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A NEW METHOD FOR EARLY DETECTION OF *Ceratocystis* spp. ON VARIOUS HOSTS

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Ceratocystis wilt is a lethal disease to several hosts. This study tested the isolation of *Ceratocystis* spp. from the leaf blade of cacao and other hosts showing yellowing and the isolates pathogenicity to their hosts. Leaves with yellowished and/or darkened areas were disinfested and placed between slices of unripe cacao pods, like a sandwich, in a BOD chamber (25º C), for four days. Ascospores were transferred to Petri dishes containing PDA and their colonies were preserved. The presence of *C. cacaofunesta* in the leaf blade of cacao tree; *C. paradoxa* in coconut palm; and *C. fimbriata* in eucalyptus, rubber, soursop, custard-apple trees and coffee plants were confirmed. To test the isolates pathogenicity, culture discs or droplets of inoculum suspensions (3×10^4 i.u./mL) were applied in two points on leaves of each host, with and without wound, and evaluated in four to seven days. Chlorosis, lesions, mycelia and perithecia development were observed in the midrib and on blades of inoculated leaves. Therefore, an early method of detection of *Ceratocystis* spp. as well as another method to evaluate pathogenicity to various hosts using whole leaves of these hosts were described in this paper.

Key words: Ceratocystis wilt, pathogenicity, leaves

Um novo método para detecção precoce de *Ceratocystis* spp. em diversos hospedeiros. A murcha de Ceratocystis é uma doença letal ao cacaueiro e diversos hospedeiros. Neste trabalho testou-se o isolamento de *Ceratocystis* spp. a partir do limbo foliar de cacaueiro e outros hospedeiros com amarelecimento das folhas e a patogenicidade dos isolados. Folhas com áreas amareladas e/ou escurecidas foram desinfestadas, colocadas entre fatias de cacau verdoengo, como sanduíche, em BOD (25º C), por quatro dias. Ascósporos foram transferidos para placas de Petri com BDA e as colônias foram conservadas. Foi confirmada a presença, a partir do limbo foliar, de *C. cacaofunesta* em cacaueiro, *C. paradoxa* em coqueiro e *C. fimbriata em* eucalipto, seringueira, gravioleira, fruta-do-conde e cafeeiro. Para testar a patogenicidade dos isolados discos das culturas ou gotas das suspensões de inóculo (3×10^4 u.i./mL) foram colocados sobre cinco folhas de cada hospedeiro em pontos, com e sem ferimento, e avaliados entre 4 e 7 dias. Nas folhas observou-se clorose, formação de lesões, micélios e peritécios nas nervuras e limbo. Portanto foram descritos um novo método de detecção de *C. cacaofunesta* e também outro método para avaliar a patogenicidade em vários hospedeiros usando folhas inteiras desses hospedeiros.

Palavras-chave: murcha de Ceratocystis, patogenicidade, folhas

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Introduction

Ceratocystis wilt is a vascular disease that causes plant death and losses in cocoa plantations, and hence it's great economic importance in cocoa production in Brazil (Bastos and Evans, 1978; Sanches, 2007) and other countries. It was first reported in Brazil in 1978 on cacao, in Rondônia State (Bastos & Evans 1978), and in Bahia State, in 1997, in grafted plants in nurseries, and in 1998 in adult cocoa trees (Bezerra, 1997; Bezerra et al., 1998). Ceratocystis-wilt, caused by *C. cacaofunesta* Engelbr. & T.C. Harr**.** 2005, is widespread in the cacao-producing region of Bahia State (Luz et al., 2013). The Ascomycota *C. cacaofunesta* and *C. fimbriata* Ellis & Halst. 1890, need wounds to penetrate into the plants shoots (Baker & Harrington, 2004; Marin *et al*., 2003; Chong, 1961; Delgado & Suárez, 2003) due to the strong mechanical barrier of the epidermis (Luz et al., 2013). *Ceratocystis* is a xylem-invading pathogen, and so the hyphal growth and production of spores in the interior of the vessels may obstruct the perforations of tracheal elements, leading to water stress in the plant. When a plant is infected by this pathogen, the first symptoms displayed are loss of the dark green color of the leaves, followed by wilt, drying out, and death, with the dry leaves remaining adhered to the plant. When the stems are cut, internal brown radial streaks from the cord to the outside of the wood can be observed at the lesion site (Ferreira and Milani, 2002; Ferreira et al., 2006). In the field, dryness can be observed in the branches progressing to the trunk and slowly killing the plant, or the infection is initiated by the plant roots, causing sudden death (Ribeiro et al, 1986). Therefore, a method that can detect the infection before the symptoms manifest and the plant dies will be of paramount importance for the disease control. The objective of this study were: i) to isolate of *Ceratocystis* spp. from the leaf blades of cacao and other host trees as an early diagnosis and ii) to test the pathogenicity of these isolates to the hosts of origin.

Materials and Methods

Searching for *Ceratocystis* spp. in the cacao region

Collections of infected plants were performed on cacao farms of some municipalities (Belmonte, Camacan, Gandú, Ilhéus, Itacaré, Mutuípe, Santo Amaro, Una and Uruçuca) of the Bahian cacao-growing region (Table 1) during three years (2010 to 2012) to detect and isolate *Ceratocystis* spp. on the cacao tree (*Theobroma cacao* L.) and other hosts with initial symptoms of *Ceratocystis* wilt.

Leaves of cacao (*T. cacao*) (Figure 1A), eucalyptus (*Eucalyptus grandis* Hill Ex Maiden) (Figure 1B), rubber [Hevea brasiliensis (Willd. ex Adr. de Juss.) Muell.-Arg.] (Figure 1C), soursop (*Annona muricata* L.) (Figure 1D), and custard-apple (*Annona squamosal* L.) (Figure 1E) trees, coconut palm (*Cocos nucifera* L.), and coffee plant (*Coffea arabica* L.) (Figure 1F), with yellowish and/or darkened areas, which can be initial symptoms of the disease, were collected. The leaves were washed in running water and sequentially placed in alcohol 70% and sodium hypochlorite 2%. Fragments of the leaves were placed on slices (approximately 10 × 2 cm) of unripe cacao fruit, like a sandwich, and the open side was sealed

Table 1. Municipality of survey, host and collection number of the *Ceratocystis* spp. isolates obtained in the cacao growing region of Bahia

Municipality	Host	Isolate nº	*Ceratocystis* spp.
Belmonte	*Theobroma cacao*	125, 126, 127	*C. cacaofunesta*
	Annona muricata	177	*C. fimbriata*
	Eucalyptus grandis	118, 119	*C. fimbriata*
	Hevea brasiliensis	178	*C. fimbriata*
Camacan	*Annona muricata*	180	*C. fimbriata*
Gandú	*Theobroma cacao*	99, 100	*C. cacaofunesta*
Ilhéus	*Theobroma cacao*	211, 224	*C. cacaofunesta*
	Hevea brasiliensis	176	*C. fimbriata*
	Cocos nucifera	227	*C. paradoxa*
	Annona muricata	187	*C. fimbriata*
Itacaré	*Cocos nucifera*	155, 156	*C. paradoxa*
	Annona squamosa	193	*C. fimbriata*
Mutuípe	*Theobroma cacao*	104, 106, 109, 165	*C. cacaofunesta*
Santo Amaro	*Theobroma cacao*	98	*C. cacaofunesta*
Una	*Theobroma cacao*	145, 146	*C. cacaofunesta*
	Hevea brasiliensis	198	*C. fimbriata*
Uruçuca	*Coffea arabica*	192	*C. fimbriata*

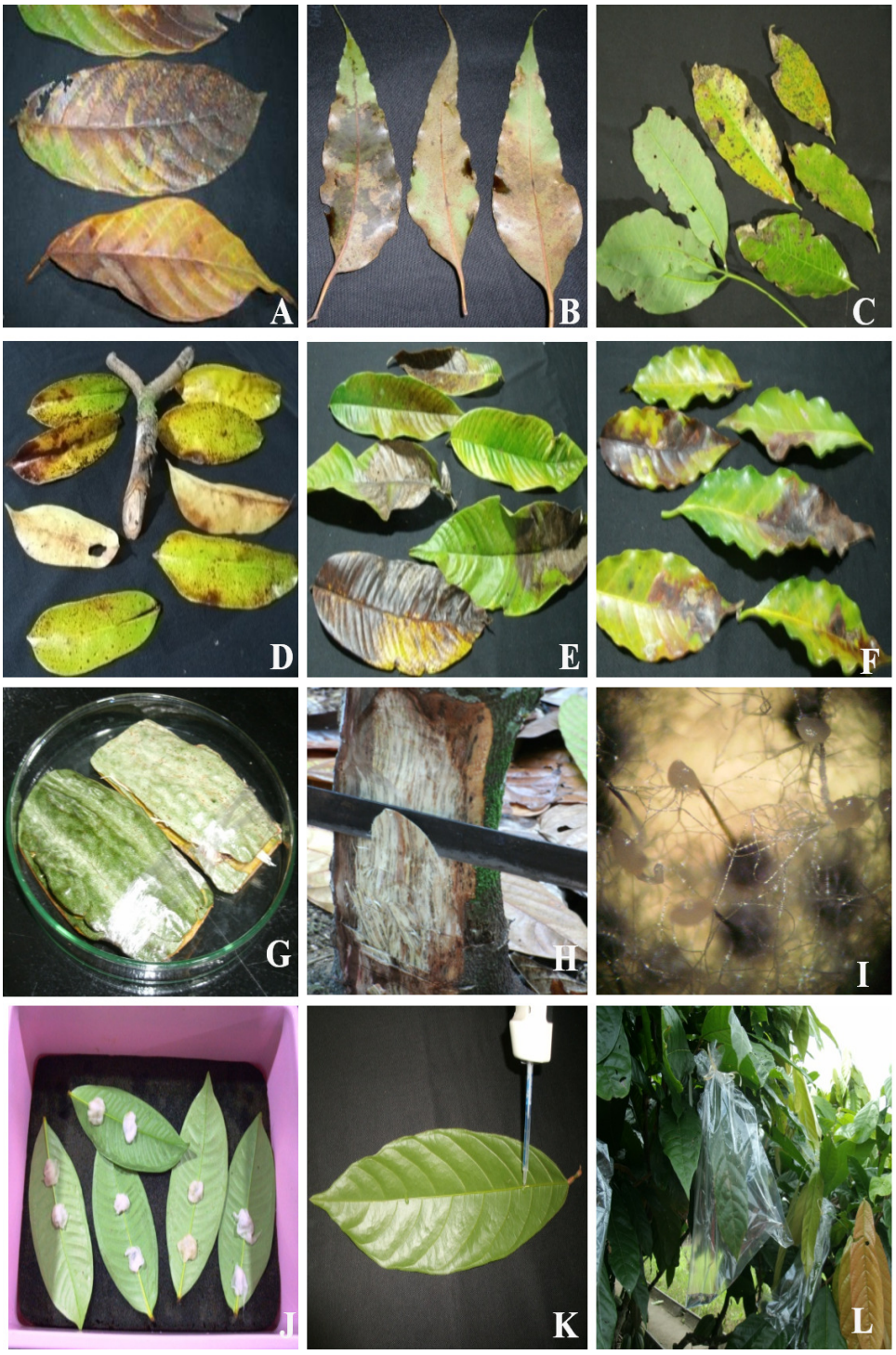

Figure 1- Leaves of the hosts with symptoms of *Ceratocystis* spp. (A-F): (A) cacao tree; (B) eucalyptus; (C) rubber tree; (D) soursop; (E) custard-apple tree; (F) coffee plant; methodologies of isolation (G,H): (G) from fragments of leaves placed between slices of unripe cacao fruits, (H) from infected trunk of cacao, (I) *Ceratocystis cacaofunesta* colony and perithecia; inoculation tests (J-L): (J) detached leaves with culture dishes, (K) detached leaves with droplets of inoculum suspension, (L) leaves of adult plants in the field.

with PVC film (Figure 1G). The "sandwiches" were incubated in plastic boxes containing foam moistened with distilled water in a BOD chamber (25º C) for four days, for production of perithecia. To confirm the presence of the pathogen on the hosts whose plants presented canker, the pathogen was also isolated in a traditional method, i.e., through fragments of infected stems (Figure 1H). Ascospores released from the perithecia formed were transferred to *potato* dextrose agar (PDA) *Petri dishes and* incubated at 25 ºC for ten days. The developed colonies (Figure 1I) were transplanted to test tubes or penicillin bottles containing PDA or sterile water and maintained in the Cacao Research Center (CEPEC) *Ceratocystis* collection culture.

Pathogenicity test

To test the pathogenicity of the isolates to stern and leaves of the original hosts experiments were done under field and laboratory conditions. Under field conditions eight wood branches with approximately 1.5 cm diameter of adult cacao, eucalyptus, rubber,

soursop and custard-apple trees and coffee plants were marked in plants of each host under field conditions at CEPLAC/CEPEC. Four of these branches received the inoculum and the other four were used as control. Superficial cuts were made horizontally with a scalpel and a droplet of 30 µL of the inoculum suspensions adjusted to 3.0×10^4 infective units/mL, in 0.3 % water agar (WA) was deposited. A cotton ball moistened with sterile distilled water was placed at the incision site and covered with a sealing tape (Silva et al., 2007). Sixty days after inoculation, the branches were cut at 20 cm below the inoculation point, labeled and brought to the laboratory where the lesion areas were measured after removing the barks off.

Young leaves of the seven hosts, including the coconut palm, were collected, washed and superficially disinfested. Discs with the cultures (0.5 cm in diameter) grown on a Petri dish containing PDA, incubated for 10 days, were placed on five clean leaves of each hosts in two points, with and without injury. A cotton ball moistened in sterile water was placed on each disc, and the leaves were maintained in a moist chamber (Figure 1J); drops of the suspension of the pathogens at the concentration of 3×10^4 cfu/mL were also utilized. In this case, the cotton was not necessary (Figure 1K). Formation of lesions and perithecia was evaluated in four to seven days after inoculation.

Leaves of health adult plants of cacao were also inoculated using culture discs or droplets of inoculum suspension of the same isolates (Figure 1L).

Results and Discussion

Ceratocystis cacaofunesta was isolated from both leaf blades and infected stems from cacao; *C. fimbriata* from eucalyptus, rubber, soursop, custard-apple, and coffee ; as well as *C. paradoxa* (Dade) C. Moreau 1952, from coconut. Due to the specificity of *C. cacaofunesta* to *T. cacao* and the literature reports attributing the infection of eucalyptus, rubber, soursop and custard-apple trees and coffee plant (Ribeiro et al., 1986; Ferreira et al., 1999; Silveira et al., 1994; Trindade & Furtado, 1997; Furtado et al., 2008; Marin et al., 2003) to *C. fimbriata* and of coconut palm to *C. paradoxa* (Pinho et al., 2013), it is assumed that the method of isolation from leaves can be utilized for the three species. These three species are those which predominate as pathogens of this genus in Brazil, with *C. cacaofunesta* causing problems in cacao plantations, *C. paradoxa* in monocotyledons, and mainly *C. fimbriata* in herbaceous and woody plants of great economic importance such as mango, rubber and eucalyptus (Bezerra, 1997; Silveira et al., 1994; Ferreira 1999).

Ceratocystis spp. is usually detected from fragments of the infected stem placed in a moist chamber in a culture medium such as PDA (Ferreira, 1989), or by the carrot-bait technique (Laia et al., 2000). There is also the technique of rapid detection of *Ceratocystis* spp. on infected stems by visualizing chlamydospores in the xylem vessels, pith and medullary rays from histopathological sections on an optical microscope (Ferreira et al., 2005). However, to perform these tests it is necessary to cut the stem in search of the lesion in dead plants. If the plant only presents leaf yellowing, with few branches affected, this technique is impracticable because it would injure the plant. Therefore, the early detection of the pathogen by isolating leaves with the initial symptoms of the disease is of paramount importance, given that it will facilitate the diagnosis and allow control decisions, removing the infected branch if the infection is localized, or the infected plant when the infection is generalized, thereby preventing the spread of the disease by vectors (Rosseto et al., 1980; Baker & Harrington, 2004; Delgado & Suárez, 2003). It will also be possible to determine whether the neighboring plants are infected.

All inoculated isolates of *Ceratocystis* spp. were pathogenic to their respective hosts, with formation of irregular brown lesions (internal streaks) on the inoculated branches of the adult plants (Figure 2A). The pathogen was re-isolated from the lesions, thus complementing Koch's postulate.

In the leaves of cacao (Figure 2B), eucalyptus (Figure 2C), rubber (Figure 2D), soursop (Figure 2E) and custard-apple trees, coffee plant (Figure 2F) and coconut palm (Figure 2G), from the fourth day after inoculation it was possible to observe yellowing and formation of brown/darkened lesions in inoculations with culture discs and droplets inoculum suspension. In the points without injury neither these lesions nor perithecia were formed, but in the points with injury, lesions with mycelial growth developed at the leaf midrib where the perithecia were formed in all hosts

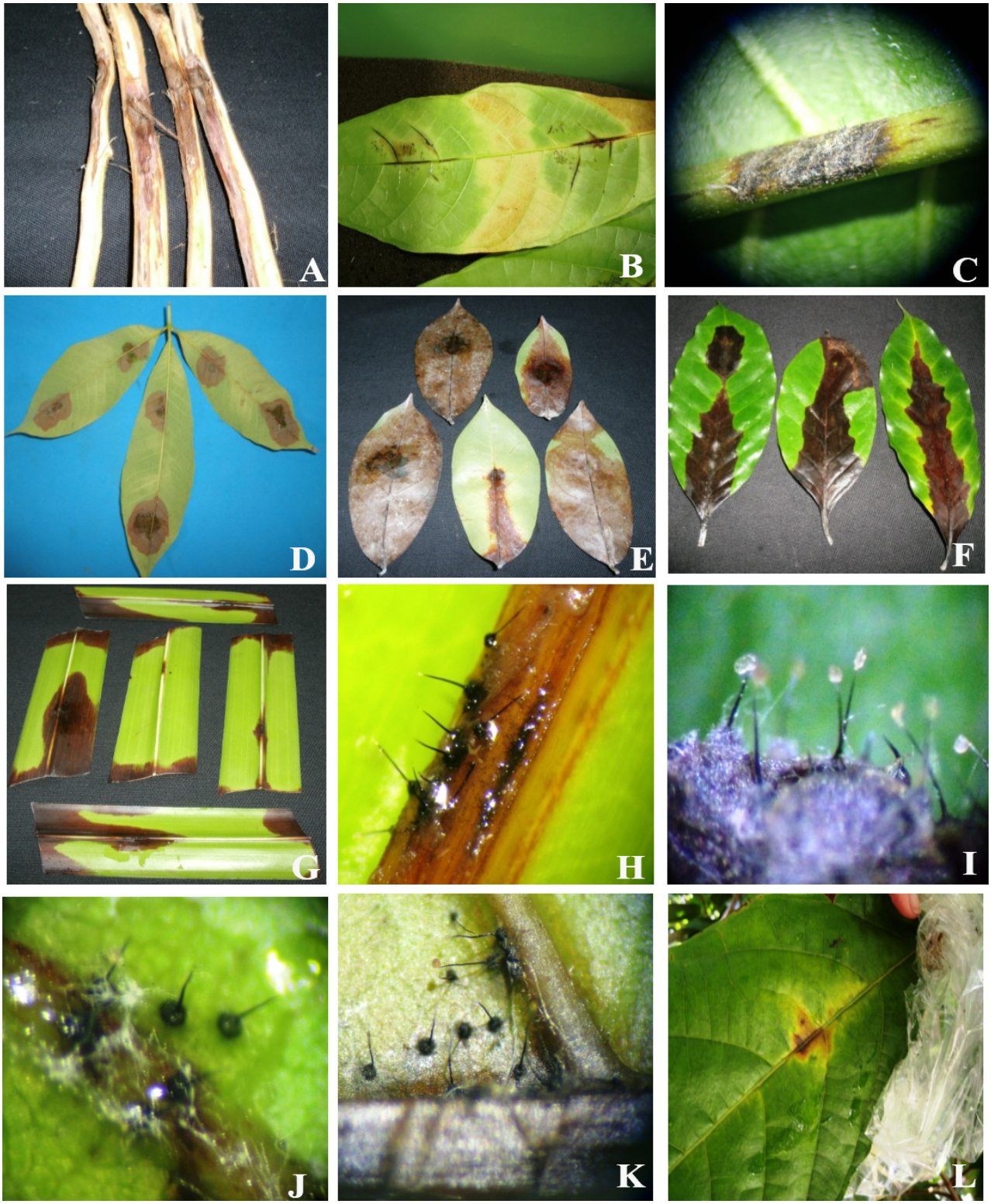

Figure 2 – Symptoms of *Ceratocystis* spp. in leaves and stems of the inoculated hosts: (A) irregular brownish lesions on stems; (B-F) yellowish and/or brownish lesion on leaves of: (B) cacao tree, (C) eucalyptus, (D) rubber tree, (E) soursop, (F) coffee plant, (G) coconut tree; (H, I) perithecia formed on the midrib of inoculated leaves; (J, k) mycelia and perithecia on leaf blade ribs; (L) lesions formed in leaf of adult cacao plants in the field.

tested (Figures 2H, I). In some cases, perithecia were formed on the leaf blade (Figures 2J, K). On leaves of health plants inoculated under field conditions, lesions and perithecia were also formed (Figure 2L).

The use of leaves for inoculation and isolation of *Ceratocystis* spp. in several hosts showed to be efficient, thus becoming an early, fast, practical and non-destructive method for detection of the pathogen under field conditions. Therefore, in this study we have reported an early method of detection of *Ceratocystis* spp. as well as another method to evaluate pathogenicity to various hosts using whole leaves of these hosts.

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