Persimmon anthracnose: a comparative study of aggressiveness on shoot and fruit among *Colletotrichum horii* isolates in southern Brazil

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ABSTRACT: *The persimmon tree is known for its rusticity and productivity and was first introduced to Brazil in the late 19th century. However, anthracnose disease is causing immature fruit drop and severe disease symptoms in persimmon fruit, shoots, flowers, and twigs. The causal agent was first described as the fungal species, Colletotrichum horii, which was first confirmed using only the ITS region. In this study, we compared the aggressiveness of 13 isolates of Colletotrichum spp. obtained from fruit and shoots of persimmon grown in the Metropolitan Region of Curitiba, Paraná State, Brazil. A multilocus molecular analysis was carried out based on ITS, GPDH, and EF genes, and we confirmed that the isolates were confirmed as C. horii. All isolates were pathogenic for unwounded and wounded persimmon fruit but differed in aggressiveness. Only one isolate was non-pathogenic when inoculated into unwounded persimmon shoots. Most isolates caused cankers and shoot death whether shoots were wounded or unwounded. In this study, we emphasized the importance of shoots as a source of primary inoculum. In future studies, it will be critical to further elucidate the epidemiological basis of anthracnose disease by conducting field studies to establish a more effective strategy for disease control.*
Key words: *canker, etiology, disease, Diospyros kaki.*

Antracnose do caquizeiro: um estudo comparativo da agressividade em ramos jovens e frutos entre isolados de *Colletotrichum horii* no sul do Brasil

RESUMO: *O caquizeiro foi introduzido no Brasil no final do século XIX. Esta planta é conhecida por sua rusticidade e produtividade. No entanto, a doença antracnose está causando queda e sintomas em frutos imaturos, ramos, flores e galhos no campo. O agente causal foi descrito inicialmente como Colletotrichum hori (confirmado usando apenas a região ITS) causando cancro de galho e sintomas de folhas. Neste estudo, comparamos a agressividade em caqui utilizando uma coleção de 13 isolados de Colletotrichum spp. obtidos de frutos e ramos de caqui da região metropolitana de Curitiba, Paraná, Brasil. Uma análise molecular multilocus foi realizada com base nos genes ITS, GPDH e EF, e os isolados foram confirmados como pertencentes a C. horii. Todos os isolados foram patogênicos em frutos não feridos e feridos, mas diferiram na agressividade. Apenas um isolado não foi patogênico quando inoculado sem ferimento em ramos. A maioria dos isolados foi capaz de causar cancro e morte de ramo, independentemente do ferimento. Neste estudo, enfatizamos a importância das novas brotações como fonte de inóculo primário. Para um próximo estudo, ainda é necessário ampliar as bases epidemiológicas com estudos em campo para estabelecer uma correta estratégia de controle desta doença.*
Palavras-chave: *cancro, etiologia, doença, Diospyros kaki.*

INTRODUCTION

The persimmon tree, *Diospyros kaki* L., was introduced to Brazil in the late 19th century. However, its cultivation only expanded around 1920 with the arrival of Japanese immigrants who provided new cultivars and production techniques (NEUWALD et al. 2009). Brazil currently ranks fifth in global persimmon fruit production after China, the Republic of Korea, Japan, and Spain (FAO, 2017). In 2017, Brazil produced 192,327 t of persimmon fruit nationwide, and production was concentrated in the states of São Paulo, Rio Grande do Sul, Minas Gerais, Paraná, and Rio de Janeiro (IBGE, 2017). In Paraná State, persimmon production decreased from 22,637 t in 2005 to 1,241 t in 2017 (SEAB/DERAL, 2017).

This reduction was mainly attributed to anthracnose disease which is caused by *Colletotrichum* species. This fungus limits tree productivity and reduces fruit quality which ultimately leads to economic losses in the persimmon industry.

The main concerns associated with the effects of anthracnose on plants have been an increased number of cankers on shoots, leaves, flowers, and the drop of immature fruit (BLOOD et al. 2015). Moreover, according to previous research, *Colletotrichum* spp. can infect flowers, leaves, twigs, and fruit, which severely damage the overall plant healthy and reduce yields. Furthermore, in multiple persimmon production fields throughout Brazil, many fruit drop prematurely (BLOOD et al. 2015). In fact, several persimmon producers in the northern and metropolitan regions of Curitiba have ceased cultivation of persimmon due to the high incidence of this disease (MAY DE MIO et al. 2015; BLOOD et al. 2015).

Given the high incidence of anthracnose disease and its effects on persimmon fruit production, epidemiological studies to further understand this disease have been conducted over the last few years. For example, five fungal isolates were identified as *Colletotrichum horii* using only the ITS gene, and inoculation tests further revealed that following exposure of plants to stress, these isolates caused

necrotic spots on twigs and leaves and subsequent defoliation (MAY DE MIO et al. 2015). However, the aggressiveness and pathogenicity of fungal isolates from shoots and fruit have not been assessed by comparing unwounded and wounded shoot and fruit inoculations, and the identification of these isolates has not been confirmed using a multi-gene approach as proposed by WEIR et al. (2010). Thus, the main objective of this study was to evaluate the aggressiveness of 13 isolates of *Colletotrichum* spp. from persimmon shoots and fruits from Paraná State. Additionally, multilocus (ITS, GAPDH, and EF-1α) analyses were carried out to identify the *Colletotrichum* spp.

MATERIALS AND METHODS

Collection of fungal isolates

From 2009 to 2011, persimmon fruit and shoots of Fuyu and Kakimel cultivars showing typical anthracnose symptoms were collected from conventional and organic orchards located in four municipalities (Bocaiúva do Sul, Quatro Barras, Lapa, and Campina Grande do Sul) of the Metropolitan Region of Curitiba, Paraná State, Southern Brazil (Table 1). After collection, shoots and fruits were placed in a moist chamber for 48 h. When the affected tissues showed abundant pathogen

Table 1 - Cultural characteristics of isolates of *Colletotrichum* spp. from persimmon trees located in the metropolitan region of Curitiba, Paraná, Brazil.

Isolate	Region	Cultivar	Plant Part	Year	Culture characteristics[1]	
					Verse	Reverse
D$_k$PR09-11	BS	Fuyu	Fruit	2009	Grayish brown	Dark/concentric
D$_k$PR10-13	CP	Fuyu	Shoot	2010	Dark gray	Dark/concentric with dark spots
D$_k$PR10-14	QB	Fuyu	Shoot	2010	Grayish brown	Light, concentric and darkening on the edges
D$_k$PR11-02	CGS	Fuyu	Fruit	2011	Gray	Dark from the center to the edges
D$_k$PR11-03	CGS	Fuyu	Fruit	2011	Gray	Dark/concentric
D$_k$PR11-04	CGS	Fuyu	Shoot	2011	Grayish brown	Dark/concentric
D$_k$PR11-07	LP	Fuyu	Shoot	2011	Light gray	Light yellow, concentric
D$_k$PR11-09	CGS	Fuyu	Fruit	2011	Dark brown to gray	Dark from the center to the edges
D$_k$PR11-10	CGS	Fuyu	Fruit	2011	Grayish brown	Dark/concentric
D$_k$PR11-15	CGS	Fuyu	Fruit	2011	Light gray	Light brown concentric
D$_k$PR11-16	CGS	Fuyu	Fruit	2011	Grayish brown	Dark, concentric
D$_k$PR11-17	CGS	Kakimel	Fruit	2011	Light gray	Dark/concentric with darker edges
D$_k$PR11-18	LP	Fuyu	Fruit	2011	Light gray	Dark/concentric with darker edges

[1]In Potato-Dextrose-Agar (PDA) medium at 25 °C. CGS: Campina Grande do Sul; LP: Lapa (25° 46′ 12″ S, 49° 42′ 57″ W); BS: Bocaiúva do Sul (25° 12′ 21″ S, 49° 6′ 54″ W); CP: Campo Largo (25° 27′ 32″ S, 49° 31′ 40″ W); QB: Quatro Barras (25° 21′ 57″ S, 49° 4′ 37″ W).

sporulation, conidia masses were removed using a sterile needle and cultured in Petri dishes containing potato-dextrose-agar (PDA). Plates were incubated for 15 days in growth chambers maintained at 25 ± 2 °C with a photoperiod of 12 h, and single-spore isolates were obtained.

DNA extraction and sequencing

Fungal genomic DNA extractions were performed using an UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, USA). DNA concentration was estimated using a NanoDrop™ (Thermo Fisher Scientific Inc., Waltham, USA). A multi-gene approach was utilized to investigate the phylogenetic affinities of *C. horii* isolates within the *Colletotrichum gloeosporioides* species complex based on three gene markers used by WEIR et al. (2010). The ITS region and parts of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and translation elongation factor 1-α (TEF1) genes were amplified using the primers ITS1, ITS4 (WHITE et al. 1990), GAPDH (TEMPLETON et al. 1992), and EF1-728F/EF1-986R (CARBONE & KOHN, 1999). The PCR conditions were: 5 min at 94°C, followed by 35 cycles of 30 s at 94 °C, 1 min at an adequate annealing temperature, 1 min at 72 °C, a 3 min finishing step at 72 °C, and a cool-down step to 4 °C. The annealing temperature varied for each gene: 60 °C (GAPDH), 55 °C (ITS), and 50 °C (TEF1). Amplicons were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA). The BLAST analyses were conducted in GenBank (http://www.ncbi.nlm. nih.gov). Multilocus sequence data obtained from individual genes were combined and aligned using Muscle (EDGAR, 2004) and manually improved using MEGA ver. 7.0 (TAMURA et al. 2011) when required. Phylogenetic analysis was performed in MEGA 7.0 using the method of maximum likelihood (ML) and the best substitution model was identified by Model Test and 1.000 bootstrap replicates of the combined sequences (ITS, GAPDH, and EF1-α) to form a dataset containing 32 isolates. Sequences of type strains for *Colletotrichum* and *Colletotrichum boninense* (MAFF305972) were used as outgroup terminals, while conspecific sequences of *C. horii* were used as in group terminals. Sequences derived in this study have been deposited into GenBank (Table 2).

Aggressiveness on fruit

The experimental design involved 13 fungal isolates (treatments) with six replicates in a randomized experiment. Three inoculations were performed on 52 fruits (26 wounded fruits and 26 unwounded fruits). The distribution of the isolates was also randomized among 156 spots (13 isolates x 6 replicates x 2 inoculation methods). We used healthy and unwounded Fuyu persimmon fruit; this cultivar is more susceptible to disease and is commonly used in persimmon production. Fruits were collected from an organic orchard at Campina Grande do Sul. Persimmon fruits were disinfected with 70% ethanol solution for 1 min, immersed in 1% sodium hypochlorite for 1 min, and then washed three times in sterile distilled water. Next, the surface-sterilized fruit were air-dried for 30 min and placed individually in plastic boxes (8 cm diameter) containing dampened sterile filter paper. The persimmon fruits were pinpricked to a 3.0 mm depth at a medium portion of each fruit, and a 3 mm the mycelial plugs obtained from 15 day-old colonies of each of the 13 isolates was placed onto the wounded surface and on a pre-marked surface of the unwounded fruit. Fruits were subsequently transferred to plastic boxes containing humidified filter paper at 25 ± 2 °C with a photoperiod of 12 h and humidity between 90% and 100%. Fruits were evaluated daily until the 12th day after inoculation. The incubation period (IP) for each isolate was considered as the time between inoculation and symptom onset in 50% of the fruit.

Following onset of anthracnose symptoms, the pathogens were re-isolated through the removal of fruit pieces (3 mm × 3 mm) from margins of the lesion and the healthy tissue. These pieces were placed in Petri dishes with PDA medium and incubated at 25 °C with a photoperiod of 12 h. Fruit pieces were incubated until colony growth to confirm Koch's postulates.

Aggressiveness on shoots

The experimental design was 13 fungal isolates (treatments) with 3 replicates in a randomized experiment. Eight three-year-old asymptomatic potted Fuyu persimmon plants were selected and pruned to allow the release of new shoots. Three shoots (approximately 30 days old) were selected and randomized per isolate. Each shoot was inoculated with each of the 13 isolates at two positions: 4 cm above the shoot base (wounded inoculation) and 10 cm above the shoot base (unwounded inoculation). Shoots were wounded using a sterile needle to create a lesion (approximately 3 mm long and 1 mm deep).

Table 2 - Collection details and GenBank accession numbers of isolates included in this study.

Species	Culture collection	Host	Origin	------------GenBank accession number------------		
				ITS	EF	GAPDH
C. horii	D_kPR09-11	*Diospyros kaki*	Brazil	JX486017	MH445526	MH445540
	D_kPR10-13	*D. kaki*	Brazil	KJ925141	MH445524	MH445538
	D_kPR10-14	*D. kaki*	Brazil	JX486019	MH445523	MH445537
	D_kPR11-02	*D. kaki*	Brazil	KJ925136	MH445532	MH445546
	D_kPR11-03	*D. kaki*	Brazil	KJ925137	MH445531	MH445545
	D_kPR11-04	*D. kaki*	Brazil	KJ925138	MH445530	MH445544
	D_kPR11-07	*D. kaki*	Brazil	JX486016	MH445529	MH445543
	D_kPR11-09	*D. kaki*	Brazil	KJ925139	MH445528	MH445542
	D_kPR11-10	*D. kaki*	Brazil	KJ925140	MH445527	MH445541
	D_kPR11-15	*D. kaki*	Brazil	KJ925142	MH445522	MH445536
	D_kPR11-16	*D. kaki*	Brazil	KJ925143	MH445521	MH445535
	D_kPR11-17	*D. kaki*	Brazil	KJ925144	MH445520	MH445534
	D_kPR11-18	*D. kaki*	Brazil	KJ925145	MH445519	MH445533
	C1069	*D. kaki*	Japan	GCU79682	GQ329692	GQ329685
	ICMP: 10492	*D. kaki*	Japan	GQ329690	JQ071901	GQ329681
	C1177	*D. kaki*	New Zealand	GQ329689	GQ329691	GQ329683
	C1180	*D. kaki*	Japan	NR119754	GQ329693	GQ329681
	MM150	*D. kaki*	New Zealand	GQ329688	GQ329695	GQ329684
	TSG002	*Mangifera indica*	China	AY791890	GQ329696	GQ329680
	TSG001	*D. kaki*	China	AY787483	GQ329697	GQ329682
C. gloeosporioides	ICMP:12071[*]	*Malus domestica*	New Zealand	GU174542	GU174565	GU174553
	ICMP:17797[*]	*M. domestica*	New Zealand	GU174548	GU174571	GU174559
	ICMP:17785[*]	*M. domestica*	New Zealand	GU174549	GU174572	GU174560
	ICMP:17941[*]	*D. kaki*	Japan	GU174547	GU174569	GU174557
	ICMP:17972[*]	*D. kaki*	New Zealand	GU174544	GU174566	GU174554
	ICMP:17973[*]	*D. kaki*	New Zealand	GU174545	GU174567	GU174555
	ICMP:17974[*]	*D. kaki*	New Zealand	GU174543	GU174568	GU174556
C. fragariae	ICMP:17814[*]	*Fragaria x ananassa*	USA: Florida	GU174546	GU174576	GU174564
C. kahawae	ICMP:17816[*]	*Coffea arabica*	Kenya	GU174550	GU174574	GU174562
C. musae	ICMP:12930[*]	*Musa sp.*	New Zealand	GU174552	GU174570	GU174558
C. xanthorrhoeae	ICMP:17903[*]	*Xanthorrhoea preissii*	Australia	GU174551	GU174575	GU174563
C. boninense	MAFF305972[*]	*Crinum asiaticum*	Japan	AB051400	GU174577	JX009905

GenBank accession numbers highlighted in bold have been generated in this study, [*] = Type strain. [*] GZAAS Guizhou Academy of Agricultural Sciences herbarium, China; ICMP International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; MTCC Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

One plug of mycelium (Ø 3.0 mm) from each isolate was carefully placed onto the wounded/unwounded part of the shoot and it was further used a parafilm to maintain humidity. Inoculated plants were then incubated at 23 °C ± 2 with a 12 h photoperiod. Fruits were evaluated daily by measuring the longitudinal lesion size until the 14th day after inoculation.

Statistical analyses

For aggressiveness tests, the design was completely randomized with three and six replicates for persimmon shoots and fruits, respectively. Analysis of variance (ANOVA) was conducted in the R program to analyze aggressiveness data and averages were compared using the Scott-Knott (SCOTT & KNOTT, 1974). Generated data from the wounded fruit were transformed using $\sqrt{x}$, while data from the unwounded fruit were transformed using $\sqrt{x+1}$.

Data regarding the diameter of the lesions on fruit from different evaluation dates were integrated in time and transformed into area under the disease-progress curve (AUDPC). The following

equation was used to calculate AUDPC: AUDPC = $\sum^{n-1} [(X_i + X_{i+1})/2] (t_{i+1} - t_i^{i=1})$, where X is the average aggressiveness of the disease by isolate; $X_1 = x (t_1)$, with n as the number of evaluations; and $(t_{i+1} - t_i)$ as the interval between two consecutive evaluations (SHANER & FINNEY, 1977).

RESULTS

The growth pattern of all colonies was radial with concentric circles. The colonies had a similar appearance, with the mycelium varying from grayish to dark brown in color with a velvet-like texture. The reverse color of colonies was light to dark yellow (Table 1).

It was confirmed that each fungal strain belonged to the *Colletotrichum* using the BLAST tool, which revealed that there was high similarity between strains characterized as *C. horii* (97.6 to 100%) in the GenBank database. These new sequences were deposited into GenBank (Table 2). The topology of the trees of individual gene regions was consistent; thus, the three loci were combined.

A multi-gene approach was utilized to investigate the phylogenetic affinities of *C. horii* isolates within the *C. gloeosporioides* species complex based on three gene markers used by WEIR et al. (2010). We reported that the examined isolates were separated within the same clade as the *C. horii* representatives (Figure 1) by clustering them with the reference isolates on the phylogenetic tree generated from maximum likelihood analysis based on the general time-reversible model. The tree with the highest log-likelihood (-4467.2619) is shown (Figure 1). Initial trees for the heuristic search were automatically obtained by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then by selecting the topology with superior log-likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.4840)]. The rate variation model permitted some sites to be evolutionarily invariable [(+I), 23.0808% sites]. The analysis involved 33 nucleotide sequences (32 in groups and 1 outgroup). All positions with less than 95% site coverage were eliminated; that is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 1,091 positions in the final dataset. The 14 *C. horii* isolates and the reference isolates clustered together with 93% bootstrap support. Further, the clades of *C. gloesporioides*

isolates, including their reference isolates, were also well supported by bootstrap values of 97%.

In the aggressiveness test, all isolates showed 100% infection on the wounded shoots. In unwounded shoots, 31% of the isolates did not cause infection (Table 3). Among the isolates, 38% and 23% caused the death of wounded and unwounded shoots, respectively. Figure 2 shows canker symptoms and shoot death after the inoculation of seedlings with *C. horii*.

Regarding persimmon fruits, the D_kPR11-09, D_kPR11-15, D_kPR10-14, D_kPR11-10, D_kPR10-13, and D_kPR09-11 isolates had the highest AUDPC, and these isolates were the most aggressive when inoculated into wounded or unwounded tissues. The D_kPR10-13 isolate was less aggressive and produced
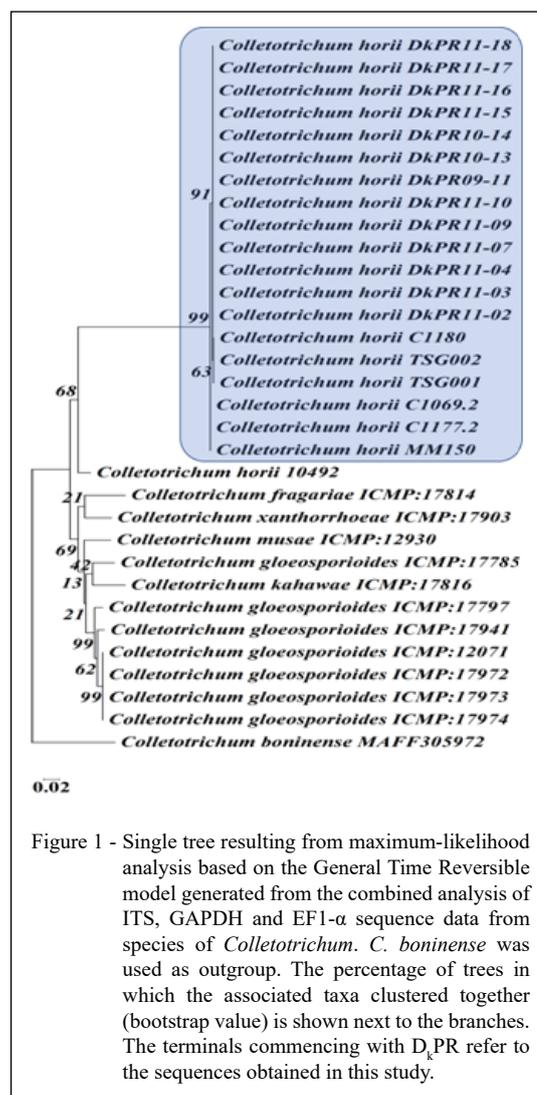

Figure 1 - Single tree resulting from maximum-likelihood analysis based on the General Time Reversible model generated from the combined analysis of ITS, GAPDH and EF1-α sequence data from species of *Colletotrichum*. *C. boninense* was used as outgroup. The percentage of trees in which the associated taxa clustered together (bootstrap value) is shown next to the branches. The terminals commencing with D_kPR refer to the sequences obtained in this study.

Table 3 - Canker lesions of Colletotrichum horii isolates on shoots after 14 days of inoculation in persimmon potted plants. Curitiba, Paraná, Brazil.

Isolates	---Length of the lesions on shoot (mm)---									
	---------------------------Wounded---------------------------					---------------------------Unwounded---------------------------				
	1	2	3	Average1		1	2	3	Average1	
DkPR11-15	10[*]	20[*]	11	13.7	a	12[*]	15[*]	11[*]	12.7	a
DkPR10-14	12[*]	14	12	12.7	a	9[*]	13[*]	19	13.7	a
DkPR11-09	11	11	13	11.7	a	0	0	0	0.0	b
DkPR11-03	11[*]	15	7	11.0	a	0	0	0	0.0	b
DkPR09-11	13	10	8	10.3	a	10	4	0	4.7	b
DkPR11-10	8	10	9	9.0	b	3	11	14	9.3	a
DkPR11-02	7[*]	6	11	8.0	b	1	0	0	0.3	b
DkPR11-04	7	10[*]	6	7.7	b	0	0	0	0.0	b
DkPR11-18	11	6	6	7.7	b	1	0	0	0.3	b
DkPR11-16	7	8	6	7.0	b	14[*]	1	1	5.3	b
DkPR11-17	6	8	6	6.7	b	6	2	0	2.7	b
DkPR10-13	6	8	6	6.7	b	1	1	0	0.7	b
DkPR11-07	6	7	6	6.3	b	0	0	0	0.0	b
Average				9.1	A				3.8	B

[1]Averages followed by the same letter, lowercase in the column and capital on the line, do not differ between themselves by the Scott-Knott test at a 5% probability level. To wounded shoot data was transformed by $\sqrt{x. 2}$ and to unwounded shoot data transformed by $\sqrt{x+1}$. [*]Canker in the shoots causing wilt of the shoots.

smaller lesions on wounded fruits and no symptoms in unwounded fruits (Table 4). Moreover, the D_kPR11-07 isolate was non-pathogenic in unwounded fruits (Table 3 and 4).

The incubation period varied between 3 and 4 days for most isolates infecting fruits, except D_kPR11-02, D_kPR11-03, and D_kPR11-17, which exhibited an IP of 6 days (Table 4).
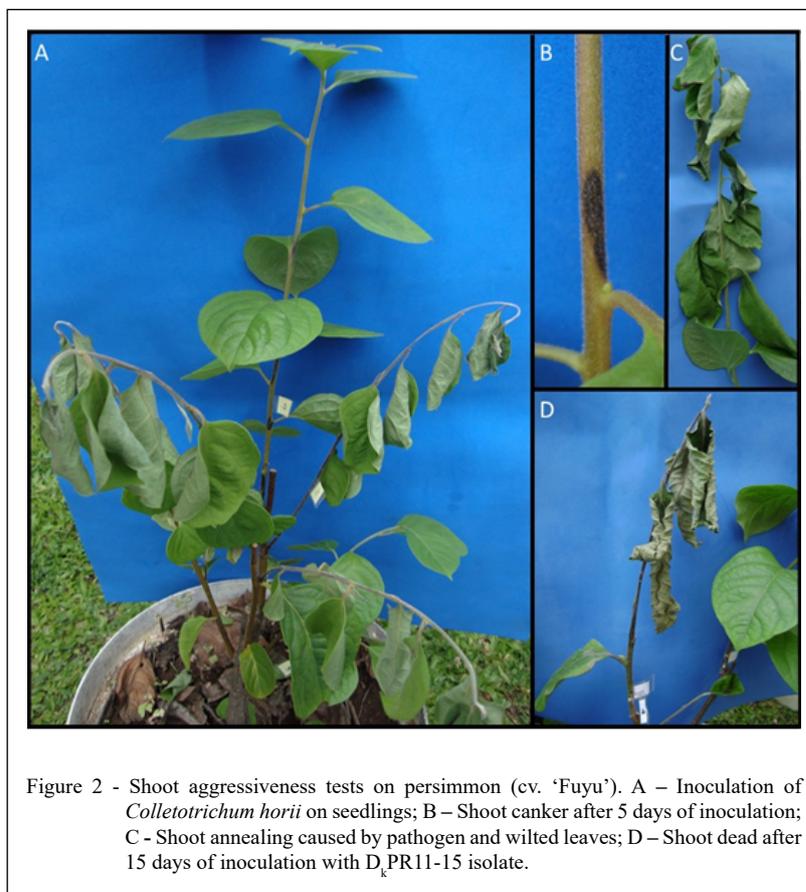
DISCUSSION

In this study, we provided new etiological information regarding *Colletotrichum horii* by comparing its aggressiveness in different parts of persimmon. This information will facilitate the development of an important and effective epidemiological strategy to manage this disease, especially given that cultural control by pruning infected shoots is necessary to reduce primary inoculum to avoid infection and continuous inoculum within the plant.

Anthracnose of persimmon is an emerging disease in Brazil, resulting in significant crop losses due to fruit drop and disease symptoms in fruit (BLOOD et al. 2015, MAY DE MIO et al. 2015). Diagnosis and control of this disease have thus gained significant attention. However, the taxonomy of *Colletotrichum* spp. has been revised (WEIR et al. 2012; DAMM et al. 2012), the morphological identification of *Colletotrichum* species has always been problematic. Morphological characteristics are complementary to molecular analyses, with many exhibiting plasticity that may vary with methodological approaches and experimental conditions (HYDE et al. 2009).

In this study, fungal colonies were predominantly grayish and velvet-like in appearance with a dark and concentric reverse side. XIE et al. (2010) described colonies on PDA as velvety, floccose, gray to dark gray, with large numbers of yellowish-orange conidial masses, edge regular and reverse dark gray to dark brown, with concentric zonation. Additionally, they showed no signs of sporulation or the presence of setae. XIE et al. (2010) further observed these same characteristics in *C. horii* isolates obtained from *D. kaki* cv. Wuheshi that was grown in China. Weir and Johnston (2010) characterized and neo-typified the fungus, *C. horii*, proposing a new delimitation of *Colletotrichum* species introduced into the "Genealogical Concordance Phylogenetic Species Recognition" (GCPSR) concept. It is complicated to separate these species based on a few

Figure 2 - Shoot aggressiveness tests on persimmon (cv. 'Fuyu'). A – Inoculation of *Colletotrichum horii* on seedlings; B – Shoot canker after 5 days of inoculation; C - Shoot annealing caused by pathogen and wilted leaves; D – Shoot dead after 15 days of inoculation with D_kPR11-15 isolate.

distinguishing morphological characteristics. Thus, researchers have used several other characteristics to distinguish these species, such as culture morphology, conidium size, mycelial growth rate, optimum growth temperature, and fungicide sensitivity (ADASKAVEG & FÖRSTER, 2000; PERES et al. 2005). Therefore, taxonomical and phenotypical identification methods must be used with caution (DAMM et al. 2012; WEIR et al. 2012) and supported by molecular characterizations (WEIR et al. 2012). In the current study, strains grouped in the *C. horii* clade exhibited no genetic divergence of sequences in the ITS, GAPDH, and EF1-α regions, suggesting that these regions were ideal for the identification of *C. horii*.

In the present study, collection of fungal isolates (2009-2011) was a comprehensive effort to identify *C. horii* associated with persimmon trees in the metropolitan region of Curitiba, Brazil. Phylogenetic analysis revealed that these isolates occur in the same clade as the neotypic of *C. horii* (WEIR & JOHNSON, 2010) and other strains from different regions and plants (ZHANG, 2008; YU et al.

2015). However, researchers recently collected fungal isolates during 2017-2018 season from persimmons in several parts of Brazil and identified, in addition to *C. horii,* new species within the *Colletotrichum* genus such as *C. fructicola*, *C. nymphaeae,* and *C. melonis* (CARRARO et al. 2019). Moreover, different species of *Colletotrichum* have been responsible for persimmon anthracnose worldwide, including *C. gloeosporioides* (Penz.) Penz & Sacc*., C. acutatum* J. H. Simmonds*, C. horii*, *C. karstii*, *C. dematium,* and *C. siamense* (WILLIAMSON & SUTTON, 2010; XIE et al. 2010; KWON & KIM, 2011; KWON et al. 2013; PALOU et al. 2015; JEON et al. 2017; HASSAN et al. 2018; LEE & JUNG, 2018). Furthermore, Deng et al. (2019) conducted a novel analysis of the genetic diversity of *C. horii* isolates infecting persimmon in China using an SSR approach. Based on cluster analysis of the SSR data, the 23 *C. horii* isolates collected from different provinces showed partial relationships among groups and regional origins.

Regarding the aggressiveness test, there was no specificity between the shoot D_kPR10-14

Table 4 - Incubation period (IP), area under the disease progress curve (AUDPC), and diameter of the anthracnose lesion caused by *Colletotrichum horii* isolates on fruit of Fuyu cultivar. Curitiba, Paraná, Brazil.

Isolates	--------------------------Wounded--------------------------			--------------------------Unwounded--------------------------		
	IP	AUDPC [3]	Diameter of the lesion [1,4]	IP	AUDPC [3]	Diameter of the lesion [2,4]
D_kPR11-09	4	126.3 a	19.3 a	6	36.3 a	5.0 a
D_kPR11-15	3	123.2 a	17.4 a	4	36.5 a	4.8 a
D_kPR10-14	3	117.8 a	17.5 a	6	37.7 a	5.6 a
D_kPR11-10	3	114.6 a	16.3 a	4	37.5 a	4.6 a
D_kPR10-13	4	111.5 a	16.8 a	nd	0 b	0 c
D_kPR09-11	3	109.8 a	15.1 a	6	35.6 a	5.1 a
D_kPR11-18	4	100.2 b	17.4 a	12	4.8 b	2.4 b
D_kPR11-04	4	100 b	19.0 a	12	11.1 b	2.5 b
D_kPR11-16	4	92.9 b	13.5 b	nd	1.8 b	0.9 c
D_kPR11-03	6	77.8 b	15.8 a	nd	9.5 b	1.9 b
D_kPR11-17	6	59.8 c	9.9 b	6	17.5 b	2.5 b
D_kPR11-02	6	51.9 c	9.4 b	nd	2.1 b	0.8 c
D_kPR11-07	12	12.2 d	3.5 c	nd	0 b	0 c
Average		84.5 A	14.7 A		17.7 B	2.7 B

Averages followed by the same letter, lowercase in the column and capital on the line, do not differ between themselves by the Scott-Knott test at a 5% probability level. [1]Data transformed by $\sqrt{x}$. [2]Data transformed by $\sqrt{x+1}$. [3]Area under the Disease-Progress Curve. [4] Diameter (mm) measured 12 days after inoculation. nd-not determined (12 days of assessment)

isolate, and the fruit D_kPR11-15 isolate as they were both able to induce symptoms in both parts of the plant and cause large lesions on wounded and unwounded plant parts. It was also evident that the injury promoted the infection process, and its absence likely impeded infection, as occurred in the isolates D_kPR11-09, D_kPR11-03, D_kPR11-04, and D_kPR11-07, which did not cause lesions on unwounded shoots. However, Xie et al. (2010) reported intense aggressiveness of *C. horii* isolates inoculated into both wounded and unwounded shoots of persimmon. The implication of the aggressiveness of *C. horii* on shoot and fruit in the field could be further related to the natural conditions of the plant, as for example the nutrition, aged, spacing between plants, cultural and phytosanitary measures. In addition, the frequency of new species can be influenced by all those factors.

A similar result was observed for the fruit aggressiveness test in which the same isolates (D_kPR10-14 and D_kPR11-15) caused large lesions and high AUDPC values in both wounded and unwounded fruit. However, 100% of a virulent isolates inoculated into unwounded fruits came from twigs or shoots and showed some differences when inoculated into fruits. Further, most isolates (69%) inoculated into wounded fruits had an IP of approximately 3-4 days, by Xie et al. (2010), who reported a three days incubation

period of *C. horii* isolates inoculated into persimmon tree branches.

In contrast, all isolates produced disease symptoms in persimmon fruits regardless of whether they were wounded or unwounded, and there were no relations between wounded and unwounded groups. Besides that, the wounded tissues inoculated showed more severe symptoms than unwounded tissues as pathogenicity assay on shoots as pathogenicity on fruit, causing more disease incidence and large lesions diameter; consequently, *Colletotrichum* isolates were more agressive when it was inoculated on wounded tissues, as related in other studies (MOREIRA et al. 2020; DE SILVA et al. 2019). This could be also associated with plant mechanism defense (cuticle and epidermis), if the tissue is wounded, it will be disrupted and susceptible to infection, because the cuticle action is considered a barrier to infection by *Colletotrichum* spp. (AUYOUNG et al. 2015).

According to the difference response about persimmon tissue pathogenicity among *Colletotrichum* isolates, we suggested that a comparative study should be conducted between the isolates of different plant parts as well as between different populations and different regions of persimmon production in Brazil, especially considering the recent detection of new

Colletotrichum species causing anthracnose in persimmon (CARRARO et al. 2019). This kind of information would be highly advantageous for developing future strategies to control this disease.

CONCLUSION

In conclusion, this study revealed that *C. horii* was the primary causal agent of anthracnose in persimmon shoots and fruits of the metropolitan region of Curitiba, Brazil, during the persimmon production seasons (2009-2011). The 13 *C. horii* isolates in this study exhibited no genetic divergence, and most colonies were grayish with mycelial growth occurring at approximately 25°C. The severe symptoms observed in wounded and unwounded shoots highlighted the importance of epidemiological studies to verify the importance of pruning shoots before blooming to avoid primary inoculum. Overall, all isolates were pathogenic for the shoots and fruits of *D. kaki* cv. Fuyu. However, there was variability in aggressiveness in that two isolates (one from the shoot and one from the fruit) showed significant differences in aggressiveness compared with other *C. horii* isolates.

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DECLARATION OF CONFLICT OF INTERESTS

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

Renato R. Y. Blood: Conceptualization, Methodology, Formal Analysis, Data Curation, Writing – Original Draft, Thiago A. Carraro: Writing- Reviewing and Editing. Josiane G. Figueiredo: Conceptualization, Methodology, Visualization, Writing- Reviewing and Editing. Louise Larissa May De Mio: Conceptualization, Supervision, Project Administration, Visualization, Funding Acquisition, Writing- Reviewing and Editing.

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