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Characterization of the brown leaf spots pathosystem in Brazilian pecan orchards: pathogen morphology and molecular identification

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Abstract Due to the increase in pecan nuts demand, plantation areas are expanding around the world and more frequent epidemics caused by fungal pathogens may occur in orchards and nurseries. *Ragnhildiana diffusa* is a pathogenic fungus reported to cause brown leaf spots on pecans in numerous countries. The scarcity of comprehensive information in symptoms on the host and morphology of the fungus lead this disease to be initially incorrectly identified in Brazil. In this study, we employed different approaches to characterize the pathogen morphology and pathogenicity and to molecularly identify the organism causing brown leaf spots in southern Brazil. A phylogenetic analysis based on the internal transcribed spacer (ITS) and the large subunit (LSU) gene sequences confirmed *R. diffusa* as the causal pathogen of the disease. Inoculation tests on healthy leaflets confirmed pathogenicity isolates, although some variation in their virulence was observed. Variation in the morphology of the asexual stage was observed among and within isolates. This study brought unprecedented morphological, genetic and pathogenic information that aids elucidating the disease. The accurate and prompt identification of the disease may assist in controlling further spread of the pathogen into orchards and nurseries still free of the disease in South America.

Keywords: Ascomycota; Mycosphaerellaceae; Plant protection; Tropics and Subtropics.

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Introduction

There is a positive forecast for continuous growth of the global demand for pecan nuts [*Carya illinoinensis* (Wangenh.) K. Koch] up to year 2030. Increasing pecan production in non-traditional countries including the South Africa, Australia, and China is also a major considerable factor for the growth of pecan market (PMR 2020). In South America, northeastern Argentina and southern Brazil are the main areas commercially producing pecan nut. In both countries, plantations of this crop are expanding annually, aiming to supply the intern and extern demands of pecan nuts (Madero et al. 2016, Trabichet et al. 2016, Poletto et al. 2018, Nagel et al. 2020, de Oliveira et al. 2021). As a result of the increase in nuts demand (around 1.5-fold up to year 2030, PMR 2020), plantation areas will further expand around the world, and more frequent epidemics caused by fungal pathogens may occur in both orchards and nurseries.

According to the International Association for the Plant Protection Sciences, "a fundamentally important starting point for plant protection is the ability to anticipate the emergence and spread of noxious organisms and to prevent their introduction and spread before they become agricultural pests in specific crops and regions" (www.plantprotection.org). Thus, the process of plant protection starts with the accurate diagnosis of the problem, by correctly identifying the organism responsible for the observed symptoms and injuries.

In Mexico, South Africa, and in the United States, *Ragnhildiana diffusa* (synonym: *Sirosporium diffusum*) has been reported causing brown leaf spots in pecan (Chupp 1953, Crous & Braun 2003). In Brazil, this disease was firstly observed in pecan in 2016 (Poletto et al. 2017), causing leaf spots and subsequent defoliation.Due to the lack of information, brown leaf spots in southern Brazilian pecan orchards were initially attributed to cercosporiosis, due to the similarity in symptoms on the host and morphology of the fungus. The symptoms of this disease can also be confused with those of Gnomonia leaf spots, a disease caused by *Gnomonia dispora* (Kluepfel et al. 2014). The occurrence of *R. diffusa* as the causal agent of brown leaf spots was confirmed in one pecan orchard in southern Brazil shortly after the first register of the disease (Poletto et al. 2017).

To properly perform a primary evaluation and characterization of pathogens, various aspects must be assessed including morphological characteristics of isolates of the fungus in culture, spore production, and mycelial growth (Dhingra & Sinclair 1995). Although the occurrence of *R. diffusa* was confirmed in one orchard, and a severity scale has been proposed for the disease (Poletto et al. 2020), there is scarce information on the symptoms associated with infection, or the morphological diversity exhibited by this pathogen in southern Brazil. This information shortage limits the management of the disease in the country and in neighbour lands where pecan is cultivated.

This study aimed at characterizing this pathosystem in different pecan orchards in southern Brazil, where plants symptomatic to brown leaf spots were reported. The morphological characteristics, the pathogenicity, and the virulence of each isolate was investigated, and the identity of the pathogens was verified based on the sequence of two intergenic nuclear regions.

Material and methods

Origin of pathogenic isolates

Leaflets with symptoms of brown leaf spots were collected from ten pecan orchards established in the State of Rio Grande do Sul, Brazil (Table 1). The leaves were placed separately in boxes containing 20 g of silicon dioxide (SiO_2) prior to isolation of the pathogen, in order to preserve fungal structures

and avoid unwanted saprophytic fungal growth on the leaves. Symptomatic leaflets from five different trees were collected in each orchard.

Table 1 Isolates of *Ragnhildiana diffusa* obtained from pecan trees in different orchards in Rio Grande do Sul State, southern Brazil. The geographic coordinates, and SMDB Herbarium and GenBank accession numbers are indicated.

Isolates	Geographic coordinates of orchard	SMDB Accession	GenBank Accession	
			ITS[a]	LSU[b]
SS-TP-1	30°36'43" S, 51°34'49" W	16.431	MG786511	MG786501
IL-GG-1	28º54'36" S, 52º 07'08" W	16.428	MG786520	MG786510
CZ-AL -1	29°30'49" S, 51°59'29" W	16.430	MG786516	MG786506
EN-MR-1	29°12'39" S, 51°53'57" W	16.432	MG786518	MG786508
EN-MR-2	29°12'39" S, 51°53'57" W	16.433	MG786519	MG786509
DR-PZ-1	29°05'11" S, 51°59'42" W	16.425	MG786517	MG786507
AM-EG-1	29°19'39" S, 55°51'04" W	16.429	MG786515	MG786505
AG-RM-1	28°53'59" S, 52°02'55" W	16.434	MG786513	MG786503
AG-ZI-1	28°53'40" S, 52°02'23" W	16.426	MG786514	MG786504
AG-NT-1	28°53'30" S, 52°01'13" W	16.427	MG786512	MG786502

[a] Internal transcribed spacer region of the 5.8S nrRNA gene
[b] Large subunit of the 28S nrRNA gene

Direct isolation of the pathogen was performed from lesions producing spores of the fungus. Conidia were transferred to Petri dishes containing carrot agar (CA) medium (200 mL carrot juice, 20 g agar, and 800 mL distilled water) and incubated at 20±1 ºC under a 24 h photoperiod for 56 days (Poletto et al. 2017). Monosporic isolates were obtained following established technique (Fernandes 1993). The isolates (Table 1) were lyophilized and deposited in the fungal collection of the Laboratory of Plant Pathology, and in the Herbarium of the Department of Biology (SMDB) of the Federal University of Santa Maria, Brazil.

Pathogenicity tests

To ensure healthy, disease-free foliage, central pairs of mature leaflets of 12-year-old trees of pecan cv. 'Barton' growing in commercial orchards were collected during mid-summer for pathogenicity tests. The healthy detached leaflets were droplet-inoculated with 100 µL of a 2 × 05 spores mL^{-1} suspension, at six points on the abaxial portion of each leaflet (Walker et al. 2018). Sterilized distilled water was used as a control. The conidial suspension was prepared from a 56-days old culture of the fungus grown in CA culture medium as described above, by scraping the surface of the medium with the aid of a Drigalski handle. The mycelium was transferred to individual Petri plates and 4 mL of sterile distilled water was added to the plates. The suspension was filtered through a double layer of gauze and the spore concentration was determined using a light microscope with aid of a Neubauer hemocytometer. Inoculated leaflets were incubated in boxes containing moistened paper and moistened cotton to prevent dehydration. Leaflets were incubated at 23 ± 1 ºC with a 12 h photoperiod for 12 days. The paper sheets and cotton were remoistened every two days. Pathogenicity tests were performed after each individual sampling and posteriorly repeated

for all isolates at the same time with eight replicate leaflets used for each isolate.

The development of symptoms was monitored microscopically using a stereomicroscope (Model SZX7, Olympus Inc., Japan). The test was considered positive for disease if the symptoms appeared associated with at least one of the droplet inoculations sites on the leaflet. A scale with seven levels (0%, 16.7%, 33.3%, 50%, 66.7%, 83.3%, and 100%) based on the number of inoculation sites with symptoms on a leaflet (0, 1, 2, 3, 4, 5 or 6 sites, respectively) was used to quantify the virulence of each of the 10 pathogen isolates (Table 2). To confirm pathogenicity, the fungus was re-isolated to fulfil Koch's postulates.

Table 2 Percentage scale relative to number of lesions used to assess the virulence of *Ragnhildiana diffusa* on leaflets of pecan (cv. 'Barton')

No. of lesions	Virulence (%)
0	0.0
1	16.7
2	33.3
3	50.0
4	67.7
5	83.3
6	100

Morphological analysis

Colony characterizations for each isolate were performed on cultures grown on CA medium, incubated at 20±1 ºC with a 24 h photoperiod. Six replicate cultures were used for each isolate. The diameter of each colony was measured orthogonally each week using a digital caliper (Digimess, SP, Brazil), and the colony color was described following the descriptors of the Munsell Soil Color Chart (Munsell Color, Grand Rapids, MI, USA). Measurements were used to calculate the mycelial growth rate index (MGRI) as MGRI = Σ ((D-Da)/N), where D is the current mean diameter of the colony and Da is the mean diameter of the colony from the previous week (Maguire 1962).

For measurements of conidia, samples of the sporulating colonies were prepared in sterilized distilled water and a droplet placed on a microscope slide under a coverslip. Length and width of the conidia, and the number of septa were determined from a random sample of 50 conidia from the spore suspension examined using a light microscope. Measurements were performed with a 10× magnification eyepiece YS-Cf 10X/18 (Nikon Inc., Japan) coupled to an optical BX41 phase-contrast microscope (Olympus Inc., Japan) using a 40× objective (400× magnification).

The morphological variables were subject to analysis of variance ($\alpha = 0.01$). The data were further submitted to a multivariate analysis, tested for multicollinearity, and adjusted using the Mahalanobis distance matrix.

Scanning electron microscopy

A culture of isolate AG-RM-01 was grown on CA medium at 20±1 °C, under a 24 h photoperiod for 56 days. Samples were prepared for scanning electron microscopy (SEM) analysis by taking discs containing structures of the fungus and placing them in SiO2 for seven days for dehydration. Dehydrated discs were mounted on a stub using double-sided carbon tape, metalized with silver in a metallizer Bal-Tec SCD 050 (Capovani Brothers Inc., NY, USA) for observation using a SEM JSM 6060 (JEOL Inc. Japan) at an accelerating voltage of 5 kV.

DNA isolation, amplification and sequencing of the ITS and LSU regions

Genomic DNA was isolated from mycelia scraped from the colonies, using the CTAB method (Doyle & Doyle 1991). The internal transcribed spacer (ITS) region of the 5.8S nrRNA gene and the large

subunit (LSU) of the 28S nrRNA gene were amplified using the primer pairs ITS1 (5'-TTCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for the ITS region (White et al. 1990) and LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') for the LSU region (Vilgalys & Hester 1990). The PCR mix contained approximately 30 ng of DNA, 5× buffer, 200 µM of each dNTP, 1.5 mM MgCl2, 200 nM of each primer, and 1 U of GoTaq DNA polymerase in a total volume of 25 µL. A thermal cycler PTC-100 (MJ Research, Inc., Canada) was used for the PCR reaction with an initial denaturation step of 94 ºC for 2 min, 40 cycles of 94 ºC for 10 s, 54 °C for 30 s and 72 °C for 45 s, and a final elongation step of 72 ºC for 4 min. A negative control without DNA was also run as negative control. PCR products were purified by precipitation with PEG 6000 (Schmitz & Riesner 2006) and sequenced using an ABI 3500 XL sequencer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The nucleotide sequences obtained from the isolates were compared to those employed by Videira et al. (2017) in the phylogenetic study of Mycosphaerellaceae. This study is the most complete and up-to-date analysis of the phylogenetic relationship among species of this family and all sequences are deposited in the GenBank database. Comparisons were performed using the Basic Local Alignment Search Tool (BLAST) procedure and subject sequences that showed the greatest coverage (> 80%) and similarity (> 95%) were selected to perform the phylogenetic analysis. Nucleotide sequences were aligned using the ClustalW algorithm with the BioEdit software (Hall 1999).

Phylogenetic analyses

Appropriate genetic evolution models were selected using MrModeltest V2.3 (Nylander 2004) and applied to each gene partition. A Bayesian inference was performed on CIPRES (Miller et al. 2010), using MrBayes V3.2.1 (Ronquist et al. 2012) applying the SYM+I+G base substitution model for the locus ITS and the GTR+I model for the LSU region. Sequences of *Amycosphaerella africana* strains CBS 110500, CBS 111996, CBS 134927, and CBS 680.95 were used as outgroups (Genbank IDs KF901837, MF 951124, MF951125, and KF902048 for the LSU region and AY725531, EU707855, MF951289, and KF901701 for the ITS region, respectively).

Posterior probabilities were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes V3.2.1. Six simultaneous Markov chains were run for 10,000,000 generations and trees were sampled every 100 generations until convergence was achieved. Trees were visualized in Geneious V8 (https://www.geneious.com). Sequences derived in this study were deposited in GenBank (Table 1). The alignments and trees were deposited in TreeBASE (http://www.treebase.org).

Results

Disease symptoms and morphological characterization of the pathogen

The disease symptoms in the field were first observed at the end of the spring as small irregular dark spots. Later, these spots became well-delimited lesions of reddish-brown color with a dark halo, measuring 0.5 to 1.5 cm in diameter (Figure 1A), coalescing with progress of the disease. The disease continued to progress while foliage remained on the tree, and eventually resulted in severe defoliation. On the lesion surface, the fungus produced abundant conidiophores and conidia, mainly on the abaxial side of the leaves.

One isolate of the pathogen was obtained from each of the ten collection sites (Table 1). On CA medium, all 10 isolates had colonies that presented light brown, aerial mycelium, with septate hyphae. The conidia were originated

from short light brown conidiophores, single or grouped in fascicles, and slightly geniculated (Figure 1E-F). At the extremities of the conidia, there were minute rounded scars originating from attachment points (Figure 1J arrow). Immature conidia were single, cylindrical, straight, hyaline, and had few to no septa (Figure 1E, H, L arrow). When mature, the conidia were cylindrical to oblong-cylindrical, curved, tapered towards the apex, strongly constricted at the septa, with a thick, brown cell wall (Figure 1G, I-K).

The colonies of all isolates exhibited very slow mycelial growth. After 56 days of incubation, the colonies had not grown to the edge of the Petri dish (Figure 1D). The total diameter of the colonies varied from 18.7 mm (isolate EM-MR-1) to 26.2 mm (DR-PZ-1) (Table 3). The daily mycelial growth (DMG) varied, with isolates SS-TP-1 and DR-PZ-1 being faster (0.47 mm/day) compared to isolates EN-MR-1 and EN-MR-2 that had the slowest growth (0.33 and 0.35 mm/day, respectively).

The isolates showed differences in sporulation, with most sporulation observed for isolate CZ-AL-1 (22.9 × 105 spores mL^{-1}) and AM-EG-2 (20.2 × 105 spores mL-1). The colonies formed sectors, i.e., portions of the colony with different morphological characteristics where fruiting was more abundant as single conidiophores or grouped in fascicles. Mean conidia length ranged from 73.4 µm to 90.5 µm, depending on the isolate. However, a large range was observed for all strains; MS-2 presented most variation in conidia length (15 to 247.5 µm), and width (2.5 to 6.3 µm). Conidial septation was variable between isolates and within each isolate. For example, isolate EM-MR-2 had zero to 30 septa, probably due to the range in maturity of the conidia.

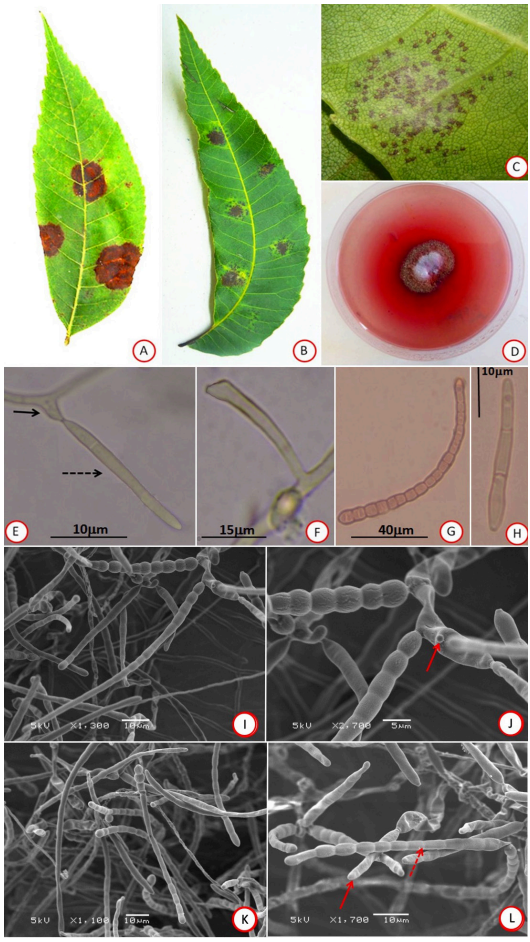


Figure 1 (A) Symptoms of brown leaf spots caused by *Ragnhildiana diffusa* on a leaflet observed in the field; **(B)** symptoms on an inoculated leaflet; **(C)** detail of the initial lesions that developed during the pathogenicity tests; **(D)** colony of one isolate on CA medium with a diffuse red halo in the center and around the colony; **(E)** conidiophore (arrow) and conidium (dash arrow); **(F)** conidiophore. **(G)** mature conidium; **(H)** immature conidium. **(I-L)** Fungal structures of *Ragnhildiana diffusa* visualized under scanning electron microscopy. **(I)** conidiophore and conidia; **(J)** scar (arrow) on conidiophore and constriction on conidia septa; **(K)** mature conidia; **(L)** immature (arrow) and mature (dashed arrow) conidia.

The color of the colonies on the reverse side of the Petri dish was constant, with all the isolates having a similar reddish-brown color. However, a red pigmentation diffused into the culture medium around the colonies with varied intensity depending on the isolate (Figure 2).

The multivariate analysis based on the morphological characteristics of the 10 isolates revealed MGRI as the main morphological characteristic distinguishing the *Ragnhildiana* isolates, representing 40.2% of the total variation. DMG, mycelial growth, conidia width, number of septa, sporulation and length of conidia represented 19.5%, 13.7%, 13.7%, 6.0%, 3.8%, and 3.0% of the total variation, respectively.

Table 3 Morphological characteristics of different isolates of *Ragnhildiana diffusa* collected from leaflets of pecan with symptoms of brown leaf spot in southern Brazil. The fungus was cultured for 56 days on carrot agar with photoperiod of 24 h at 20± 1°C.

Isolates	Diam (mm)	DMG[a] (mm/day)	MGRI[b]	Sporulation ($\times 10^5$ mL^{-1})	Conidia		
					Length (µm) (range)	Width (µm) (range)	Septa (range)
SS-TP-1	26.1	0.47	1.36	19.4	73.4 (15 - 150)	4.2 (2.8 - 5.3)	9 (0 - 26)
IL-GG-3	25.8	0.46	1.26	11.1	73.4 (17.5 - 165)	4.6 (2.5 - 5.5)	10 (0 - 26)
CZ-AL -1	23.9	0.43	1.29	22.9	75.4 (20 - 130)	3.7 (2.5 - 5)	9 (0 - 18)
EN-MR-1	18.7	0.33	0.91	15.6	79 (45 - 137.5)	4.3 (3 - 5)	11 (5 - 19)
EN-MR-2	19.8	0.35	0.86	11.4	84.0 (15 - 247.5)	3.7 (2.5 - 6.3)	7 (0 - 30)
DR-PZ-1	26.2	0.47	1.49	16.4	69.1 (17.5 - 120)	3.4 (2.5 - 5)	4 (0 - 11)
AM-EG-1	23.7	0.42	1.17	20.2	90.5 (20.0 - 237.5)	4.0 (2.5 - 5)	9 (0 - 18)
AG-RM-1	20.1	0.36	1.06	19.7	74.5 (22.5 - 162.5)	4.2 (2.5 - 5)	8 (0 - 21)
AG-ZI-2	20.8	0.37	1.00	17.5	68 (17.5 - 152.5)	3.5 (2.5 - 5)	8 (0 - 20)
AG-NT-1	20.9	0.37	1.07	10.0	60.5 (22.5 - 120)	3.9 (2.5 - 5)	7 (0 - 20)

[a] Daily mycelial growth.

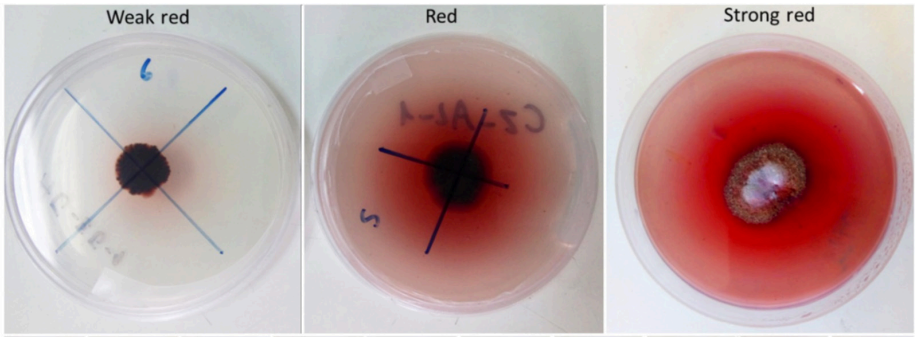
[b] Mycelial growth rate index.

Pathogenicity analysis

All isolates of *Ragnhildiana* were pathogenic on pecan, with some differences observed in the characteristics of the pathogenicity among the isolates. Symptoms on leaves first appeared eight days after inoculation, starting as small necrotic spots that corresponded to the inoculation sites and subsequently coalescing and forming larger areas of necrotic tissue (Figure 1B, C). Many fruiting bodies were observed on the lesions, which were composed of conidiophores, single and grouped in fascicles, with conidia. The symptoms matched the typical development of the disease as observed in the field. Subsequently, the same fungus was re-isolated from the lesions and grown on CA medium, fulfilling Koch's Postulates. There were no symptoms of disease on the controls.

Except for isolate SS-TP-1, which had a lower incidence (87.5%), all other isolates caused an incidence of 100% leaflets diseased in all replicates (Figure 2). Virulence varied among isolates; 100% of infection sites resulted in disease with DR-PZ-1, AG-RM-1, and AG-ZI-1, followed by isolates IL-GG-3, AG-NT-1, EM-MR-2, and AM-EG-2, which had intermediate virulence. The isolates with fewest inoculation sites resulting in disease

were CZ-Al-1 and SS-TP-1 (Figure 2). With time, colonization of the host extended beyond the inoculation sites with most isolates, resulting in larger areas of necrotic tissue.



Isolate:	SS-TP-1[a]	CZ-AL-1[a]	EM-MR-2[b]	AM-EG-1[b]	IL-GG-3[c]	EM-MR-1[c]	DR-PZ-1[c]	AG-RM-1[c]	AG-ZI-2[c]	AG-NT-1[c]
Incidence (%):	87.5	100	100	100	100	100	100	100	100	100
Virulence (%):	43.8	72.9	89.6	85.4	93.8	93.8	100	100	100	93.8

Figure 2 Reddish pigment diffused by different isolates of *Ragnhildiana diffusa* in carrot:agar culture medium. The intensity of the pigmentation (a weak red, b red and c strong red) in the Petri plates is indicated, as well as the pigmentation produced by each of the ten isolates collected from different orchards of pecan in southern Brazil. Pathogenicity (incidence) and virulence of isolates when inoculated on leaflets of pecan (cv. 'Barton') is presented. Turnover in macrofungal species composition before and after treatments

Phylogenetic analysis

The DNA sequence used for the phylogenetic analysis consisted of 1183 bases (455 bases of the ITS and 728 bases of the LSU regions). A total of 30 individuals with the outgroups were included in the analysis. The alignment represented 76 unique site patterns, with 53 for the ITS sequence and 23 for LSU sequence. After topological convergence of the Bayesian runs, 1035 phylogenetic trees were generated and 828 subsequently sampled (using a burn-in fraction of 0.25) to generate the Bayesian phylogeny (Figure 3). The resulting phylogenetic tree showed consistent clustering of the isolates. Using *Amycosphaerella africana* as outgroup, *A. kaniensis* formed a basal branch on the tree. Species from the genera *Phaeoramularia* and Ragnhildiana formed a cluster with high posterior probability support (PP = 1.0). Three specimens of *Phaeoramularia* formed a sister cluster to the *R. diffusa* / *R. ferruginea* clade, with a PP = 0.93. *Ragnhildiana ampleopsidis*, *R. gnaphaliaceae* and *R. perfoliati* (PP = 0.94) formed a sister cluster to the *Phaeoramularia*/*R. diffusa*/*R. ferruginea* clade with high support (PP = 1.0). All 10 isolates obtained in this study grouped with the representative of the type species of *R. diffusa* (strain CBS 106.14) with high posterior probability support (PP = 1.0).

Discussion

In this study, we characterized the symptoms of the brown leaf spot in southern Brazilian

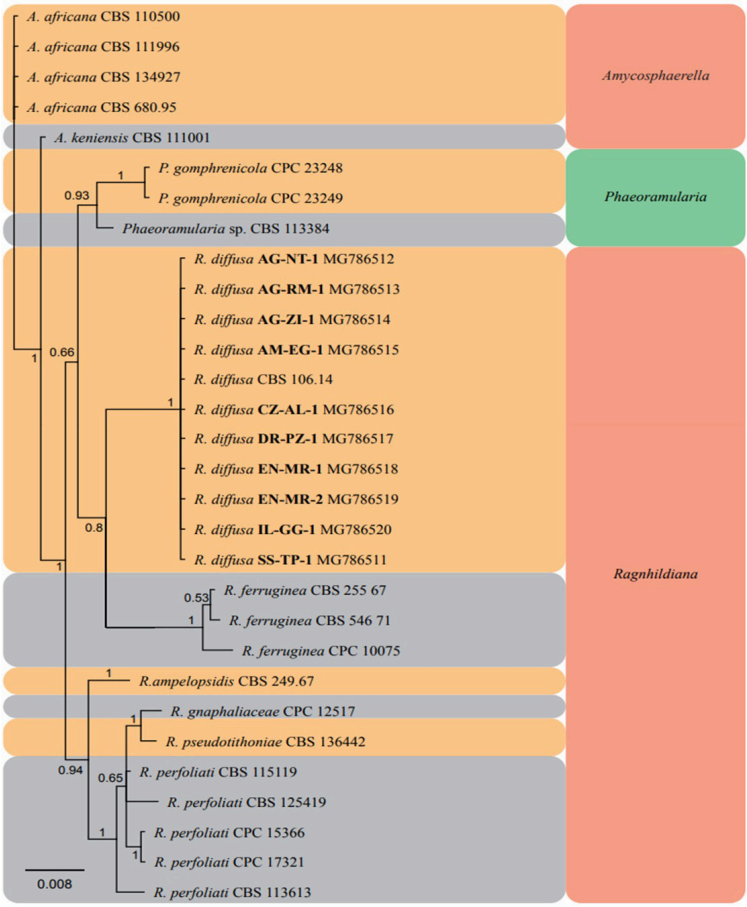


Figure 3 Consensus phylogram (50% majority rule) of the Bayesian inference of the combined gene sequence alignment for sequence of the internal transcribed spacer (ITS) region of the 5.8S nrRNA gene and the large subunit (LSU) of the 28S nrRNA gene. Posterior probabilities are indicated left of each node. The scale bar indicates 0.008 expected changes per nucleotide site. Species clades are delimited in colored boxes. Brazilian isolates of *Ragnhildiana diffusa* are indicated in bold. The tree was rooted to equivalent sequence data for *Amycosphaerella africana* (CBS 110500, CBS 111996, CBS 134927 and CBS 680.95)

pecan orchards, as well as the morphology and pathogenicity of fungal isolates collected from symptomatic plants. The symptoms observed for brown leaf spots in this study agree with those described for pecan trees growing under the climatic conditions of the U.S.A. (Chupp 1953, Ellis 1976, Stein et al. 2012). In the U.S.A. brown leaf spots is more prevalent in areas where rainfall is abundant and humidity is high, or in orchards with nutritional deficiencies (Kluepfel et al. 2014). In southern Brazil, summers are generally hot and wet, a factor that likely favors the occurrence of fungal diseases in pecan.

Of the ten isolates of *R. diffusa* that were collected and used for inoculation, nine resulted in 100% of inoculation sites developing symptoms of brown leaf spots and Koch's postulates were fulfilled for all 10 isolates, with *R. diffusa* re-isolated from the inoculated

leaves. There was some indication of a range in virulence among the ten isolates, with 43.8% to 100% of inoculation sites resulting in disease symptoms. Virulence is a component of pathogenicity and has a quantitative character (Shaner et al. 1992), associated with the biochemical properties of the pathogen and related to the production of toxins that contribute to the increase of disease severity. Toxins are considered virulence factors since they contribute to the severity of the disease without being essential for their production (Pascholati 2011). However, it is not yet known if such factors affect the virulence of *R. diffusa.*

Toxins are products of microbial pathogens that can be produced both in affected tissues and in culture media (Bach et al. 2005). Toxins can act in different sites at the cellular level, changing the permeability and/or the potential of membranes, impairing plant physiology (Bach & Kimati 1999). Bach et al. (2005) demonstrated that the isolated application of toxins produced by *Exserohilum turcicum* (which causes northern corn leaf blight) caused necrosis in corn leaves. Reino et al. (2004) showed that there is a correlation between the production of toxins *in vitro* and the degree of virulence for eleven strains of *Botrytis cinerea*. Isolates with low toxin production were less virulent on bean (*Phaseolus vulgaris*) in comparison to those with higher toxin production (Reino et al. 2004).

All ten isolates of *R. diffusa* produced a red pigmentation in culture that diffused in the culture medium. For some isolates the color was more intense compared to others (Figure 2). The production of a red pigment in the culture medium around colonies is common in species of the genus *Cercospora* and was reported in *C. beticola* (Lynch & Geoghegan 1979), *C. piaropi* (Jiménez et al. 2010) and *C. kikuchii* (Upchurch et al. 1991). In these cases, the red pigment is a toxin, cercosporin, considered crucial in plant pathogenesis (Upchurch et al. 1991). Almeida et al. (2005) reported a correlation between cercosporin content and virulence of *C. kikuchii* in *Glycine max* (L.) Merril; the strains with more pigmented colonies were the most virulent. Although the pigmentation associated with colonies of isolates of *R. diffusa* were not characterized, there may be some association between virulence and pigmentation. The colonies producing weak red pigmentation appeared to have lowest virulence (43.8% and 72.9%). Colonies producing red pigmentation revealed somewhat higher virulence (85.4% and 89.6%), while the colonies producing intense red pigmentation had virulence ranging from 93.8% to 100% (Figure 2). Further research is needed to both characterize the red pigment and establish the range in virulence of *R. diffusa*, since it is possible that the variation in virulence among strains may be associated with the pigmentation produced by the colonies.

Ragnhildiana diffusa was previously identified as the cause of brown leaf spots of pecan in one orchard in southern Brazil (Poletto et al. 2017). In the present study, the consensus Bayesian phylogeny based on the alignment of the combined sequences of the ITS and LSU genes clustered the ten Brazilian strains of *R. diffusa* within a single clade, grouped with *R. diffusa* strain CBS 106.14. Moreover, our combined ITS/LSU alignment monophyletically segregated *R. diffusa* from other species within the same genus, supporting the distinctness of the species and the equivalence of the isolates (Figure 3).

Conclusions

In this study, we confirmed *R. diffusa* as the causal pathogen of brown leaf spot in pecan orchards in Southern Brazil, despite the variation in the morphology of the asexual stage observed among isolates. Inoculation tests on healthy leaflets confirmed that all sampled isolates were pathogenic, with some variation observed in their virulence. Thus,

the accurate and prompt identification of the disease may assist controlling further spread of the pathogen into orchards and nurseries still free of the disease. In addition, the reddish pigment produced in the culture medium deserves attention and deep characterization, since it may play an important role in the pathogenesis.

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