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TWIG BLIGHT AND DEFOLIATION CAUSED BY *Colletotrichum horii* IN PERSIMMONS IN BRAZIL[1]

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ABSTRACT - Persimmon anthracnose has been a great concern to Brazilian producers. This study aimed to identify and characterized the causal species from Brazilian persimmons by assessing morphological and molecular characteristics and pathogenicity tests. Five fungal isolates obtained from diseased twigs and fruits were identified as *Colletotrichum horii*, based on morphological characteristics and nucleotide sequences of ITS region. Inoculation tests revealed that the fungal isolates caused necrotic spots followed by defoliation of leaves, blight of twigs and buds of potted persimmon plants.
Index terms: Anthracnose, *Colletotrichum gloeosporioides, Diospyros*, Koch's postulate.

QUEIMA DOS RAMOS E DESFOLHA CAUSADA POR *Colletotrichum horii* EM CAQUIZEIRO NO BRASIL

RESUMO- Antracnose do caqui tem sido uma grande preocupação para os produtores brasileiros. O objetivo deste estudo foi identificar e caracterizar as espécies causadoras de antracnose avaliando as características morfológicas, moleculares e testes de patogenicidade. Cinco isolados, obtidos a partir de ramos e frutas doentes, foram identificados como *Colletotrichum horii* com base nas características morfológicas e sequências de nucleotídeos da região ITS. Inoculações revelaram que os isolados causaram manchas necróticas seguido de desfolha, queima de ramos e gemas em plantas de caqui cultivadas em vaso.
Termos de indexação: antracnose, *Colletotrichum gloeosporioides*, *Diospyros*, postulado de Koch.

The causal agent of persimmon anthracnose was previously identified as *Colletotrichum gloeosporioides* (ZHANG et al. 2005) based on morphological characteristics. Recently, Weir and Johnston (2010), studied isolates of *C. gloeosporioides* group species from New Zealand, characterized and neotypified as *Colletotrichum horii* based on Genealogical Concordance Phylogenetic Species Recognition (GCPSR). Anthracnose disease caused by *C. horii* results in considerable economic damage to sweet persimmon (*Diospyros kaki* L.) in Japan, China and in southern Korea yearly (KWON et al. 2013; WEIR e JOHNSTON, 2010; XIE, et al. 2010; ZHANG, 2008). In China, Zhang (2008) reported persimmon anthracnose causing twig blight, leaf defoliation and tree death in the Chongan area of Zhejiang province.

In 2009, Brazil came in the fourth place in the world rankings with 173,300 tons, behind China, Japan and South Korea (VIEITES, 2012). The major production states in Brazil are São Paulo, Rio Grande do Sul, Paraná and Rio de Janeiro (PEREIRA; KAVATI, 2011).

The Plant Pathology Laboratory of the Federal University of Paraná obtained samples of anthracnose on persimmon twigs and fruits with the same symptoms described by Xie (2010), which was not common until that year (2006) in Brazil. Local growers were unfamiliar with those symptoms and reported reduction in the local persimmon production, causing serious economic losses in several municipalities of the metropolitan region of Curitiba, state of Paraná.

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Although this disease has been present in Brazil since 1940, to our knowledge, no studies have been published regarding the pathogenicity of the isolates on twigs and leaves or identifying the species of the pathogen in Brazil. The correct identification of this pathogen will be useful for future epidemiological studies aimed at managing the disease in the field. The goal of the present study was to confirm identification of Colletotrichum species and to prove pathogenicity of the isolates causing twig blight and defoliation in Brazil.

Five single-spore isolates were obtained from anthracnose lesions on the persimmon twigs and fruits cv. Fuyu collected from different municipalities of the state of Paraná, Brazil. The isolates were named as EMCo7 (collected at 25° 46′ 12″ S, 49° 42′ 57″ W - Lapa), EMCo11 (25° 12′ 21″ S, 49° 6′ 54″ W - Bocaiúva do Sul), EMCo12 (25° 21′ 57″ S, 49° 4′ 37″ W - Quatro Barras), EMCo13 (25° 27′ 32″ S, 49° 31′ 40″ W - Campo Largo) and EMCo14 (Quatro Barras). Isolates EMCo7, EMCo12, EMCo13 and EMCo14 were derived from the diseased twigs, while isolate EMCo11 was from the diseased fruit. Isolated fungi were grown on PDA medium for 7 days at 25ºC and 12 h light/dark photoperiod.

Morphological characteristic of each isolate was performed to determine the species according to the methods described by Weir and Johnston (2010). Colonies on the PDA plate with margin initially white to grey and dark grey over time, fluffy surface and cottony aerial mycelium (Figure 1A). Regular margins that formed concentric zonation showed olivaceous black on reverse. After one week, the colonies reached a diameter of 45 – 65 mm, hyaline, cylindrical with obtuse apex, non-septate, smooth, size ranging from 16-22.5 x 4.5-5.5 (Figure 1B). The optimal temperature for the mycelium growth was approximately 25°C, and higher temperatures inhibited its growth. Colonies of *C. horii* were typically gray or pale gray in appearance (XIE et al. 2010; WEIR; JOHNSTON, 2010). The results of culture studies showed no distinct differences in characteristics among the isolates tested in this study. The conidial size of *C. horii* has varied in previous studies from 18.5-21.5×5.7 µm (XIE, et al. 2010), 15-21×4-5.5 µm (WEIR; JOHNSTON, 2010).

For nucleotide sequencing analysis of the causal fungus, genomic DNA of each isolate was extracted using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to the manufacturer's protocol.

PCR amplification was carried out using the following primers: ITS1F (CTT GGT CAT TTA GAG GAA GTA A) (GARDES; BRUNS 1993) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (White et al. 1990) as described by Weir e Johnston (2010). Amplicons were sequenced using both PCR primers and DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Biosciences). Sequences were manually aligned using MEGA v.5 software (TAMURA, et al. 2011) by inserting gaps. The obtained sequences were aligned according to existing sequences in the NCBI database though the BLASTn program. Consequently, nucleotide sequences of the ITS region of four the isolates (Accession numbers from JX486016 to JX486019) showed 100% homology with that of *C. horii* (No. GQ329687). These results agreed with those of previous reports that stated that *C. horii* causes persimmon anthracnose in China (XIE et al. 2010) and New Zealand (WEIR; JOHNSTON, 2010).

Pathogenicity tests were performed using 5 healthy 1-year-old persimmon plants cv. Fuyu, inoculating a suspension of 10^4 conidia/ml of isolate EMCo11 prepared from colony growed on PDA medium for 7 days at 25ºC and a 12 h light/dark photoperiod. The conidia were sprayed on the plants using a manual microspray (Famastil Taurus®) until the suspension ran off. The incidence and severity of the disease on the leaves were assessed daily. The severity of the disease was assessed using WinRhizo® when leaves fall down using a sample of 5 leaves per plant.

As a result, the lesions on the leaves and petioles appeared as small, circular necrotic black spots (Figures 1C and 1D). On the leaves the lesions appeared after five days of the inoculation and they are elongated to a round shape reaching 1-10 mm in diameter, and the lesions increased in size over time to a maximum diameter of 35 mm and the leaves fell down when the severity reach 23%. Along with tissue necrosis, conidial masses formed that exhibited a bright orange color. When the plants went into dormancy and during the assessment in the following year, no symptoms of the disease on the twigs had been detected for the first inoculation test.

In March of 2009, second pathogenicity tests were performed using 10 healthy, one-year-old persimmon plants cv. Fuyu that were being maintained in a 5 L container of sterilized soil at a greenhouse. The plants were inoculated by the same methodology as described for the first test. Each isolate was inoculated into two plants. After inoculation, the plants were immediately placed in a moist chamber composed of plastic bags in a greenhouse at 25 °C under natural light conditions for 48 h to provide optimal infection conditions. The

incidence of disease on the leaves and twigs was assessed during the first two months (before natural defoliation) and after 410 days (one month before the next natural defoliation) in May 2010. The incidence was calculated by mean of leaves with any symptoms of the disease related the total number of the leaves on the plant. For the twig blight it was counted the number of twigs of one year or two years-old per plant with disease symptoms in a total of twigs per plant. It was calculated incidence of twig canker.

All the inoculated plants exhibited small spots in areas on the leaf surface, mainly on veins and petioles, as observed in the first test, but no symptoms were observed on the twigs until at the beginning of winter. Nevertheless, in the following year, symptoms were observed on the leaves, twigs and buds of the inoculated plants. The incidence of symptoms on the leaves ranged from 3-35% (Table 1). On the twigs, infections resulted in round or oval lesions with black streak (Figure 1E) extended into the xylem, resulting in collapse with longitudinal cracking (Figure 1F). The average incidence of the symptoms appeared on 2-year-old twigs was 37.2 %, while that of 1-year-old twigs was 17.7%. On the buds, the symptoms were only observed on 4 buds of 2-year-old twigs.

We hypothesized that the pathogen survived during our experiment as an epiphyte or remained latent on buds or twigs after inoculation. The symptoms of the diseases on twigs were probably intensified by stresses caused by the high temperature and lack of water in the greenhouse during two weeks at winter time. Further studies on the development of the disease in different field conditions are mandatory to better understand this pathosystem.

Anthracnose caused by *C. horii* is an emerging disease that may threaten the profitability of persimmons in Brazil. It is necessary extensive research on controlling this disease, including breeding programs for resistant cultivars to anthracnose, additional studies on the etiology and epidemiology of persimmon anthracnose are required to improve disease management strategies. Also future research should investigate isolates of *C. horii* and its relationships with the host.

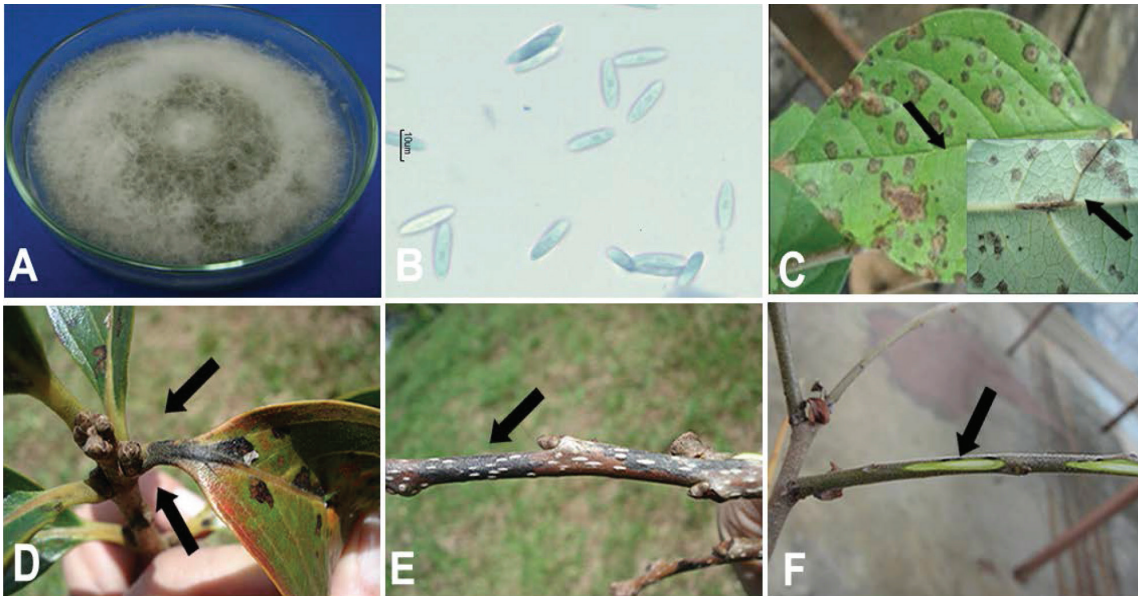

FIGURE 1 – A: Colony morphology of *Colletotrichum horii* on PDA + lactic acid 0.1%; **B:** Conidia in lactophenol cotton blue **C**; Small, concentric black spots depressed on leaves and a lesion on the vein in detail **D:** Black streak on petiole; **E:** Symptoms in 2-year-old twigs **F:** Blight formation in the twigs of persimmon cultivar 'Fuyu' in the field;

TABLE 1- Lesions on 1- and 2-year-old twigs and buds and incidence on the leaves of persimmon cultivar Fuyu after inoculation by spraying[x] five isolates of *Colletotrichum horii*.

Isolate[y]	Plant	2-year-old twig			1-year-old twig			Leaf		
		No. of twigs	With symptom		No. of twigs	With symptom		No. of leaves	With symptom	Incidence %
			Twig	Bud		Twig	Bud			
EMCo7	1	3	1	0	9	1	0	162	5	3.09
	2	4	2	1	13	1	0	106	16	15.09
EMCo11	3	5	1	0	11	2	0	98	3	3.06
	4	4	1	3	11	1	0	122	12	9.84
EMCo12	5	4	2	0	19	10	0	114	2	1.75
	6	4	3	0	26	1	0	122	10	8.20
EMCo13	7	4	1	0	19	1	0	147	7	4.76
	8	4	1	0	16	4	0	104	17	16.35
EMCo14	9	7	4	0	25	2	0	109	38	34.86
	10	4	0	0	20	7	0	127	5	3.94
Mean incidence (%)			**37.2**			**17.7**				**9.50**

[x]10^4 conidia.mL^{-1} until run off.
[y]EMCo7 (from Lapa), EMCo11 (from Bocaiúva do Sul), EMCo12 (from Quatro Barras), EMCo13 (from Campo Largo) and EMCo14 (from Quatro Barras). Isolates EMCo7, EMCo12, EMCo13 and EMCo14 were isolated from twigs, EMCo11 was isolated from fruit.

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