was considered germinated when the length of its primary germ tube was at least half of its width. The experiment was performed twice.

### Experimental design and data analysis

The mycelial growth and germination at different temperatures experiments were set in a completely randomized design, in a 15 (small-spored *Alternaria* isolates) x 5 (temperature) factorial treatment design, with four replicates (plates). A replicate was considered as a combination of isolate and temperature. The sporulation at different temperatures was set in a completely randomized design, in a 15 (small-spored *Alternaria* isolates) x 2 (sealed and unsealed plates) x 5 (temperature) factorial treatment design, with four replications. All experiments were subjected to analysis of variance with software R version 4.2.2 (R Development Core Team, 2021).

## Results

**Mycelial growth at different temperatures.** Mycelial growth of small-spored *Alternaria* isolates was analyzed after 7 days of incubation at all temperatures. Temperature influenced the mycelial growth rate of representative isolates (**Figure 2**). All isolates grew on PDA when incubated at temperatures ranging from 15 to 30 °C, and then decreased sharply with increasing temperature. Isolates of *A. alternata* showed different average growth than *A. arborescens* at all evaluated temperatures. At 25 °C, UFVAssp-75 had the highest mean colony diameter among the *A. alternata* isolates, 55.6 mm, whereas for *A. arborescens* UFVAssp-239, the mean colony diameter was 30.2 mm. Maximum values of colony diameter for *A. alternata* and *A. arborescens* were recorded at 25 or 30 °C.

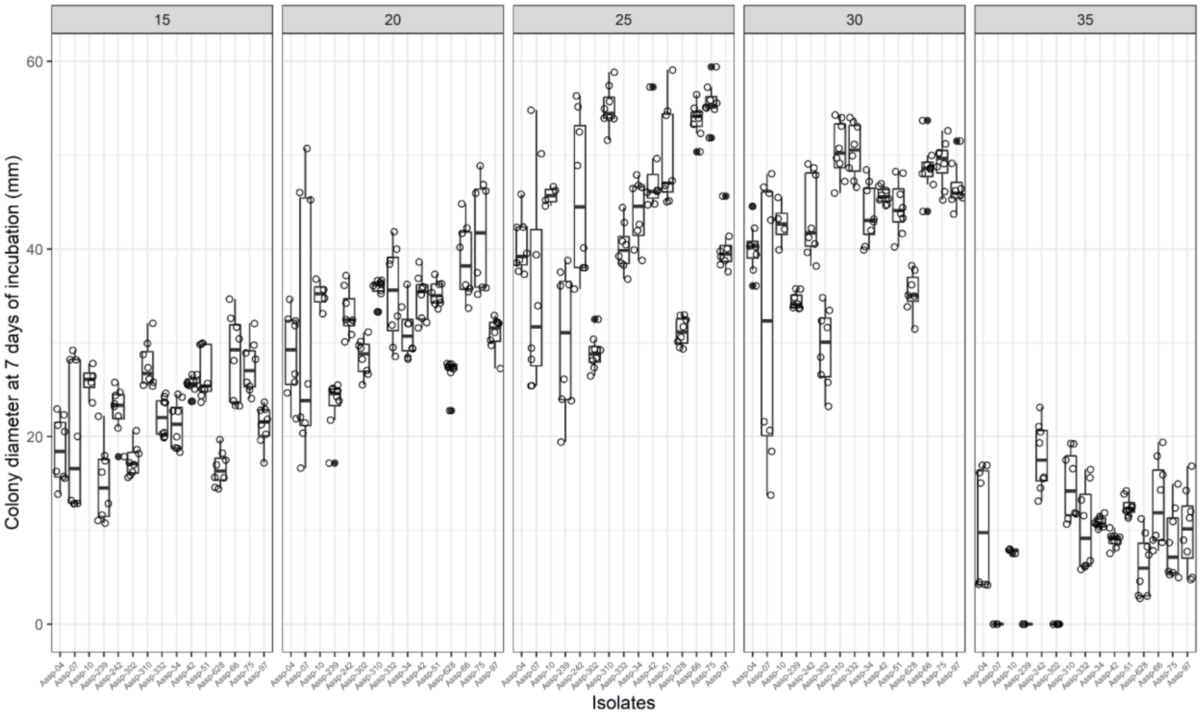

**Figure 2**: Boxplots for the values of colony diameter measured at seven days of incubation for small-spored *Alternaria* isolates grown at different temperatures. Data from the two experiments were pooled for analysis.

**Sporulation at different temperatures.** In two experiments, the highest sporulation under discontinuous incubation was observed at 15°C (**Figure 3**). In the "continuous incubation" experiment, greatest sporulation was observed at 25°C and 30°C (**Figure 4**). Regardless of the incubation condition, no sporulation was found at 35°C. In all experiments, the mean sporulation of *A. alternata* was higher than that of *A. arborescens*.

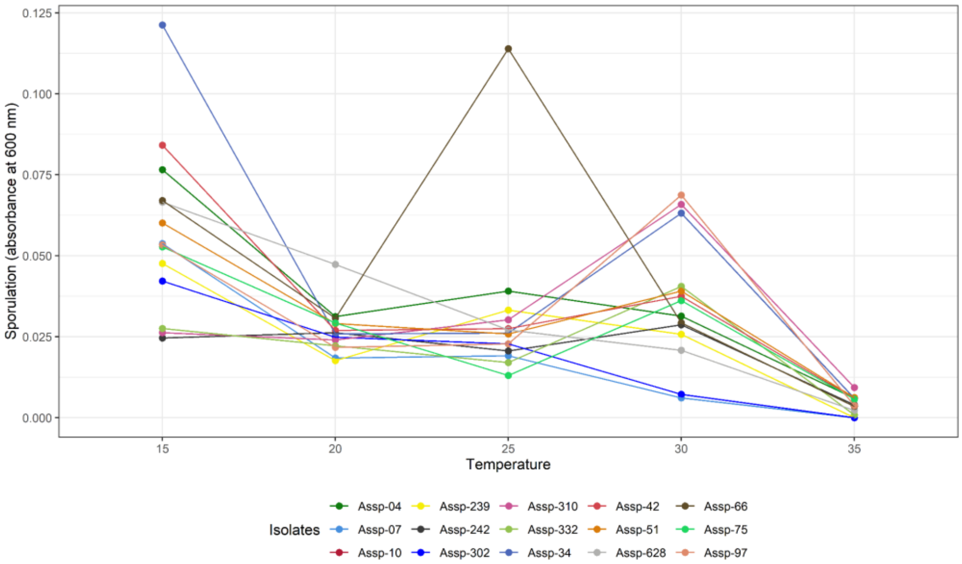

**Figure 3.** Sporulation of small-spored *Alternaria* isolates under discontinuous incubation at different temperatures. The points represent the average of sporulation in relation to the interaction between the variables.

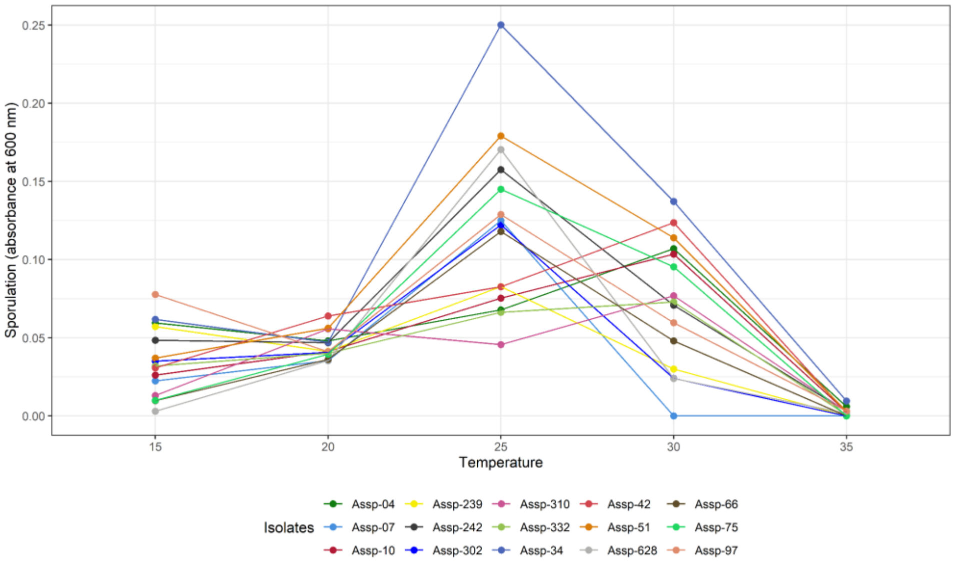

**Figure 4.** Sporulation of small-spored *Alternaria* isolates under continuous incubation at different temperatures. The points represent the average of sporulation in relation to the interaction between the variables.

**Germination at different temperatures.** The germination of the conidia of the representative isolates occurred in 4 h at all temperatures. The germination percentage was affected by the incubation temperature (**Figure 5**). The percentage of germinated conidia increased at 25°C until 30°C and reduced after this point. All representative isolates responded similarly to temperature and no differences were observed between *A*. *alternata* and *A*. *arborescens* isolates.

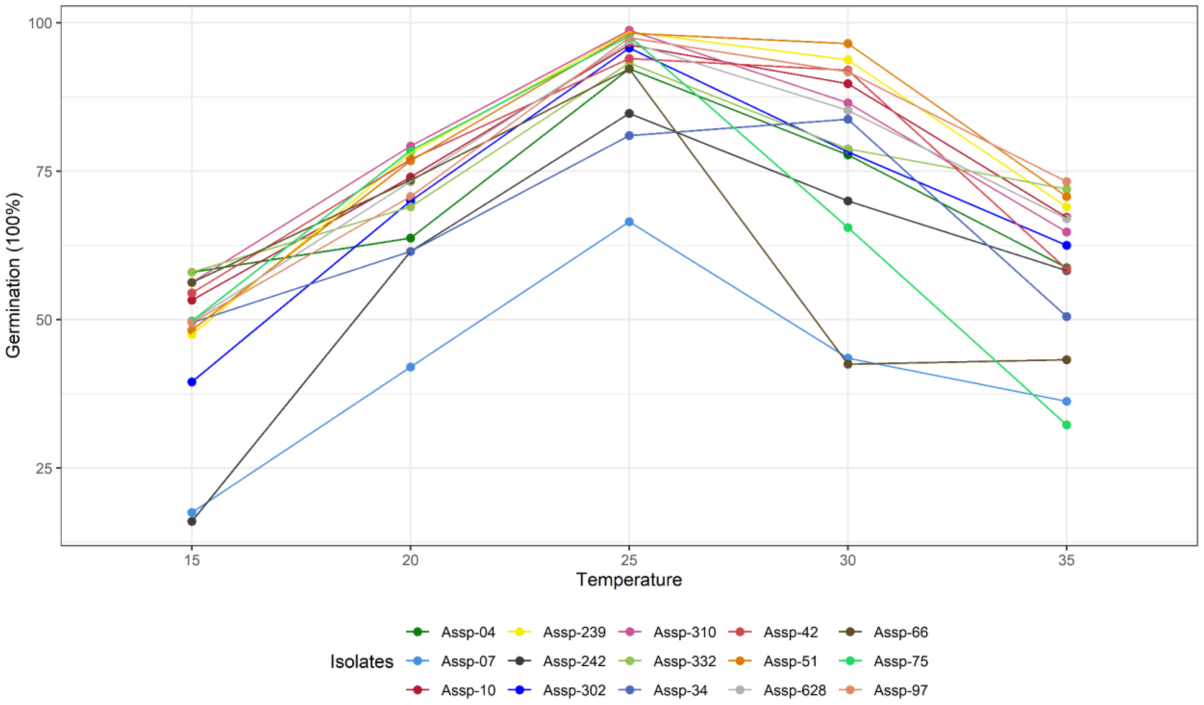

**Figure 4.** Germination of small-spored *Alternaria* isolates after incubation at different temperatures. The points represent the average germination percentage of each isolate, at each temperature.

## Discussion

Climate change is predicted to have direct effects on disease severity in crops, with implications for food security (Gautam et al. 2013). Epidemics caused by *Alternaria* species may be strongly influenced by environmental conditions (Camilleti et al. 2021). This study provides preliminary evidence that small-spored *Alternaria* species that cause brown spot on potatoes in Brazil are favored by relatively high temperatures. Even though experiments were conducted under controlled conditions, the present study is the first epidemiological investigation ever done with *Alternaria* spp. causing brown spot in the country.

The response of the mycelial growth to different temperatures is a criterion used to characterize *Alternaria* species (Andersen et al. 2005; Oviedo et al. 2010; Pose et al. 2009; Zhu and Xiao 2015; Camiletti et al. 2022). For *A. alternata*, the reported optimal temperature for growth varies between 25 and 30 °C (Rotem 1994; Pose et al. 2009). Similar response pattern and similar temperature range were reported in the current study and suggest that small-spored *Alternaria* species collected in different

Brazilian regions are capable of growing at slightly high temperatures. Isolates obtained from Bahia state, 13.00º S latitude, to further south in Rio Grande do Sul State, 28.00º S, appear not to be differentially affected by temperature.

A contrasting response in the growth rates of Brazilian isolates of *A. alternata* and *A. arborescens* were detected when isolates were kept at different temperatures. *A. alternata* grew faster than *A. arborescens* and this response was similar to that described in other studies in which individuals of the two species were analyzed (Wang et al. 2021; Harteveld et al. 2014; Zhu and Xiao 2015; Camiletti et al. 2022). Higher growth percentages of *A*. *alternata* at a wide range of temperature may be associated with the broad distribution of this species. The high prevalence of *A*. *alternata* in brown spot lesions is reported in many areas, including in Brazil, as revealed in the present study. Furthermore, the capacity to grow faster under wide temperature conditions may contribute to better compete with and even displace other small-spored *Alternaria* species pathogenic to potato.

The sporulation percentage of small-spored *Alternaria* isolates was directly influenced by both temperature and incubation mode. Limited information is available in the literature on the effect of temperature on sporulation. In general, the optimal temperatures for sporulation between different *Alternaria* species vary from 15 to 30 °C (Rotem, 1994). The results presented herein did not demonstrate a clear differentiation regarding the optimum temperature in the sporulation of *A. alternata* and *A. arborescens*, since the incubation method significantly altered the behavior of sporulation in response to different temperatures. However, sporulation of *A. alternata* was more abundant than *A. arborescens*, which may explain its higher prevalence in potato crops in different regions, although more studies are needed to confirm this hypothesis.

Temperature is one of the most important environmental factors influencing the germination of *Alternaria* conidia. Temperature affects the percentage of conidia that germinate as well as the time required for germination and the elongation of the germ tube (Vloutoglou et al. 1996). The effects of temperature on the germination percentage of conidia of other *Alternaria* spp. were studied in detail, *in vitro* or *in vivo*, (Degenhardt et al. 1982; Strandberg 1988; Rotem 1994; Biswas 2013), demonstrating that the optimal temperature for germination of *Alternaria* spp. is around 25°C (Rotem, 1994). For *A. alternata* isolates, optimal germination temperatures were found to vary from 25 to 30 °C (Hatzipapas et al. 2002). In our results, the highest germination rates were observed at these temperatures.

Under optimum temperatures, germination of conidia of most *Alternaria* species begins within 1 to 3 h (Vloutoglou et al. 1996; Hatzipapas et al. 2002). The germination of conidia of representative isolates of small-spored *Alternaria* was evaluated after 4 h of incubation at all temperatures studied. The relatively rapid germination process of the conidia of *Alternaria* may be advantageous for the pathogen since penetration and infection establishment can occur in a few hours. This may be even more relevant when the duration of the leaf wetness period is limited. If leaves are wet only for up to three or four hours, germination can occur and spores can infect the plant.

In conclusion, small-spored *Alternaria* isolates obtained from different states had similar response to temperature. For all phenotypic traits evaluated the ideal temperature varied between 25 and 30°C, except when incubation is under fluctuating conditions. Although estimated *in vitro*, these temperatures correspond to those typically found during the day, in the summer crop season, in regions where potatoes are grown in Brazil. Understanding the response of small-spored *Alternaria* species to temperature and the implications of this response to brown spot severity is important for developing brown spot management strategies. The growing number of new small-spored *Alternaria* species associated with potato brown spot could lead to more severe epidemics against a backdrop of rising global temperatures.

## General Conclusions

We demonstrated that more than one species of small-spored *Alternaria* can cause brown spot in Brazil. This finding emphasizes the complexity of the pathogenicity of these small-spored species and the need to understand the diversity of pathogens involved.

Variation in brown spot severity depends on the geographic distribution of small-spored *Alternaria* species in different potato-producing regions in Brazil. This variation in small-spored species distribution needs to be considered when planning and implementing disease control strategies in order to optimize the effectiveness of these measures.

Temperature has a significant impact on the biological characteristics of these pathogenic species, influencing mycelial growth, sporulation and germination of small-spored *Alternaria* isolates. This relationship between temperature and pathogen biology is valuable information for developing management strategies based on specific climatic conditions. Overall, this dissertation provides valuable information on the factors that influence the occurrence and severity of potato brown spot.

## Supplementary material

**Table S1:** Small-spored *Alternaria* isolates used in the study

| Isolate[a] | Species | Location (Municipality - State) | Date of Collection |
|---|---|---|---|
| UFVAssp-04 | *A. alternata* | Chapada Diamantina - BA | June 2020 |
| UFVAssp-07 | *Alternaria* sp. | Ponta Grossa - PR | October 2020 |
| UFVAssp-10 | *A. alternata* | Patrocínio - MG | February 2020 |
| UFVAssp-34 | *Alternaria* sp. | Uberaba - MG | February 2020 |
| UFVAssp-42 | *Alternaria* sp. | Perdizes - MG | February 2020 |
| UFVAssp-51 | *A. alternata* | São Gotardo - MG | February 2020 |
| UFVAssp-66 | *A. alternata* | São Francisco de Paula - RS | January 2020 |
| UFVAssp-75 | *A. alternata* | Guarapuava - PR | January 2020 |
| UFVAssp-97 | *Alternaria* sp. | Morrinhos - GO | June 2020 |
| UFVAssp-239 | *A. arborescens* | Água Doce - SC | February 2021 |
| UFVAssp-242 | *Alternaria* sp. | Divinolândia - SP | February 2021 |
| UFVAssp-302 | *Alternaria* sp.<br>new species | Mucugê - BA | June 2021 |
| UFVAssp-310 | *A. alternata* | Leme - SP | June 2021 |
| UFVAssp-332 | *A. alternata* | Cristalina - GO | July 2021 |
| UFVAssp-628 | *A. alternata* | Rio Paranaíba - MG | March 2017 |

[a] UFVAssp = code of the culture collection "*Alternaria* small spored" (UFVAssp) of the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil).

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