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CROP PROTECTION

Morphological characterization and molecular identification of *Colletotrichum* species associated to sweet persimmon anthracnose in Southern Brazil

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ABSTRACT: *The highlands of Southern Brazil contribute with 40% of Brazilian persimmon production. Although expanding, persimmon production faces major problems caused by anthracnose disease (black spot), including fruit rot and necrosis of leaves. Several Colletotrichum species (C. horii, C. gloeosporioides, among others) are implicated in persimmon anthracnose around the world. To identify Colletotrichum species associated with persimmon anthracnose in the highlands of Southern Brazil, 34 isolates were analyzed by ITS-rDNA partial region, GAPDH, and TUB2 partial gene sequences, morphological characteristics, and virulence on persimmon fruits and leaves. Data showed a high prevalence of C. horii (85.3%), that associated with its high virulence on fruits and leaves, confirm a considerable degree of host preference. Moreover, other species C. aenigma, C. asianum, C. fructicola, and C. nymphaeae, were identified, but the last three ones exhibited low virulence on fruits and were not able to produce symptoms on leaves. As far as we know this is the first reference on C. asianum in persimmon. The present data may contribute to a better understanding of the etiology of anthracnose in sweet persimmon in Southern Brazil, and it will be useful for epidemiological studies and the development of disease management measures.*
Key words: *black spot, molecular identification, virulence.*

Caracterização morfológica e identificação molecular de espécies de *Colletotrichum* associadas à antracnose de caqui doce no Sul do Brasil

RESUMO: *As terras altas do sul do Brasil contribuem com 40% da produção brasileira de caqui. Embora em expansão, a produção de caqui enfrenta grandes problemas causados pela antracnose (mancha preta), incluindo o apodrecimento dos frutos e necrose das folhas. Várias espécies de Colletotrichum (C. horii, C. gloeosporioides, entre outras) estão envolvidas com a antracnose do caqui em todo mundo. Para identificar espécies de Colletotrichum associadas à antracnose de caqui nas terras altas do sul do Brasil, 34 isolados foram analisados através da região parcial de ITS-rDNA e sequências parciais dos genes GAPDH e TUB2, características morfológicas e virulência em frutos e folhas de caqui. Os dados mostraram uma alta prevalência de C. horii (85,3%), que associada à sua alta virulência em frutos e folhas, confirma um grau considerável de preferência pelo hospedeiro. Além disso, foram identificadas outras espécies C. aenigma, C. asianum, C. fructicola, e C. nymphaeae, mas as três últimas exibiram baixa virulência nos frutos e não foram capazes de produzir sintomas nas folhas. Até onde sabemos, esta é a primeira referência sobre C. asianum em caqui. Os presentes dados podem contribuir para uma melhor compreensão da etiologia da antracnose em caqui doce no sul do Brasil e isso pode ser útil para estudos epidemiológicos e para o desenvolvimento de medidas de controle da doença.*
Palavras-chave: *mancha preta, identificação molecular, virulência.*

INTRODUCTION

Persimmon (*Diospyros kaki* L.) was introduced in Brazil by Japanese immigrants at the beginning of the 20th century, and its growing area has expanded considerably since the 1920s. Nowadays, Brazil is the fourth world producer of persimmon with a planted area of 8,148 ha, and a production that reaches 157 thousand tons/year (IBGE, 2018). The highlands of Southern Brazil, which account for most of the Brazilian production, are part of a subtropical and temperate macroregion with altitude from 500 to 680 m, a mean annual temperature of 17°C, and a precipitation of 1908 mm/year.

The most prominent persimmon cultivars in Southern Brazil are Fuyu, Giombo, and Kyoto (FIORAVANÇO & PAIVA, 2007). Although, growing at rates that reach 10% per year in Southern Brazil,

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persimmon culture faces a significant problem of increasing anthracnose damage in fruits leaves and branches, caused by *Colletotrichum* species, which are responsible for important losses in highly infected fields (MAY DE MIO et al., 2015; CARRARO et al., 2019).

Persimmon anthracnose in new twigs appears as small dark elliptic spots that enlarge during time and can coalesce. Leaves infected by *Colletotrichum* show small circular to elliptic purple to dark brown spots, and fruit anthracnose appears as small sunken and dark spots that progress to larger dark brown lesions (>20 mm) with grey center and orange conidia. Early fruit infections cause premature fruit drop. Moreover, the disease progress during ripening and after harvest, leading to important losses during storage, that can reach 50 to 90% (ZHANG, 2008; DAMM et al., 2010; PALOU et al., 2013).

The causal agent of persimmon anthracnose was first classified at the beginning of the 20th century as *Gloesporium kaki*, and later as *Colletotrichum kaki* (XIE et al., 2010). Based on molecular data and phylogenetic analysis, WEIR & JOHNSTON (2010) reclassified this pathogen as *Colletotrichum horii*, one of the species of the *Gloeosporioides* complex. Since this report, *C. horii* has been confirmed as the most prevalent species associated with anthracnose in leaves, twigs and, fruit in Japan, China, Southeast Brazil, and Korea (YU et al., 2013; KWON et al., 2013; MAY DE MIO et al., 2015; HASSAN et al., 2018; JEON et al., 2017; ASANO & HIRAYAMA, 2019; CARRARO et al., 2019). However, other *Colletotrichum* species have been reported causing anthracnose in persimmon leaves, twigs or, fruits, like *C. acutatum* in the USA (WILLIAMSON & SUTTON, 2010), *C. gloeosporioides* in Spain (PALOU et al., 2013), *C. karstii* in China (WANG et al., 2016), *C. kakivorum* in Korea (LEE & JUNG, 2018), *C. fructicola, C. nymphaeae, C. meloni* in Paraná, Brazil (CARRARO et al., 2019).

Aiming to expand the knowledge of anthracnose etiology in southern Brazil, the study characterized *Colletotrichum* isolates associated with the disease from the region based on the molecular, morphological, and pathogenic analysis and identified the species. Furthermore, the present survey can give subsidies for further epidemiological studies and disease management of this devastating disease.

MATERIALS AND METHODS

Samples

Persimmon fruits showing typical symptoms of anthracnose were collected from commercial orchards of the highlands of Southern Brazil (Table 1). Fruit skin tissues of approximately 5 mm in diameter, were collected, surface-sterilized with 1% NaClO for 1 min, washed twice with sterile distilled water, and partially dried with filter paper. The tissues were placed on PDA (potato-dextrose-agar) amended with gentamicin (50 mg/L) and cefotaxime (100 mg/L). The plates were incubated at 27°C for 4 days. Single-spore cultures were maintained in PDA slants and stored at -80°C in cryotubes with 25% glycerol.

DNA extraction and PCR amplifications

DNA was extracted from fungal mycelia using the method proposed by TAPIA-TUSSELL et al. (2006). DNA was quantified by absorbance at 260 nm, and the quality was estimated by 260/280 ratio and agarose gel electrophoresis.

PCR amplification of the ITS1-5.8S-ITS2 rDNA, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) intron, and the TUB2 (β-tubulin) gene of fungal isolates was performed using the primers described by WHITE et al. (1990), TEMPLETON et al. (1992), O'DONNELL & CIGELNIK (1997), respectively. PCR amplifications (25 µl) included a denaturation at 94°C for 60 min, annealing at 55°C (ITS and TUB2) or 59°C (GADPH) for 1 min, and extension at 72°C for 1.5 min, with an initial denaturation of 5 min at 94°C and a final extension of 5 min at 72°C. The amplifications were confirmed in 2% agarose gels with TBE (Tris-Borate-EDTA), stained with GelRed (Uniscience), and visualized under UV light.

DNA sequencing of the amplicons

Amplification products were enzymatically purified with EXOI/SAP (USB) following manufacture instructions. Purified amplicons (50-60 ng) were sequenced using Big Dye Terminator V.3.1 sequencing kit (Thermo) and analyzed with a 3500 Genetic Analyzer (Thermo). Data were collected by Data Collection software (Thermo).

Molecular classification and phylogenetic analysis

The DNA sequences were compared with those deposited in GenBank using the BLAST similarity test. Alignment of the ITS, GAPDH and, TUB2 sequences of the *Colletotrichum* isolates, references of *Colletotrichum* species, and *Monilochaetes infuscans* (outgroup) retrieved from NCBI database was performed with CLUSTAL X. Concatenated sequences were used for phylogenetic analysis using the maximum parsimony of MEGA 6.0 (TAMURA et al., 2013).

Table 1 – *Colletotrichum* isolated from persimmon anthracnose in the state of Rio Grande do Sul - Brazil.

Code	Geographical Origin	Persimmon variety	Code	Geographical Origin	Persimmon variety
19.1	Alto Feliz	Giombo	DCFR6	Farroupilha	Kyoto
19.18	Alto Feliz	Giombo	DCFR7	Farroupilha	Kyoto
19.19	Alto Feliz	Giombo	DCFR8	Farroupilha	Kyoto
19.21	Alto Feliz	Giombo	DCU3	Farroupilha	Fuyu
19.23	Alto Feliz	Giombo	LMFC.19.3	Alto Feliz	Giombo
B5	Caxias do Sul	Kyoto	LMFC.19.9c	Alto Feliz	Giombo
B6	Caxias do Sul	Kyoto	LMFC.19.17	Alto Feliz	Giombo
BFR6	Farroupilha	Kyoto	LMFC.19.18	Alto Feliz	Giombo
C2	Caxias do Sul	Kyoto	LMFC.19.19	Alto Feliz	Giombo
CCF1	Alto Feliz	Giombo	LMFC.19.20	Alto Feliz	Giombo
CF1	Alto Feliz	Giombo	LMFC.19.21	Alto Feliz	Giombo
CF4	Alto Feliz	Giombo	LMFC.19.22f	Alto Feliz	Giombo
CF6	Alto Feliz	Giombo	LMFC.19.23	Alto Feliz	Giombo
CRC3	Caxias do Sul	Kyoto	PPFO.19.5	Caxias do Sul	Kyoto
CRF2	Caxias do Sul	Kyoto	PPFR.19.21	Caxias do Sul	Kyoto
CRF3	Caxias do Sul	Kyoto	PPFR.19.22	Caxias do Sul	Kyoto
DCFR5	Farroupilha	Kyoto	VF	Vacaria	Kyoto

Clade stability was determined by 1,000 bootstrap replicates.

Morphological characteristics of the species

Radial mycelial growth was recorded daily by two perpendicular measures, for 7 days on PDA cultures at 28°C with 16 h photoperiod, initiated from 5 mm diameter mycelium plugs. Colony appearance and conidial production were evaluated on the seventh day. Conidia were recovered after 7 days on PDA plate by scraping with saline, followed by filtration. Conidial shape and size were microscopically evaluated at x 400 magnification using a Nikon E200 microscope coupled with a CCD camera. Conidial appressoria were evaluated as proposed by LIU et al. (2016).

Pathogenicity test

The pathogenicity of representatives of the *Colletotrichum* species (five species and a total of 11 isolates) isolated from persimmon fruits was evaluated on leaves and fruits of Kyoto cultivar. Intact leaves were inoculated with 0.5 cm plugs of fungal mycelia obtained from 7 days cultures on PDA medium. The necrotic spots diameter was evaluated 7 days after incubation at 28°C on 99% humidity chambers. Persimmon fruits (without lesions) were inoculated with 10 µL conidial suspensions (1 x 10^6 conidia/mL) freshly prepared and incubated on 99% humidity chambers for 7 days. The lesion diameters were determined using a digital calypter. In each assay, a total of eight repetitions (fruit or leaves) were utilized.

Statistical analysis

Results were analyzed statistically by analysis of variance (one-way ANOVA), and Tukey's test. The statistical analyses were performed using IBM-SPSS Statistics - version 22 software, and statistical significance was attributed to values of $P \leq 0.05$.

RESULTS AND DISCUSSION

A total of 34 *Colletotrichum* were isolated from 36 persimmon fruits with symptoms of anthracnose collected on the highlands of Southern Brazil (Table 1). These isolates were all pathogenic to

persimmon fruits and 60% of the leaves presented the anthracnose symptoms.

Isolates were evaluated by their ITS (514 bp), GAPDH (235 bp), and TUB2 (520 bp) sequences, and classified by comparison with reference sequences deposited on GenBank (Table 2). As can be observed in figure 1, 97% of the isolates were included in the Gloeosporioides clade. Among these, 85.3% (29/34)

Table 2 – Identification of *Colletotrichum* isolates and their GenBank accession code for ITS, GAPDH and TUB2 partial sequences.

Species	Isolate	----------GenBank accession number----------		
		ITS	GAPDH	TUB2
C. horii	19.1	MT583946	MT587620	MT587587
C. horii	19.18	MT583947	MT587621	MT587588
C. horii	19.19	MT583948	MT587622	MT587589
C. horii	19.21	MT583949	MT587623	MT587590
C. fructicola	19.23	MT583950	MT587624	MT587591
C. horii	B5	MT583951	MT587625	MT587592
C. horii	B6	MT583952	MT587626	MT587593
C. asianum	BFR6	MT583953	MT587627	MT587594
C. horii	C2	MT583954	MT587628	MT587595
C. horii	CCF1	MT583955	MT587629	MT587596
C. horii	CF1	MT583956	MT587630	MT587597
C. horii	CF4	MT583957	MT587631	MT587598
C. horii	CF6	MT583958	MT587632	MT587599
C. horii	CRC3	MT583959	MT587633	MT587600
C. horii	CRF2	MT583960	MT587634	MT587601
C. horii	CRF3	MT583961	MT587635	MT587602
C. nymphaeae	DCFR5	MT583962	MT587636	MT587603
C. aenigma	DCFR6	MT583963	MT587637	MT587604
C. horii	DCFR7	MT583964	MT587638	MT587605
C. horii	DCFR8	MT583965	MT587639	MT587606
C. horii	DCU3	MT583966	MT587640	MT587607
C. horii	LMFC.19.3	MT583967	MT587641	MT587608
C. horii	LMFC.19.9c	MT583968	MT587642	MT587609
C. horii	LMFC.19.17	MT583969	MT587643	MT587610
C. horii	LMFC.19.18	MT583970	MT587644	MT587611
C. horii	LMFC.19.19	MT583971	MT587645	MT587612
C. horii	LMFC.19.20	MT583972	MT587646	MT587613
C. horii	LMFC.19.21	MT583973	MT587647	MT587614
C. horii	LMFC.19.22f	MT583974	MT587648	MT587615
C. fructicola	LMFC.19.23	MT583975	MT587649	MT587616
C. horii	PPFO.19.5	MT583976	MT587650	MT587617
C. horii	PPFR.19.21	MT583977	MT587651	MT587618
C. horii	PPFR.19.22	MT583978	MT587652	MT587619
C. horii	VF	MT583979	MT587653	MT629930

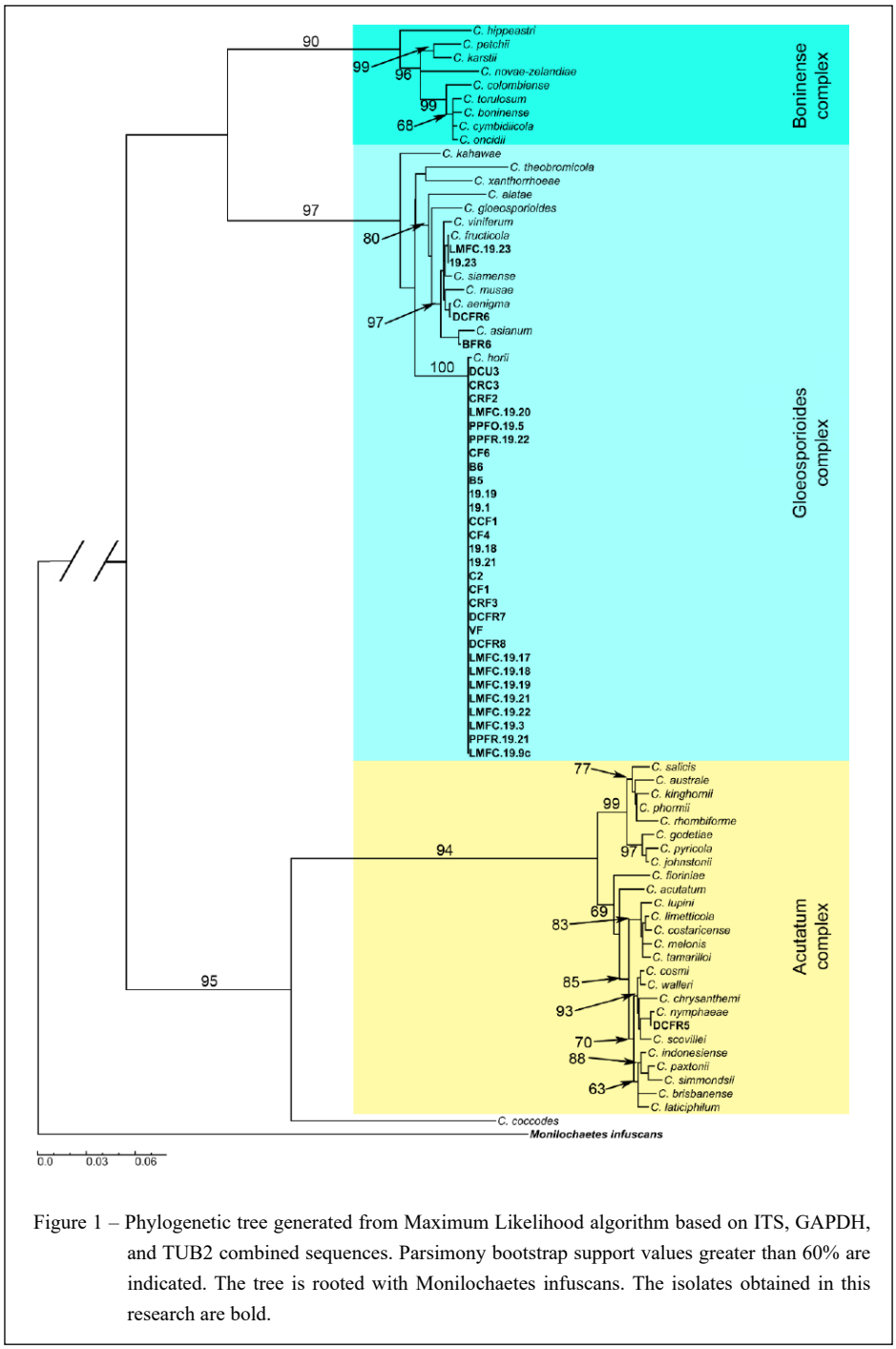

Figure 1 – Phylogenetic tree generated from Maximum Likelihood algorithm based on ITS, GAPDH, and TUB2 combined sequences. Parsimony bootstrap support values greater than 60% are indicated. The tree is rooted with Monilochaetes infuscans. The isolates obtained in this research are bold.

were highly related with *Colletotrichum horii* reference, 5.9% (2/34) were similar to *C. fructicola*, one isolate was closely related with *C. asianum* (2.9%), a sister species of *C. fructicola*, and one was classified as *C. aenigma* (2.9%). Just one isolate was classified as *C. nymphaeae*, a member of the Acutatum clade.

Colletotrichum species associated with anthracnose disease in persimmon varied depending on the classification methods. ZHANG et al. (2008), based on morphological characteristics classified persimmon anthracnose associated fungi as *C. gloeosporioides*, while WEIR & JOHNSTON (2010) classified New Zealand isolates as a new species *C. horii*, within the Gloeosporioides clade. The present study, based on phylogenetic characterization, corroborated the high prevalence of *C. horii* associated with persimmon

anthracnose, previously reported in both leaves and fruit diseases of persimmon in New Zealand, China, Korea, Japan, and Brazil (WEIR & JOHNSTON, 2010; YU et al., 2013; KWON et al., 2013; MAY DE MIO et al., 2015; HASSAN et al., 2018; JEON et al., 2017; ASANO & HIRAYAMA, 2019; CARRARO et al., 2019). *Colletotrichum horii* is highly host-specific (WEIS et al., 2012) which explains its high prevalence even in a region where crops such as apple and grapes, committed by *C. fructicola* and *C. viniferum*, are grown side by side with persimmon.

Although in a low-frequency, other species of *Colletotrichum* were isolated from persimmon fruits with anthracnose symptoms. *C. fructicola* has been reported as a causal agent of fruit anthracnose in apple (VELHO et al. 2015), grapes (ECHEVERRIGARAY et al., 2020), and other crop fruits in Brazil, and was first reported in persimmon in Parana State (Southern Brazil) by CARRARO et al. (2019), and *C. aenigma* was previously associated to sweet persimmon anthracnose in Korea (HASSAN et al., 2018), and grape ripe-rot disease in Southern Brazil (ECHEVERRIGARAY et al., 2020). Moreover, *C. asianum* is associated with anthracnose in coffee (PRIHASTUTI et al., 2009) and mango (SHARMA et al., 2015, VITALE et al., 2020), but this is the first report of this species on persimmon.

Just one isolate (2.9%) was classified as *C. nymphaeae* (Acutatum clade), and references to this species associated with persimmon anthracnose were cited in Korea (HASSAN et al., 2018) and Brazil (CARRARO et al., 2019).

Six representative isolates of *C. horii* and all the isolates of the other species identified by sequencing were further evaluated for their growing and morphological characteristics. As can be observed in figure 2 and table 3, the Gloeosporioides clade species (*C. horii, C. fructicola, C. aenigma,*

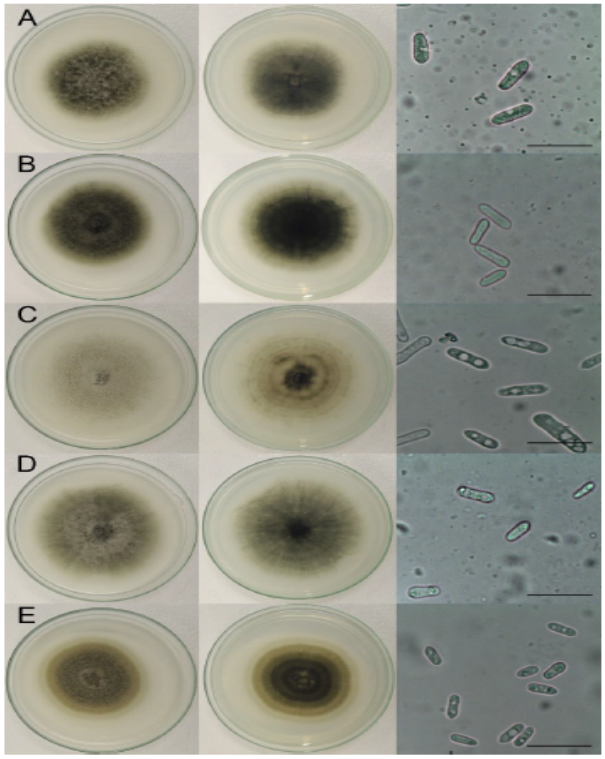

Figure 2 – Colony morphology and conidial shape of Colletotrichum species isolated from persimmon anthracnose. A – C. horii; B – C. fructicola; C – C. aenigma; D – C. asianum; E – C. nymphaeae. Colonies on PDA from above (left) and below (middle), and conidia (right). Scale bar: 20 µm.

Table 3 – Morphological characteristics (colony, conidia, and appressoria morphology) of *Colletotrichum* species Isolated from persimmon anthracnose in Southern Brazil.

	----------Gloeosporioides complex----------				
Species	*C. horii*	*C. fructicola*	*C. aenigma*	*C. asianum*	*C. nymphaeae*
N° Isolates evaluated	6	2	1	1	1
Colony morphology	grey-green, reverse light grey with darker grey sectors	pale grey with concentric grey sectors, reverse light grey with concentric dark grey sectors	White to pale grey, reverse white with concentric pale grey sectors	dark green, reverse pale grey with concentric dark grey sectors	light grey/green, reverse white with concentric grey/green sectors
Growth rate (mm/day)	9.8 ± 1.3 bc	10.5 ± 0.5 b	10.0 ± 0.5 b	11.3 ± 0.1 a	9.3 ± 0.2 c
Conidia length (µm)	18.1 ± 2.2	13.9 ± 1.5	15.1 ± 2.4	14.9 ± 1.5	10.2 ± 1.4
Conidia width (µm)	5.5 ± 0.4	5.1 ± 0.8	5.2 ± 0.7	4.8 ± 0.6	4.6 ± 0.7
Conidia shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical	Cylindrical
Conidial appressoria	Dark brown, ovoid to cylindrical	Dark brown, ovoid or slightly irregular	Very few, dark brown, and ovoids	Dark brown, ovoid	Light brown, elliptical or slightly irregular

* Different letters in the same line correspond to significant different mean values at P ≤ 0.05 by Tukey test.

and *C. asianum*) showed similar growing culture patterns on BDA medium, but the *C. nymphaeae* isolate exhibited a lower growth rate than the species of Gloeosporioides clade. Although, considered an important character of fungi, the growth rate is not directly related to fungal pathogenicity. As can be observed in figure 2 and table 3, *C. horii*, and *C. asianum* showed similar growth color and behavior defined as grey-green, with a reverse light grey with darker grey sectors. Conversely, *C. aenigma* had white to pale grey colony with concentric pale grey sectors, and *C. nymphaeae* showed light grey/green colonies with reverse white with concentric grey/ green sectors. The morphological characteristics of the persimmon isolates obtained in this research are similar to those described for these species (WEIS et al., 2012; DAMM et al., 2012).

All isolates produce hyaline conidia with a classical cylindrical shape with broadly rounded ends (Figure 2 and Table 3). However, as expected, species classified within the Gloeosporioides clade (*C. horii, C. fructicola, C. aenigma,* and *C. asianum*) showed longer and wider conidia than those of the *C. nymphaeae* isolate (Acutatum clade). Moreover, among the Gloeosporioides clade species, the conidia of *C. asianum* was somehow shorter than the other species of the clade, confirming the data published by WEIR et al. (2012).

Conidial appressoria were produced by all the isolates and species but were particularly scarce in the *C. aenigma* isolate. All of them exhibited the characteristic light brown to brown color determined by the accumulation of melanin pigments (KUBO & FURUZAWA, 1991). Conidial appressoria showed a similar size (14.1 ± 1.3 x 7.3 ± 1.4 µm) and did not differ significantly within and among species.

Pathogenicity tests on fruits and leaves of persimmon (vr. Kyoto) showed that all the species were able to infect fruits (Figure 3), and *C. horii* isolates cause larger lesions than the other species, indicating that the aggressiveness to persimmon varied among species, and can be responsible for the high prevalence of *C. horii* in anthracnose symptomatic fruits. Moreover, the six isolates of *C. horii* evaluated developed larger necrotic spots on persimmon leaves, reinforcing the high pathogenicity and host-specificity of *C. horii* on persimmon (XIE et al., 2010; MAY DE MIO et al., 2015). Moreover, just one of the two isolates of *C. fructicola*, and none of the isolates of *C. aenigma*, *C. asianum*, and *C. nymphaeae* developed necrotic regions on persimmon leaves.

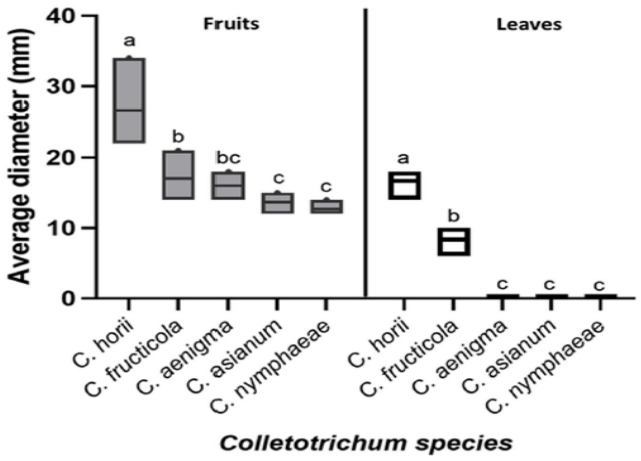

Figure 3 – Box plot summarizing pathogenicity test on fruits and leaves of persimmon cv. Kyoto. The results are the average lesion diameter of Colletotrichum horii (n=6), C. fructicola (n=2), C. aenigma (n= 1), C. asianum (n=1), and C. nymphaeae (n=1) isolates, all of them with 8 replications, after 7 days of incubation (28°C; 99% humidity). Bars with the same letters were not significantly different by the Tukey test ($P \leq 0.05$).

In conclusion, based on morphological data and molecular classification, five species of *Colletotrichum* were identified as causal agents of anthracnose in persimmon in the Southern Brazil region. These species belong to two different complexes (gloeosporioides and acutatum), with a high prevalence of *C. horii*. Although, all the species were able to infect and develop typical anthracnose symptoms in fruits of persimmon of Kyoto cultivar, the *C. horii* isolates were the most virulent, and together with *C. fructicola* were the only species that infected the persimmon leaves.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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