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Characterization of *Ceratocystis fimbriata* from passion fruits

Caracterização de **Ceratocystis fimbriata** *de frutos de maracujazeiro*

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ABSTRACT: Passion fruits (*Passiflora edulis*) were found with symptoms of rot in the field, in the city of Tanhaçu, Bahia. After isolating the pathogen associated with this rot, in the present study we aimed to characterize the *Ceratocystis* isolate from passion fruit for better understanding this pathosystem. Molecular characterization was done based on the region ITS-5.8S rDNA. Pathogenic characterization was carried out for seedlings and fruits of passionflower. Passion fruit colonization was monitored by means of scanning electron microscopy techniques (SEM). DNA analysis of the *Ceratocystis* isolate from passionflower pointed out that this species belongs to *Ceratocystis fimbriata*. The inoculated passionflower seedlings showed injury at 30 days post-inoculation, but no inoculated plant showed wilt or died. Considering fruits, no differences were found for lesions caused by this fungus among cultivars, and lesions had average diameters of 1.0 and 2.2 cm at 7 and 11 days, respectively. The analysis using SEM indicated fungus spore germination and penetration in the fruit between 2 and 6 hours post-inoculation. At 12 and 24 hours post-inoculation, fruit colonization was noted both externally and internally, while fruit wall degradation started at 48 hours post-inoculation. At 90 hours post-inoculation, formation of new perithecia was observed inside and outside the fruit. This study complements the available information about the interaction of this fungus with passion fruit.

KEYWORDS: rot; species; microscopy.

RESUMO: Frutos de maracujá (*Passiflora edulis*) foram encontrados com sintomas de podridão no campo, na cidade de Tanhaçu, Bahia. Após isolamento do patógeno associado a essas podridões, o presente trabalho teve como objetivo realizar a caracterização de um isolado de *Ceratocystis* de maracujá para melhor compreender esse patossistema. A caracterização molecular foi realizada com base no sequenciamento da região ITS-5.8S rDNA. Realizou-se a caracterização patogênica em mudas e frutos de maracujá. A colonização dos frutos de maracujá foi acompanhada com técnicas de microscopia de varredura (MEV). A análise do DNA do isolado de *Ceratocystis* mostrou que este pertence à espécie *Ceratocystis fimbriata*. As mudas de maracujá inoculadas apresentaram lesão 30 dias após a inoculação, e não foram observadas murcha nem morte das plantas inoculadas. Nos frutos não foram constatadas diferenças nas lesões causadas por esse fungo entre os cultivares, com médias de lesões de 1,0 a 2,2 cm de diâmetro, aos 7 e 11 dias, respectivamente. Nas análises realizadas em MEV, foram observadas a germinação dos esporos e a penetração do fungo nos frutos no período entre 2 e 6 horas após a inoculação. Doze e 24 horas após a inoculação foi visualizada a colonização do fruto, tanto externa como internamente, e 48 horas após a inoculação se notou o início da degradação da parede externa da casca do fruto. Noventa horas após a inoculação, observou-se a formação de novos peritécios, tanto na parte interna como na parte externa do fruto. Este estudo vem complementar as informações relacionadas à interação desse fungo com frutos de maracujazeiro.

PALAVRAS-CHAVE: podridão; espécies; microscopia.

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INTRODUCTION

The genus *Ceratocystis* covers several fungal species distributed among different places of the world. In Brazil, there are reports of few species belonging to this genus, including *Ceratocystis cacaofunesta*, *Ceratocystis paradoxa* and the most important species *Ceratocystis fimbriata*. Considering woody plants, *C. fimbriata* is a pathogen typical of xylem and its marked symptoms are dark radial striae from the medulla to the outer part of the xylem (Ferreira; Milane, 2002; Baker; Harrington, 2004). Generally, a plant infected with such a pathogen presents symptoms like leaf wilting and, consequently, drought. Cultures of *C. fimbriata* give off a smell of a ripe fruit. These volatile substances play an important role in the epidemiology of this disease since they attract the vector insect.

In Brazil, up to the middle of the 1990s, *C. fimbriata* was only considered a problem for crops of mango (*Mangifera indica* L.). Currently, this fungus has been shown harmful to other cultures like cacao (*Theobroma cacao*) (Bezerra, 1997), fig (*Ficus carica*) (Valarini; Tokeshi, 1980), teak (*Tectona grandis*) (Firmino et al., 2012b), atemoya (hybrid of *Annona cherimola* and *Annona squamosa*) (Firmino et al., 2012a), and, recently, yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) (Firmino et al., 2013). In this last case, the fungus causes rot to the fruits without causing any damage to the plant.

According to Harrington et al. (2011), the genus *Ceratocystis* can be divided into four distinct clades, or groups: Latin America, North America, Asia and Africa. Within the Latin American clade, Baker et al. (2003) studied different isolates of cacao, *Herrania* sp., sweet potato, *Platanus* sp., coffee, mango, *Annona* sp., eucalyptus and *Gmelina* sp. They verified, based on a pathogenicity test, that there is a specialization level within this host clade. Thus, Baker et al. (2003) hypothesized that local populations of *C. fimbriata* are host-specialized. Based on these studies, it was suggested that the evolution and divergence of species of *Ceratocystis* may have been conducted by host specialization, since there is little morphological differences between species of these fungus (Ferreira, 2009). Analysis on electronic scanning microscope, that were aimed to monitor the colonization of five *Ceratocystis* isolates from different hosts (eucalyptus, cocoa, mango, teak and atemoya) on the surface of eucalyptus plants to show that all isolates were capable of germinating, penetrating and developing in the vessel elements of eucalyptus plants within 6 hours, demonstrated that these fungal isolates, even from other hosts, are capable of developing in the xylem of eucalyptus plants (Firmino et al., 2015).

Report of *Ceratocystis* causing rot to passion fruits is something new. So far, the reported occurrence of this disease has been limited to drought symptoms related to xylem invasion by this fungus. Thus, the present study aimed to conduct molecular and pathogenic characterization of this fungus in plants and fruits of passionflower to improve the understanding of this pathosystem.

MATERIALS AND METHODS

Fruits of passionflower showing holes and rot were found in the field, in the city of Tanhaçu, Bahia, Brazil. These fruits had perithecia typical of *Ceratocystis*, which was isolated and deposited in the mycology collection located at the Laboratory of Forest Pathology, of the School of Agronomical Sciences of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), in Botucatu, São Paulo, Brazil (Firmino et al., 2013). For its molecular characterization, the isolate was recovered, removed from the oil and cultured on PDA medium (potato, dextrose and agar) for DNA extraction. The DNA was extracted according to the method developed by Murray e Thompson (1980), with modification. This DNA was used to amplify the region ITS-5.8S rDNA based on the protocol described by Johnson et al. (2005). The obtained DNA was sequenced and edited by using the software BioEdit Sequence Alignment Editor (1997–2005). After edition, that sequence was used to search for similar sequences by adopting the software Basic Local Alignment Search Tool (Blastn) of the National Center for Biotechnology Information (NCBI). The obtained sequences were aligned and processed with the software Mega 5.05, so that a phylogenetic tree could be built, using the method "Tamura-3-parameter" (Tamura, 1992). The distance matrix was constructed based on the Neighbor-Joining method. A bootstrap was applied with 10,000 replicates.

This *Ceratocystis* isolate from passionflower plants (one month of age) was subjected to pathogenic characterization by adopting two methods: inoculation with mycelial disks of this fungus, as described by Silveira et al. (2006), and deposit of a suspension of 10^8 cylindrical spores on the stem of the plant (Zauza et al., 2004). Evaluation occurred at 30 days post-inoculation. Seedlings were transversally sectioned in the stem to monitor the fungal invasion through the xylem. This fungal invasion into the xylem has as characteristic the vessel discoloration and darkening due to the collapse of tissues invaded by this fungus. Thus, the invasion could be measured from the inoculation site with the aid of a ruler. Five plants were employed for each inoculation method.

The isolate from fruits underwent pathogenic characterization by using six cultivars of yellow passion fruit (BRS Sol de Cerrado, BRS Ouro Vermelho, BRS Gigante Amarelo, Afruvec, FB 100 and FB 200). The fruits were collected from

an experimental orchard located in São Manuel, São Paulo, Brazil, when approximately 75% of their shell was yellow, washed with neutral soap and dried at room temperature in the laboratory. Inoculation was done by depositing 40 µL of the spore suspension (10^5 conidia/mL) on the equatorial region of the fruit, perforating 5 mm of it, through the drop, with a histological needle. Then, after 24 hours in a moist chamber, the fruits were kept for 11 days, at 25ºC, in biochemical oxygen demand (BOD). Rot severity was evaluated by perpendicularly measuring the lesion at 7 and 11 days. Six fruits were adopted per cultivar and the experiment was repeated once.

This characterization in fruits was complemented with colonization analyses by means of scanning electron microscopy. In this case, besides the isolate from fruits of passionflower, monitoring of the colonization of these fruits was done for other five *Ceratocystis* isolates from different host species: mango (ACF1), cacao (ACF15), atemoya (ACF24), eucalyptus (ACF38) and teak (ACF50). Thus, their capability to cause injury to passionflower could also be verified. For this, passion fruits were washed with neutral soap and sanitized with hypochlorite at 2% for 30 minutes. After sanitization, fruits were allowed to dry at room temperature for more than 12 hours. Once they were completely dried, fruits were placed on plastic trays, separating one tray for each studied isolate. On each tray, eight fruits were deposited. Each fruit received five drops of a spore suspension of the *Ceratocystis* isolate. No injuries were done to the fruit in this inoculation.

Following inoculation, the trays containing the fruits were kept in a moist chamber at 25ºC, in the dark. Disks were collected from the inoculation site in the fruit (around 5 mm diameter) at pre-determined intervals (2, 4, 6, 12, 24, 48, 72 and 96 hours) and fixed in "Karnovsky" solution (glutaraldehyde at 2.5%, paraformaldehyde at 2.0%, phosphate buffer at 0.05M, pH 7.2) during a minimal period of 24 hours for preparation and analysis under a scanning electron microscope. Each inoculated fruit represented one collection time. For each collection, five disks were removed from the fruit.

Fragments from the inoculated fruits were transferred from "Karnovsky" fixative to 1.5 mL microtubes containing sodium phosphate buffer, 0.05 M, where they were kept for 10 minutes. Then, the samples were dehydrated in a series of increasing acetone concentrations (30, 50, 70 and 90% for 10 minutes each, and 100% for three times of 10 minutes). Following this step, they were taken to the critical point device to complete the drying. Once dehydration was completed, the samples were mounted on stubs and covered with gold. Sample preparation and observation were carried out at the Center for Electron Microscopy located at Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ) of Universidade de São Paulo (USP).

RESULTS AND DISCUSSION

The studied isolate from passionflower was identified as *C. fimbriata*, as shown in the phylogenetic tree (Fig. 1). It was close to the fungal isolates from Latin American eucalyptus, but did not group to the isolates from Uruguay or Bahia, which shows that this isolate can be different from those that attack eucalyptus in Brazil. The isolates from Brazilian mango also kept very distant from the isolate from passionflower.

Only two plants inoculated with mycelial disk died due to collar necrotic lesion. The remaining inoculated plants, regardless of the method, did not show any symptom. Symptomatic plants had slight xylem darkening at the inoculation site, not exceeding 5 mm, which did not seem to result in damage to the plants. Wilt or drought symptoms were not observed in inoculated plants, but this may be due to the short evaluation period to which plants were subjected. There are stories of susceptibility to this fungus in eucalyptus plants which, even after 30 days of inoculation, did not show external symptoms of the disease, but had significant lesions internally in the xylem (Zauza et al., 2004).

Considering the pathogenicity tests of fruits, there were no differences among cultivars, according to Tukey's test ($p<0.05$), and lesions had average diameters of 1.0 and 2.2 cm at 7 and 11 days, respectively, evidencing the lack of resistance of the tested materials (Table 1).

As shown in Figures 2 to 4, only the isolate from passionflower was capable of developing on the tested fruits. The inoculated spores of isolates from mango, cacao, atemoya, eucalyptus and teak showed no germination, even after 96 hours of inoculation in passion fruits (Fig. 3).

As regards fruit colonization by the isolate from passionflower, spore germination and penetration in the fruit wall occurred between 2 and 6 hours post-inoculation. Differently from what was observed for xylem colonization, this isolate seems to form a structure similar to an appressorium, which helps it fix and penetrate the fruit wall without the aid of any injury. After 12 and 24 hours of inoculation, it was already possible to notice fruit colonization either externally or internally, in the inner part of the shell. At 48 hours post-inoculation, the outer part of the fruit shell started to undergo degradation due to the increased mycelium quantity in this region (Fig. 4). This fact became more frequent with time, and at 96 hours post-inoculation formation of new perithecia could already be seen, both in the inner and in the outer part of the fruit shell (Fig. 2). These new perithecia could be seen with the naked eye as small black dots.

It is important to highlight that, in the last evaluation period, it was already possible to note the rupture caused by the fungus on the outer part of the shell to allow the

exit of the new structures, both sexual and asexual (Fig. 2). These findings are of great importance since, as proven in the pathogenicity test, the isolate from passionflower is not capable of causing wilt to plants when inoculated in the xylem of this same species, differently from the tested isolates, which were capable of causing wilt to the host plants of origin. Thus, the isolate from passionflower seems to be a pathogen specific to this species, behaving more aggressively in fruits than in plants of passionflower. In addition, to manifest the severe symptoms observed in the

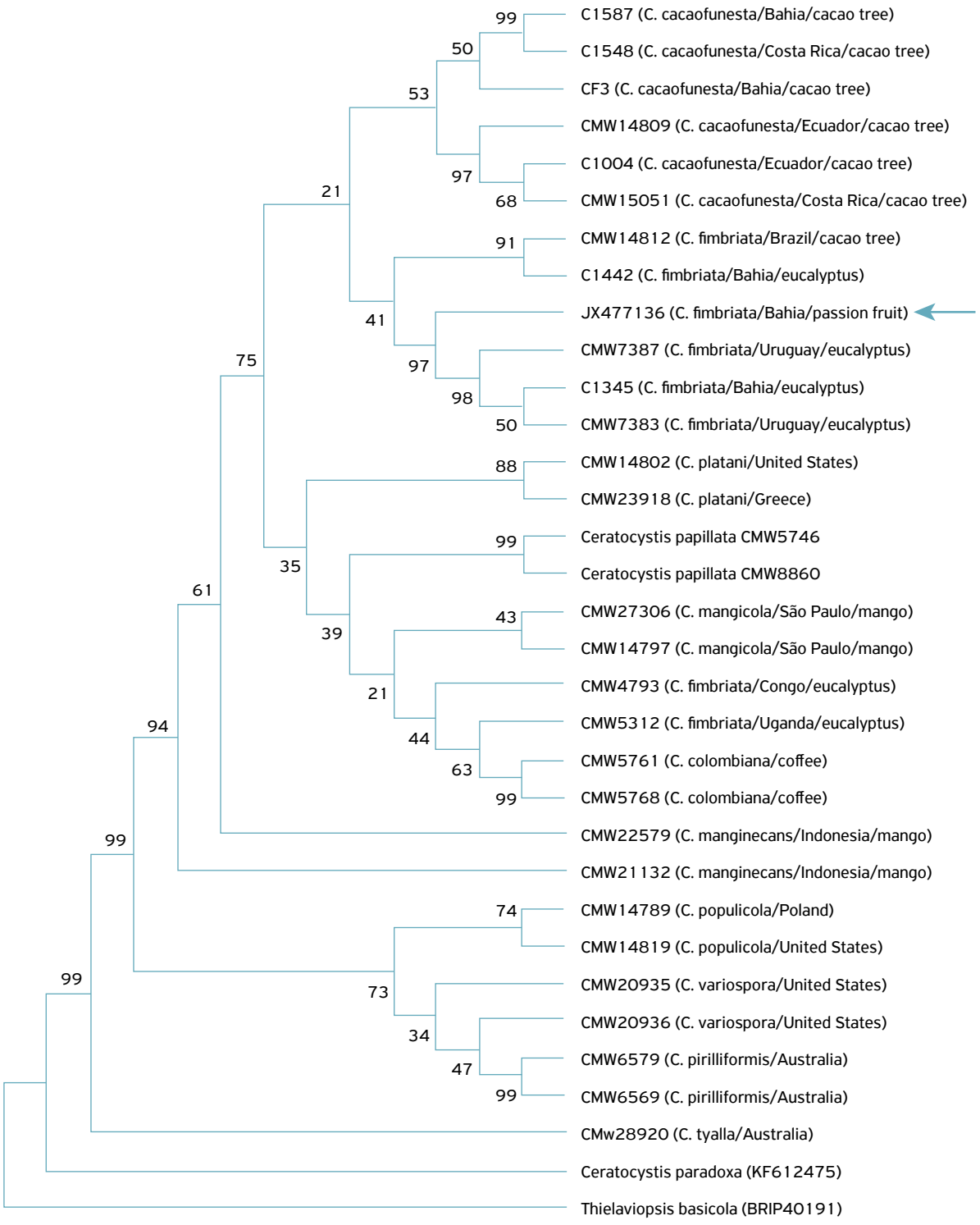
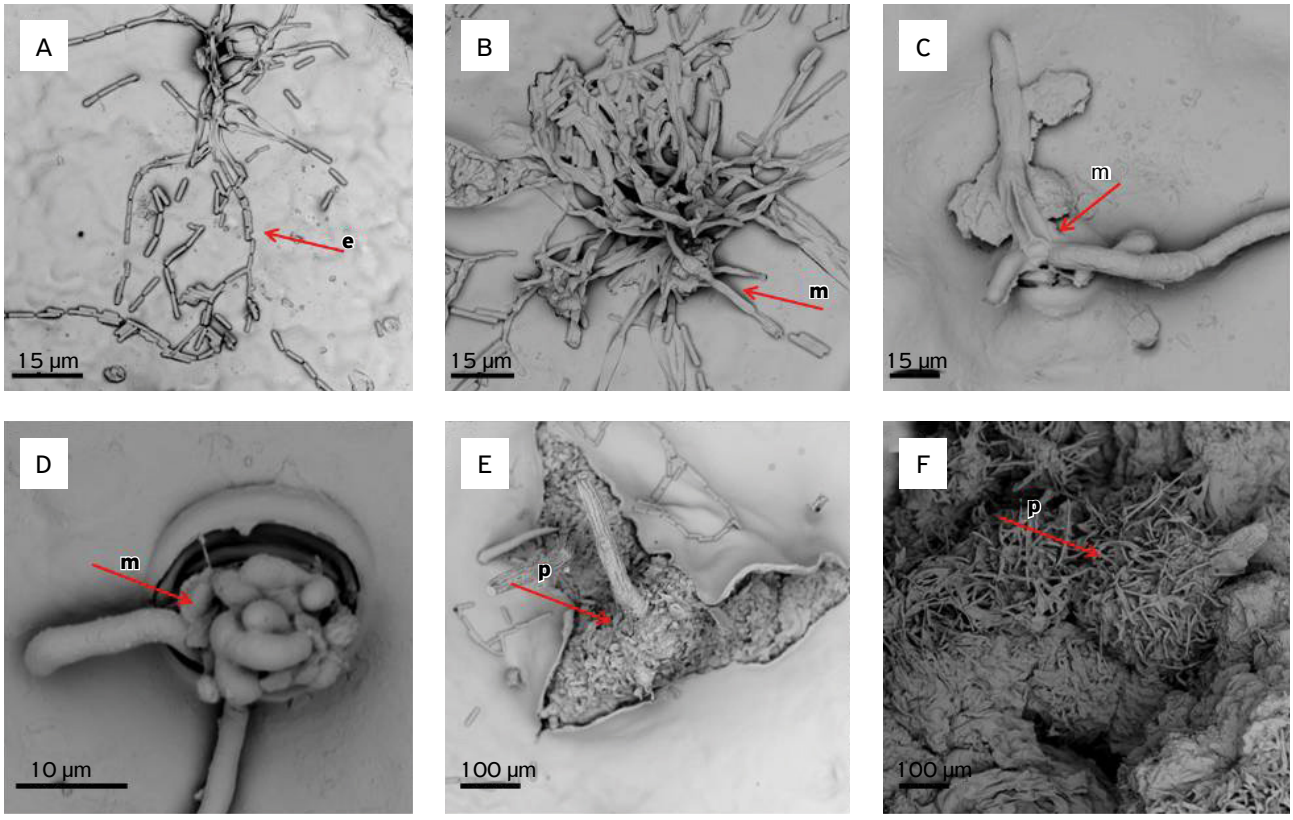


Figure 1. Phylogenetic tree, based on the ITS-5.8S region, of the *Ceratocystis* isolate collected from passionflower plants.

Table 1. Severity of rot caused by *Ceratocystis fimbriata* in cultivars of yellow passion fruit in the post-harvest.

Cultivar	Lesion diameter (cm)	
	7 days post-inoculation	11 days post-inoculation
Sol de Cerrado	1.1 a[1]	2.4 a
Ouro Vermelho	1.1 a	2.4 a
Gigante Amarelo	0.9 a	2.2 a
Afruvec	0.9 a	2.2 a
FB 100	1.0 a	2.2 a
FB 200	1.0 a	2.0 a
CV (%)	17.4	18.6

[1]Followed by the same letter in the column do not differ according to Tukey's test, at 5% significance level; CV = coefficient of variation.



E: spore; m: mycelium; p: perithecium.

Figure 2. Details of the surface (A, B, C, D and E) and the inner part (F) of the passion fruit at 96 hours post-inoculation of *Ceratocystis* isolate from passionflower.

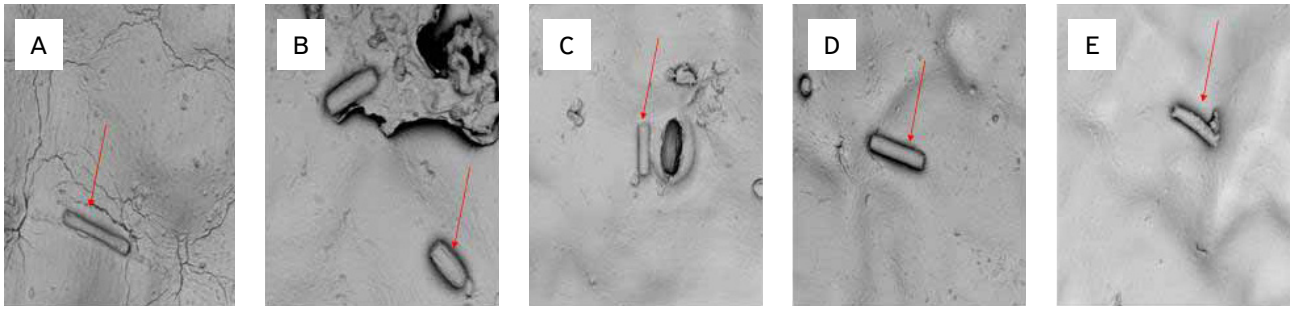
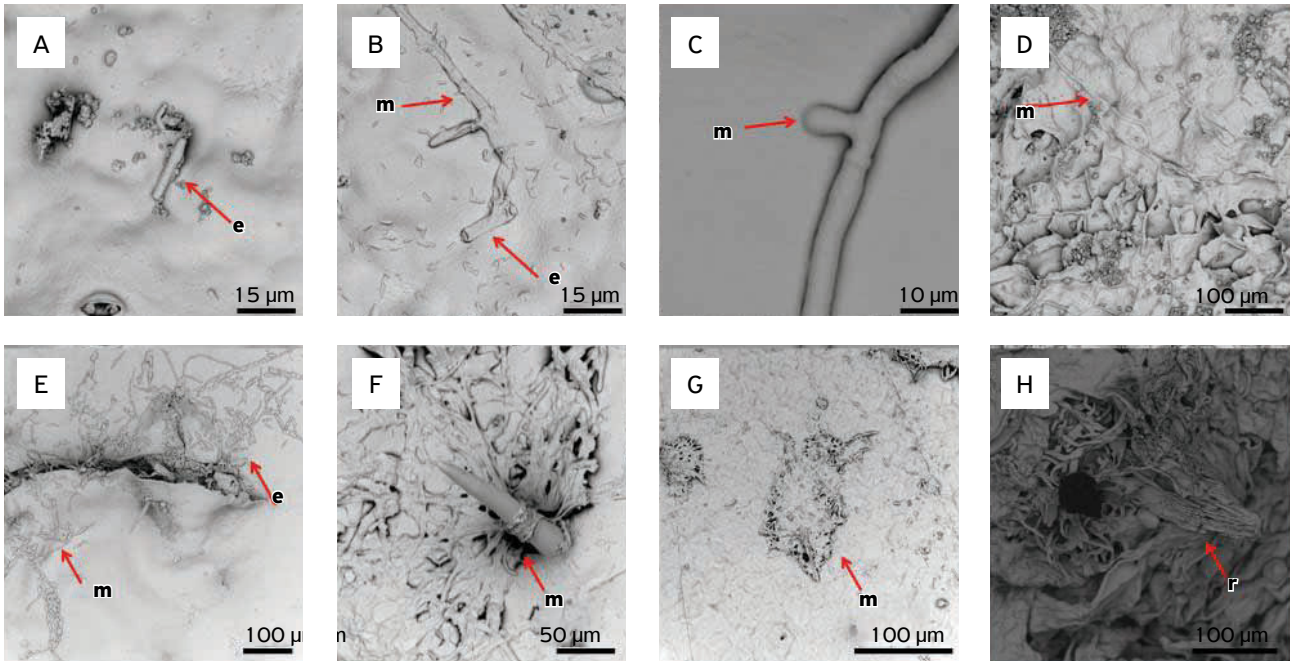


Figure 3. Spores of isolates from cacao (A), mango (B), atemoya (C), eucalyptus (D) and teak (E) on the surface of passion fruits at 96 hours post-inoculation.



E: spore; m: mycelium; r: rostrum of the perithecium.

Figure 4. Isolate from passionflower at 2, 4, 6, 12, 24, 48, 72 and 96 hours (A, B, C, D, E, F, G and H, respectively) post-inoculation in fruits.

field, it seems to require specific temperature and humidity conditions, similar to those found in its place of origin.

CONCLUSIONS

The *Ceratocystis* isolate from passion fruit can germinate and penetrate in the fruit between 2 and 6 hours post-inoculation. At 12 and 24 hours post-inoculation, fruit colonization was noted both externally and internally. At 90 hours post-inoculation, there is formation of new perithecia. The *Ceratocystis* isolated from other plant species have not penetrated passion fruit.

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REFERENCES

BAKER, C.J.; HARRINGTON, T.C. *Ceratocystis fimbriata*. In: BAKER, C.J.; HARRINGTON, T.C. *Crop Protection Compendium*. Kew: CABI Publishing, 2004. 14p.

BAKER, C.J.; HARRINGTON, T.C.; KRAUSS, U.; ALFENAS, A.C. Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. *Phytopathology*, v.93, n.10, p.1274-1284, 2003. DOI: 10.1094/PHYTO.2003.93.10.1274

BEZERRA, J.L. *Ceratocystis fimbriata* causing death of budded cocoa seedlings in Bahia, Brazil. *Incoped Newsletter*, v.1, p.6, 1997.

FERREIRA, F.A.; MILANE, D. *Diagnose visual e controle de doenças abióticas e bióticas do eucalipto no Brasil*. Mogi Guaçu: International Paper, 2002. 104p.

FERREIRA, M.A. *Estrutura genética de populações de* Ceratocystis fimbriata *e padrão espaço-temporal da murcha-de-Ceratocystis*. 2009. 107f. Tese (Doutorado em Fitopatologia) – Universidade Federal de Viçosa, Viçosa, 2009.

FIRMINO, A.C.; NOVAES, Q.S.; TOZZE JUNIOR, H.J.; ROCHA SOBRINHO, G.G.; SANTOS, A.; BEZERRA, J.L.; FURTADO, E.L. First report of *Ceratocystis fimbriata* causing fruit-rot of *Passiflora edulis* in Brazil. *New Disease Reports*, v.27, p.4, 2013. DOI: 10.5197/j.2044-0588.2013.027.004

FIRMINO, A.C.; TANAKA, F.A.O.; SILVA, S.D.V.M.; ITO, M.F.; FURTADO, E.L. Colonização do xilema de eucalipto por *Ceratocystis* spp. isolado de diferentes hospedeiros. *Summa Phytopathologica*, Botucatu, v.41, n.2, p.138-143, 2015. DOI: 10.1590/0100-5405/2061

FIRMINO, A.C.; TOZZE JUNIOR, H.J.; COSTA, P.N.; FURTADO, E.L. *Ceratocystis fimbriata* causando murcha em atemóia na região de Botucatu-SP. *Summa Phytopathologica*, v.38, n.2, p.171, 2012a. DOI: 10.1590/S0100-54052012000200016

FIRMINO, A.C.; TOZZE JUNIOR, H.J.; FURTADO, E.L. First report of *Ceratocystis fimbriata* causing wilt in *Tectona grandis* in Brazil. *New Disease Reports*, v.25, 2012b. DOI: 10.5197/j.2044-0588.2012.025.024

HARRINGTON, T.C.; THORPE, D.J.; ALFENAS, A.C. Genetic variation and variation in aggressiveness to native and exotic hosts among Brazilian populations of *Ceratocystis* fimbriata. *Phytopathology*, v.101, n.5, p.555-566, 2011. DOI: 10.1094/PHYTO-08-10-0228

JOHNSON, J.A.; HARRINGTON, T.C.; ENGELBRECHT, C.J.B. Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia*, v.97, n.5, p.1067-1092, 2005.

MURRAY, M.G.; THOMPSON, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research*, v.8, n.19, p.4321-4325, 1980.

SILVEIRA, S.F.; HARRINGTON, T.C.; MUSSI-DIAS, V.; ENGELBRECHT, C.J.B.; ALFENAS, A.C.; SILVA, C.R. *Annona squamosa*, a new host of *Ceratocystis fimbriata*. *Fitopatologia Brasileira*, Brasília, v.31, p.394-397, 2006.

TAMURA, K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution*, v.9, n.4, p.678-687, 1992.

VALARINI, P.J.; TOKESHI, H. *Ceratocystis fimbriata*: agente causal da seca da figueira e seu controle. *Summa Phytopathologica*, v.6, p.102-106, 1980.

ZAUZA, E.A.V.; ALFENAS, A.C.; HARRINGTON, T.C.; MIZUBUTI, E.S.; SILVAI, J.F. Resistance of Eucalyptus clones to *Ceratocystis* fimbriata. *Plant Disease*, v.88, n.7, p.758-760, 2004. DOI: 10.1094/PDIS.2004.88.7.758