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# *Colletotrichum* species associated with atemoya anthracnose in northeast Brazil

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**Abstract**

Anthracnose, caused by *Colletotrichum* spp., is an important disease of atemoya that can infect and cause damage to different plant organs. The symptoms include foliar anthracnose, flower abortion, branch tip bleaching, and necrotic lesions on fruits. Knowing the etiology of this disease is essential for properly developing management strategies. Therefore, this study aimed to identify *Colletotrichum* species associated with atemoya in the Northeast region of Brazil, based on multi-locus phylogeny and morphological analyses, to better understand the etiology of anthracnose in this plant species. The *Colletotrichum* isolates were obtained from symptomatic leaves and branches of atemoya collected in commercial fields in Vitória da Conquista (Bahia), Palmeira dos Índios and Rio Largo (Alagoas), located in northeast Brazil. The initial analysis of the partial sequences of the GAPDH gene was performed with nineteen *Colletotrichum* isolates obtained from leaves and branches of atemoya trees with typical anthracnose symptoms. Seventeen isolates were grouped in the *gloeosporioides* complex and two in the b*oninense* complex. Therefore, eleven isolates were submitted to concatenated phylogenetic analyses with the genes GAPDH, TUB2, and ITS region of the rDNA to confirm the identity of the isolates at the species level. The species *C. theobromicola*, *C. siamense*, *C. fructicola* and *C. karstii* were identified and are associated with atemoya anthracnose in the Northeast region of Brazil.

**Keywords:** *Annonaceae*, multi-locus, pathogenicity

## Introduction

The *Annonaceae* family is a pantropical group of trees and shrubs, comprising 2100 species in 129 genera. They are considered one of the oldest existing floral groups, with a basal lineage estimated at 112 million years (Stevens, 2016; Awachere et al., 2018). The *Annona* genus is considered the most important of the *Annonaceae* family due to its fruit economic value worldwide. Custard apple (*Annona squamosa* L.), soursop (*Annona muricata* L.), cherimoya (*Annona cherimola* Mill), and atemoya (hybrid *A. squamosa* x *A. cherimola*) are the species that stand out in the fresh consumption and the fruit processed market, and also in the bio-compounds production of medicinal, allelopathic or pesticide importance (Lemos, 2014; São José et al., 2014; Liaw et al., 2016).

The atemoya is an interspecific hybrid from the cross between the cherimoya and the custard apple (Lemos, 2014). The hybrid combines interesting traits of both species with extremely tasty fruits. Among other advantages, the fruit has a lower number of seeds, longer post-harvest shelf-life, absence of cracks, differentiated flavor, and more balanced soluble solids content, arousing the interest of some producers due to the taste and quality similar to that of cherimoya, and superior to the custard apple (Sobrinho, 2014).

Despite the high market demand for atemoya, the productivity in the Northeast region of Brazil is low (2.3 t $ha^{-1}$), which is attributed to low technology use and inadequate management of diseases (Junqueira & Junqueira, 2014; Lemos, 2014; São José et al., 2014). Among the factors associated with low productivity, anthracnose, caused by *Colletotrichum* spp., is the most important aerial part disease of atemoya (Firmino et al., 2014). Symptoms cause significant damage, such as flower abortion, bleaching of branches tip, and necrotic lesions on the fruits, compromising the plant development


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(Takahashi, 2009; Lemos, 2014).

The *Colletotrichum* genus currently comprises 257 species based on molecular data, which are grouped into 15 species complexes and 14 singleton species (Talhinhas; Baroncelli, 2021). The *C. gloeosporioides, C. boninense* and *C. acutatum* species have been associated with atemoya anthracnose in Brazil (Takahashi et al., 2009; Firmino et al., 2014). These studies were based on morphological and molecular characterization, using the ITS-rDNA region and the β-tubulin and elongation factor 1*α* genes (Takahashi et al., 2009; Firmino et al., 2014). However, the identification of the *Colletotrichum* genus has been based on more accurate studies using a multi-locus phylogenetic analysis combined with recognized phenotypic characters such as morphology, pathogenicity, and cultural characteristics (Hyde et al., 2014; Costa et al., 2019; Dammet al., 2019; Jayawardena et al., 2021; Talhinhas; Baroncelli, 2021; Wanget al., 2021).

Nevertheless, studies on the diversity of *Colletotrichum* spp. associated with atemoya in Brazil are still scarce. Therefore, this study aimed to identify *Colletotrichum* spp. associated with atemoya in the Northeast region of Brazil through multi-locus phylogeny and morphological analyses, and clarify the etiology of atemoya anthracnose in Brazil.

**Materials and methods**

*Sample collection, isolation, and preservation of Colletotrichum isolates*

The *Colletotrichum* isolates were obtained from leaves and branches of atemoya with anthracnose symptoms in commercial fields in Vitória da Conquista (Bahia), Palmeira dos Índios and Rio Largo (Alagoas), located in the Northeast region of Brazil. The leaves and branches samples were washed in running tap water and dried on paper towels. Fragments from a diseased and healthy transition lesion were cut, superficially disinfected in 70% alcohol for 30 seconds and 1% sodium hypochlorite for 1 minute, and washed twice in sterile distilled water (ADE). The fragments were transferred to Petri dishes containing potato-dextrose-agar (PDA) growth medium and incubated at 25 °C until fungal hyphae growth, where disks from the edges of the colonies were transferred to new Petri dishes containing PDA medium. The isolates were previously identified morphologically after sporulation as belonging to the *Colletotrichum* genus (Sutton, 1992; Weir et al., 2012; Damm et al., 2019).

Monosporic isolates were obtained from serial dilution of a spore suspension, up to $10^{-5}$ of the initial concentration. A 20 µL aliquot was evenly distributed into Petri dishes containing WA medium (water agar) with a Drigalski loop. After 24 hours, one germinated spore of each isolate was transferred to new Petri dishes containing PDA medium. The monosporic isolates were preserved by the Castellani method (1967) on Eppendorf tubes and by the freezing storage method in filter paper strips (Alfenas & Mafia, 2007) until the pathogenicity, morphological, cultural, and molecular characterization tests. Subsequently, they were deposited in the Collection of Plant pathogens of the Universidade Federal de Alagoas (COUFAL).

*In vitro and in vivo pathogenicity and virulence tests*

The isolates were tested for pathogenicity and virulence on detached leaves and branches of the atemoya "Gefner" variety. Each isolate was tested in the organ from which it was obtained. Asymptomatic leaves and branches were washed in running water, superficially disinfected in 1% sodium hypochlorite (NaOCl) solution for 3 minutes, washed three times in SDW, and dried with paper towels. Leaves and branches had the epidermis injured immediately before inoculation with a sterilized needle. The inoculation was carried out by adding 30µL of spore suspension ($10^6$ conidia/mL) to the surface of each organ. Three replicates for each isolate were used. The control treatment consisted of an aliquot of SDW. Leaves and branches were placed in a *gerbox* with sterile filter paper moistened with SDW and incubated in a Biochemistry Oxygen Demand (BOD) incubator at 25 °C and 12 h photoperiod. Seven days after inoculation, the lesions were measured in two diametrically opposite directions (cm) with a millimeter ruler.

For the *in vivo* assay, asymptomatic seedlings of atemoya variety "Gefner" were obtained from nurseries accredited in Bahia state. The seedlings were inoculated with PDA culture medium disks containing pathogen structures removed from the edge of seven-day-old colonies. The disks were individually fixed on the leaves' surface, which was previously injured before the inoculation with a sterilized needle. The control treatment consisted of inoculation with disks containing only PDA medium without the pathogen. After inoculation, the seedlings were wrapped in a transparent plastic bag internally moistened with sterilized distilled water to form a moist chamber and kept in a greenhouse at 25 °C and 12 h photoperiod for 48 h. The seedlings were observed daily until the first anthracnose symptoms started to appear.

A completely randomized experimental design was used, with three replicates for each *Colletotrichum* species. In both tests, the pathogen was reisolated to confirm the pathogenicity of the isolates,

according to Koch's postulate (1882).

*Molecular characterization of Colletotrichum isolates*

The mycelium for DNA extraction of the isolates was obtained through cultivation in Sucrose-Yeast Extract-Asparagine medium (Zauza et al., 2007) for five days, at 25±1 °C, without stirring and 12 h photoperiod. The obtained mycelium was washed in SDW, and the DNA was extracted following the protocol proposed by Doyle & Doyle (1987). Nucleotide sequences of four molecular markers were determined. Firstly, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using GDF/GDR primers (Templeton et al., 1992) was used for preliminary identification of isolates and assessment of species haplotypic diversity. Subsequently, representatives of each species, based on haplotypes, were amplified with the partial sequences of the β-tubulin genes (TUB2) using the T1/Bt2b primers (O'Donnell & Cigelnik, 1997; Glass & Donaldson, 1995), Mating-type locus MAT1- 2-1 (APN2/MAT-IGS) with primers CgDL_ F6/DgMAT1_F2 (Rojas et al., 2010) and rDNA Internal transcribed spacer (ITS) region with ITS1/ITS4 (White et al., 1990; Gardes & Bruns, 1993) to ensure greater reliability in the identification and taxonomic resolution of the species within the *Colletotrichum* genus. The PCR reactions were performed with 10X buffer (3 µL), 50 mM MgCl2 (0.9 µL), 10 mM DNTP (2.4 µL), 10 µM of each oligonucleotide (2 µL), 1U Taq DNA polymerase (0 .2 µL) and DNA (1µL, 25ng/µL). The final volume of the reactions was adjusted to 30 µL with autoclaved Milli-Q water.

Thermocycling conditions for the GAPDH gene amplification occurred at an initial denaturation of 95 °C for 4 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 45 s min at 72 °C, with a final extension of 7 min at 72°C. The annealing temperature differed for TUB2 at 55 °C. For the MAT1-2-1 gene, the initial denaturation started at 95 °C for 3 min, followed by 35 cycles of 30 s at 95 °C, 45 s at 62 °C, and 1 min at 72 °C, with a final extension of 10 min at 72°C. However, for the ITS region, initial denaturation started at 95 °C for 2 min was used, followed by 38 cycles at 95 °C for 1 s, 55 °C for 30 s, 72 °C for 45 s, and a final cycle at 72 °C for 10 min. (Rojas et al., 2010; Weir et al., 2012; Damm et al., 2019). After amplification, the samples were submitted to electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and observed under UV light. The PCR products were sent for sequencing at Macrogen Inc. (Seoul, South Korea).

Consensus sequences were assembled using the Staden Package software. These were initially compared to the GenBank sequence database using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov) to determine the species with which they shared the greatest sequence identity. Based on the results of the BLASTn analysis, sequence type isolates and other reference sequences of *Colletotrichum* species available on GenBank for each gene and genomic region were retrieved for phylogenetic analysis (Supplementary Table 1). Multiple sequence alignments were made using the MUSCLE algorithm (Edgar, 2004), implemented in MEGA v.11 (Molecular Evolutionary Genetics Analysis) software (https://www.megasoftware.net) for the datasets.

Bayesian Inference (BI) analyzes were performed with all sequences for the GAPDH gene (data not shown) and multi-locus for the representative isolates of the species (GAPDH, TUB2, MAT1-2-1 and ITS region) for the complex *C. gloeosporioides* and GAPDH, TUB2 and ITS region for the *C. boninense* complex. Nucleotide substitution models were estimated separately for each gene region with Mr. Model test 2.3 (Posada & Buckley, 2004) according to the Akaike Information Criterion (AIC). The clade support of each tree was determined from 1000 bootstrap generations.

BI-based phylogenetic analyzes were inferred in MrBayes v. 3.0 b4 (Ronquist et al., 2012) using the Markov Chain Monte Carlo (MCMC) method at the CIPRES web portal (http://www.phylo.org). Four MCMC chains were conducted simultaneously, starting the trees randomly up to 10 million generations for each dataset. Trees were sampled every 1.000 generations resulting in 10.000 trees. The first 2.500 trees were discarded from the analysis as a burn-in step. The posterior probability values (Rannala & Yang, 1996) were determined from a majority-rule consensus tree with 7,500 remaining trees. The trees were visualized in the FigTree v. 1.4 software(ztree.bio.ed.ac.uk/software/figtree) and edited in the Inkscape 0.91 software (https://inkscape.org/pt-br/release/inkscape-0.91). The studied sequences were deposited in GenBank (**Supplementary Table 1**). The *C. gloeosporioides* (ICMP 17821) was used as the outgroup in the analysis of the *C. boninense* complex, and the *C. boninense* (CBS123755) in the analysis of the *C. gloeosporioides* complex.

*Morphological characterization of the Colletotrichum species*

Two representatives of each *Colletotrichum* species identified in the study were selected for morphological characterization.

For the cultural characterization, a disk of the PDA culture medium containing the mycelium was removed from the edge of a seven-day-old colony and transferred individually to the center of new Petri dishes containing 20 ml of synthetic PDA medium. The diameter

of the colonies (cm) was evaluated daily, taken from the bottom of the Petri dishes by measuring, in two directions, the perpendicular diameters with a millimeter ruler. The colonies' color, appearance and development of sectors were also evaluated (Weir et al., 2012; Damm et al., 2012). A completely randomized experimental design was used, with five replicates for each isolate, consisting of a Petri dish.

In the morphological characterization the size and shape of 50 conidia and appressoria were analyzed (Dhingra & Sinclair, 1995), these were randomly chosen from colonies grown in PDA medium for seven days. In order to visualize conidia, glass slides were used, where a drop (40 mL) of SDW was deposited together with the spores; and observed under light microscope. To stimulate the formation of appressoria, a drop of conidia suspension was deposited on a sterile glass slide packed in a humid chamber (Petri dish lined with sterile filter paper moistened with SDW) for 24h, to keep the environment humid and allow the formation of appressoria. The length and width of the conidia and appressoria were measured from images captured through a digital camera (Olympus IX2-SLP) coupled to the light microscope at a 400x magnification, projected in a computer monitor, through the Cellsense Standard software (SAMSUNG SDC-415®).

**Results and Discussion**

*Isolates obtaining*

A total of nineteen *Colletotrichum* spp. isolates were obtained from leaves and branches of atemoya trees, with anthracnose symptoms, in commercial fields in the states of Alagoas and Bahia, Northeast Brazil.

*Molecular characterization of the Colletotrichum isolates*

The initial analysis of the partial sequences of the GAPDH gene was performed with nineteen *Colletotrichum* isolates obtained from atemoya leaves and branches with typical anthracnose symptoms. Ten isolates were obtained from leaves and nine isolates from branches. After comparing the sequences with the GenBank database accessions using the BLASTn algorithm and constructing Bayesian Inference trees, it was possible to group seventeen isolates in the *gloeosporioides* complex and two in the *boninense* complex (Data not shown).

After the species identification with the GAPDH gene, eleven isolates were selected and submitted to concatenated phylogenetic analyses with the GAPDH, TUB2, MAT1-2-1 genes, and the ITS region of the rDNA for the *gloeosporioides* complex (**Figure 1**) and GAPDH, TUB2 and ITS rDNA region for the *boninense* complex (**Figure 2**) to confirm the species identification.

The concatenated alignment of the *gloeosporioides* complex showed 1611 characters, of which 194 are parsimoniously informative sites, and 1271 are conserved sites. The loci boundaries in the alignments were: GAPDH: 1-308, ITS: 309-907, TUB2: 908-1629, and ApMat: 1630-2517. The *boninense* complex showed 1335 characters, of which 180 are parsimoniously informative sites, and 1011 are conserved sites. The loci boundaries in the alignments were: GAPDH: 1-281, ITS: 282-837, and TUB2: 838-1335.

Bayesian Inference concatenated phylogenetic analyses identified four species within two complexes of the *Colletotrichum* genus. Nine isolates showed similarity to species of the *gloeosporioides* complex above 99% for all genes and regions used. The isolates COUFAL0339, COUFAL0341, COUFAL0342, COUFAL0345, and COUFAL0349 were grouped with the *C. theobromicola* species, while the isolates COUFAL0337 and COUFAL0338 were grouped with *C. siamense* and the isolates COUFAL0332 and COUFAL0334 with the *C. fructicola* species.

The phylogenetic analyses of the *C. boninense* complex isolates included two isolates, COUFAL0335 and COUFAL0336, with well-supported clades with the *C. karstii* species.

*Pathogenicity Test*

Detached leaves and branches inoculated with the *Colletotrichum isolates* showed necrotic spots two days after inoculation. The *C. fructicola* isolated caused the largest lesion (2.56 cm), while the species *C. theobromicola* caused the smallest lesion (0.68 cm), differing from the other species (**Figure 3** and **Table 1**).

In atemoya seedlings inoculated, the symptoms appeared within the first 48 h after inoculation. The isolate of *C. siamense* caused the largest lesion (7.34 cm), while the *C. theobromicola* isolate caused the smallest lesion (3.15 cm), statistically differing from each other on severity levels (Figure 3 and Table 1).

*Morphological and cultural characterization*

The evaluated morphological and cultural characteristics of the *Colletotrichum* species, such as size and conidia shape, appressoria, colony aspects, and mycelial growth rate, are shown in Table 1 and Figure 3.

The cultural characteristics of the colonies within the *gloeosporioides* complex proved to be heterogeneous in the aspect and color of the aerial mycelium. The predominant color was white with changes in tonality in the center and/or on the edge of the colonies, ranging

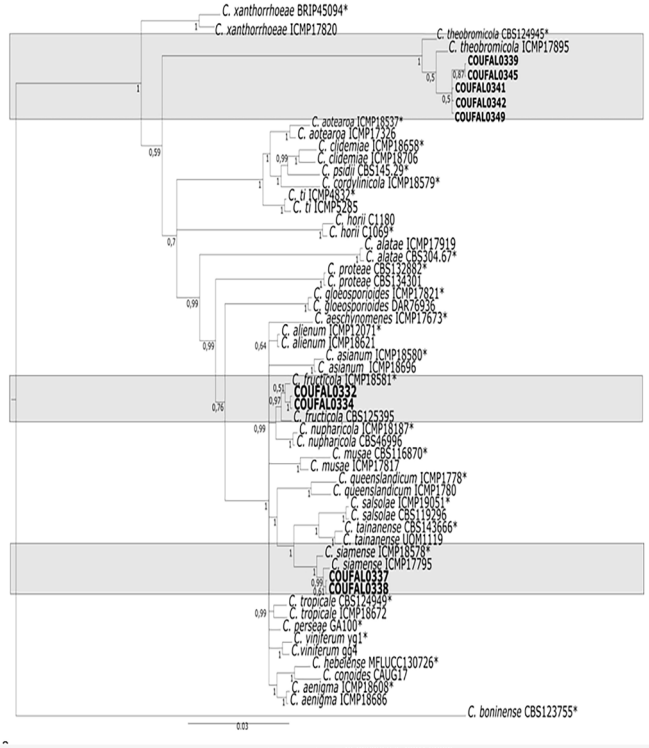


**Figure 1. Multi-locus** phylogenetic tree inferred (Bayesian inference) with the GAPDH, TUB2, ApMat and ITS region genes for species of the *Colletotrichum gloeosporioides* complex. Values at nodes indicate posterior probabilities > 0.03. Ex-type isolates are marked with an asterisk. The isolates used in the study are highlighted in bold. The tree was rooted using *Colletotrichum boninense* (CBS 123755) as outgroup.

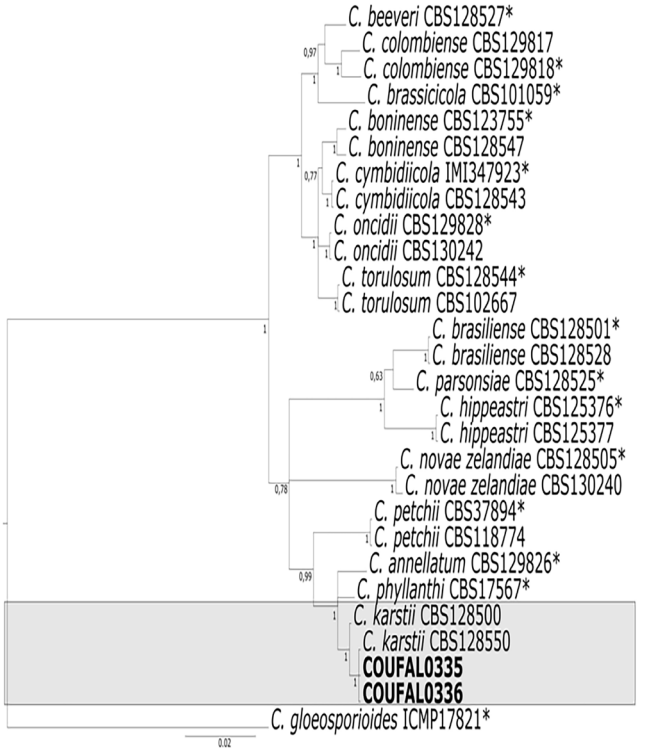


**Figure 2. Multi-locus** phylogenetic tree inferred (Bayesian inference) with the genes GAPDH, TUB2 and ITS region for species of the *Colletotrichum boninense* complex. Values at nodes indicate posterior probabilities > 0.02. Ex-type isolates are marked with an asterisk. The isolates used in this study are highlighted in bold. The tree was rooted with *Colletotrichum gloeosporioides* (ICMP 17821) as outgroup

from pink, light gray, and dark green, with and without concentric rings. The variation in the average growth rate of colonies within the complex ranged from 0.78 to 0.97 mm. day$^{-1}$. The conidia were hyaline, non-septate, and straight-cylindrical with sharp or rounded tips. The conidia length ranged from 7.85 to 8.56 µm and 2.91 to 3.39 µm in width. Appressoria were predominantly dark brown, with ovoid and irregular shapes. The appressorium length ranged from 4.63 to 5.82 µm and 3.36 to 4.05 µm in width.

In the *boninense* complex, the isolates showed colony colors ranging from pink with white edges to salmon with concentric rings. The average growth rate within the complex was 0.74 cm.day$^{-1}$. The conidia were hyaline, non-septate, and straight-cylindrical with sharp or rounded tips. The conidia length was 9.18 µm and 3.77 µm in width. Appressoria were predominantly dark brown, with ovoid and irregular shapes. The appressoria length was 6.31 µm, and 4.19 µm in width.

Our study aimed to identify *Colletotrichum* species associated with anthracnose disease in atemoya in commercial fields located in the Northeast region of Brazil, considering that this is one of the most important diseases in plants of the *Annonaceae* family, affecting branches, leaves, flowers, and fruits, both in the initial and in more advanced phenological stages, causing severe economic losses to producers (Firmino et al., 2014; Kamei et al., 2014; Costa et al., 2019).

However, despite the great importance of atemoya anthracnose in Brazil, the etiology of this disease remains unclear (Junqueira & Junqueira, 2014). Therefore, the results obtained in this study will fill in some gaps in the knowledge of atemoya anthracnose etiology.

In this study, different species of *Colletotrichum* were identified as the causal agents of this disease in atemoya leaves and branches using a multi-locus phylogeny analysis approach with the GAPDH, TUB2, MAT1-2-1 genes, and ITS region of rDNA. Together with the morphological and cultural studies, these analyses allowed the identification of four species within two complexes. Three species belong to the *gloeosporioides* complex *(C. theobromicola, C. siamense* and *C. fructicola*), and one belongs to the b*oninense* complex (*C. karstii*).

Members of the *C. gloeosporioides* and *C. boninense* species complex had already been reported in several crops of economic importance around the world (Weir et al., 2012; James et al., 2014; Rodrigues et al., 2014; Silva et al., 2017; Veloso et al., 2018; Costa et

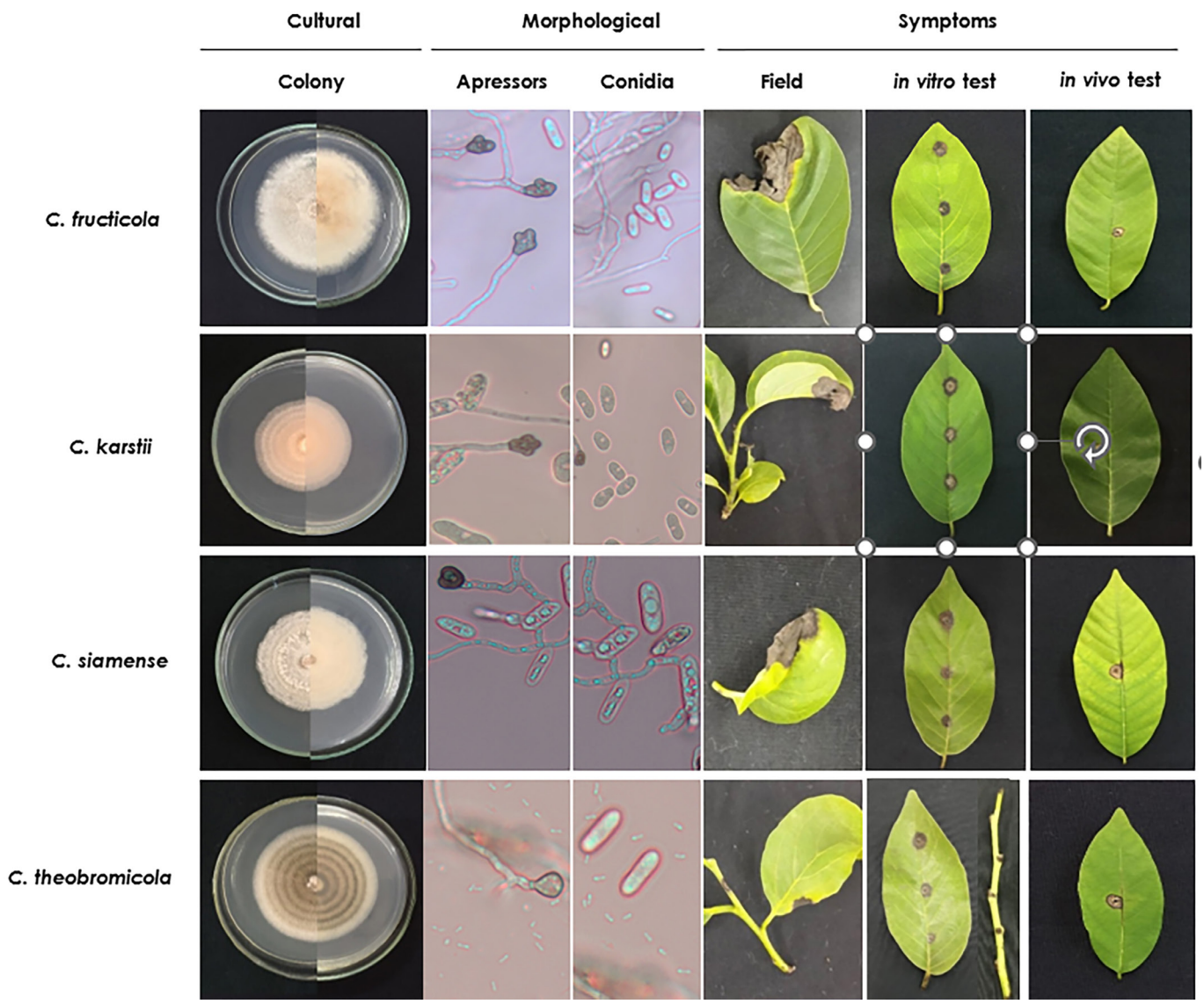


**Figure 3.** Cultural and morphological aspects of the isolates and field and pathogenicity test symptoms caused *Colletotrichum spp.*

al., 2019). However, in Brazil, the only study that identified members of the *Colletotrichum* genus associated with atemoya anthracnose was carried out by Firmino et al. (2014), where *C.acutatum* and *C. boninense* species were identified.

Reports of *C. theobromicola* causing anthracnose in leaves of *Annona muricata* L. and *Annona squamosa* L. have been published in Brazil (Costa et al., 2019) and in other *Annonaceae* species in Mexico (Villanueva-Arce et al., 2005), Panama (Rojas et al., 2010), Colombia (Álvarez et al., 2014) and Japan (Morita et al., 2015). This species is widely distributed in tropical and subtropical regions of the world, infecting a wide diversity of hosts (Tozze Júnior et al., 2010; Álvarez et al., 2014; Chung et al., 2020; Costa et al., 2019; Jayawardena et al., 2021; Soares et al., 2021; Talhinhas; Baroncelli, 2021). In our study, this was the only species observed in atemoya branches and the most frequent species associated with leaves and branches from samples ofall collected areas. However, it was significantly less virulent than the other species, both in vitro and in vivo. Despite being associated with several agronomically important *Annonaceae* species, there are no reports of *Colletotrichum theobromicola* infecting atemoya in Brazil.

The second most frequent species associated with anthracnose in the atemoya leaves was *Colletotrichum fructicola*, which was not found on branch samples. This species was the most virulent in the *in vitro* assay. However, it showed virulence levels similar to *C. kastii* and *C. theobromicola* in the *in vivo* assay. Although being reported in a wide range of hosts such as papaya (*Carica papaya* L.), pepper (*Capsicum frutensens* L.) (Phoulivong et al., 2010), cocao (*Theobromae cacao* L.) (Rojas et al., 2010), apple and strawberry (*Fragaria*

**Table 1-** Morphocultural data and pathogenicity tests of the species of *Colletotrichum* ºCOUFAL (Collection of Phytopathogens of the Federal University of Alagoas).

| Species | Conidia (µm) | | | Appressorium (µm) | | Mycelial growth (mm/day) | Colonies | Diameter of lesions (cm) | |
|---|---|---|---|---|---|---|---|---|---|
| | Length | Width | Format | Length | Width | | | in vitro | in vivo |
| *C. fructicola* (COUFAL0332; COUFAL 0334) | 7.85 (8.71-6.53) | 3.39 (3.94-2.83) | Cylindrical | 5.82 (6.94-4.80) | 4.05 (5.87- 3.15) | 0.78 | Pinkish in the center with white edges, presence of concentric rings and dense mycelium. | 2.56 (2.15 – 2.80) | 3.41 (2.85 – 4.00) |
| *C. karstii* (COUFAL0335; COUFAL 0336) | 9.18 (11.37-7.78) | 3.77 (4.17-2.94) | Cylindrical | 6.31 (8.31-4.86) | 4.19 (5.07-3.16) | 0.74 | Salmon with concentric rings and dense mycelium. | 1.36 (1.10 – 1.65) | 3.85 (3.46 – 4.10) |
| *C. siamense* (COUFAL0337; COUFAL 0338) | 7.97 (10.37-6.63) | 2.91 (3.35-2.49) | Cylindrical | 4.63 (5.82-3.92) | 3.36 (3.92-2.64) | 0.97 | Color ranging from white to light pink with dense mycelium. | 1.06 (0.50 – 1.65) | 7.34 (6.23 – 8.73) |
| *C. theobromicola* (COUFAL0342; COUFAL 0345) | 8.56 (10.30-7.36) | 2.96 (3.33-3.70) | Cylindrical | 5.17 (9.05-3.85) | 4.05 (5.14-3.10) | 0.80 | Color varying between gray and green in the center, with white edges, concentric rings, thin mycelium and may or may not have sectors. | 0.68 (0.50 – 0.90) | 3.15 (2.96 – 3.49) |

*vesca* L.) (Weir et al., 2012), citrus (*Citrus* spp.) (Peng et al., 2012), grapefruit (*Vitis* sp. L.) (Huang et al., 2013) and *Annonaceae* species (Costa et al., 2019), this is the first report of *C. fructicola* causing anthracnose in atemoya.

The *C. siamense* and *C. karstii* species were represented by only two isolates in atemoya leaves and were not found on branch samples. In the pathogenicity test *in vivo*, *C. siamense* was the most virulent species, while in vitro, it was the second less virulent whereas *C. karstii* behaved as the third most virulent in both tests. There was inconsistency in the virulence of the pathogens *in vitro* and *in vivo*. This may be related to several factors, such as favorable environmental conditions for each species, pathogen virulence, and preference for specific plant organs (Costa et al., 2019; Marins et al., 2022). However, *in vivo*tests reflect environmental conditions closer to those observed in the field.

*Colletotrichum siamense* is one of the main pathogens causing both pre and post-harvest anthracnose in fruits at local markets in Thailand, including *Annona reticulata* L. (Udayanga et al., 2013). It has a wide geographic distribution and affects numerous host species (Weir et al., 2012; Costa et al., 2019; Jayawardena et al., 2021; Soares et al., 2021; Talhinhas; Baroncelli, 2021). In Brazil, it has been found on *Annona muricata* and *Annona squamosa* fields (Costa et al., 2019) and also in other important crops such as *Capsicum* spp. (Silva et al., 2017), pomegranate (*Punica granatum* L.) (Silva-Cabral et al., 2020) and *Saccharum* spp. (Marins et al., 2022).

The *C. karstii* species was first reported in orchid leaves in China and is considered an important pathogen of the *Orchidaceae* family (Yang et al., 2012; Jadrane et al., 2012). However, *C. karstii* has been reported in several fruit trees causing significant economic losses (Damm et al., 2012; Lima et al., 2013; Vieira et al., 2014; Velho et al., 2014). In *Annonaceae* plants, *C. karstii* has been reported in cherimoya fruits in Mexico and New Zealand (Damm et al., 2012), in soursop in Colombia (Álvarez et al., 2014), and soursop and custard apple trees in Brazil (Costa et al., 2019). Nonetheless, this is the first report of *C. siamense* and *C. karstii* causing anthracnose in atemoya.

Identification of *Colletotrichum* species using the multi-locus phylogeny has proved to be an important tool, with reliable results on these species identification (Álvarez et al., 2014; Firmino et al., 2014; Kamei et al., 2014; Balendres et al., 2019; Costa et al., 2019; Chung et al., 2020; Jayawardena et al., 2021; Talhinhas; Baroncelli, 2021). Data obtained in this study expanded the knowledge of

the different *Colletotrichum* species associated with the atemoya anthracnose in Brazil.

According to Takahashi et al. (2009), only *C. gloeosporioides* was reported as the causal agent of atemoya anthracnose in Brazil. However, Firmino et al. (2014) later reported *C. acutatum* infecting atemoya leaves and *C. boninense* infecting fruits, branches, and leaves. Therefore, our study corroborates these findings and confirms the occurrence of more than one species of *Colletotrichum* causing anthracnose in atemoya.

Although *C. gloeosporioides* is considered the main causal agent of anthracnose in manyfruit trees, more than one species causing anthracnose in the same host has been frequently reported (Tozze Júnior et al., 2010; Costa et al., 2019; Silva-Cabral et al., 2020; Chung et al., 2020). Similar to the results found in our study, the occurrence of more than one *Colletotrichum* species causing anthracnose in other *Annonaceae* plants has been reported, such as *C. gloeosporioides stricto sensu*, *C. brevisporum*, *C. cliviae*, *C. fructicola*, *C. gigasporum*, *C. karstii*, *C. siamense*, *C. theobromicola* and *C. tropicale* in custard apple and soursop (Costa et al., 2019) and *C. gloeosporioides*, *C. boninense*, *C. fragariae* and *C. magna* in custard apple and soursop (Kamei et al., 2014). The same has occurred in other fruit trees, such as pomegranate, where the species *C. theobromicola*, *C. siamense* and *C. fructicola* were found (Silva-Cabral et al., 2019; 2020); Passion fruit, with the occurrence of *C. acutatum, C. boninense* and *C. gloeosporioides* (Almeida et al., 2007; Tozze Júnior et al., 2010); Avocado, being infected by*C. siamense* and *C. karstii* (Soares et al., 2021); and strawberry with *C. miaoliense* sp. nov., *C. karstii*, *C. siamense*, *C. fructicola* and *C. boninense* (Chung et al., 2020).

These are concerning results from an agronomic perspective, as the occurrence of more than one species of *Colletotrichum* infecting the same host might difficult the control of anthracnose. This raises the importance of this disease on different commercial fruit trees, given the differentiated behavior of *Colletotrichum* species, such as divergent sensitivity to fungicides (Silveira et al., 2020).

In this study, a molecular identification approach was used with the genes GAPDH, TUB2, MAT1-2-1, and the ITS region of the rDNA, and the results indicate that most species of *Colletotrichum* obtained in the atemoya samples showed high genetic diversity. The phylogenetic analysis used in this study showed that, although *C. karstii* (*C. boninense* complex) has been associated with atemoya anthracnose, the *C. gloeosporioides* species complex (*C. theobromicola, C. siamense, and C. fructicola*) had the largest number of grouped isolates (17 isolates), being, therefore, the group with the largest number of species infecting atemoya. Among the species described within the *gloeosporioides* complex, few are host-specific, which means that most of the species in this complex can be found in many crops and native species (Weir et al., 2012; Costa et al., 2019; Jayawardena et al., 2021; Soares et al., 2021). These results corroborate with other studies published recently, confirming the occurrence of several other species of the *gloeosporioides* complex associated with anthracnose in different fruit trees (Álvarez et al., 2014; Kamei et al., 2014; Costa et al., 2014; Costa et al., 2019; Chung et al., 2020; Jayawardena et al., 2021; Soares et al., 2021).

However, as observed in our study, species of the *gloeosporioides* complex were not solely found to be associated with anthracnose in *Annonaceae* and other fruit trees (Kamei et al., 2014; Firmino et al., 2014; Costa et al., 2019; Jayawardena et al., 2021; Soares et al., 2021). Hence, as most species of *Colletotrichum* do not possess single host specificity, and cultivation of different fruit trees commonly occurs in the same or geographically close areas, this increases the dispersion of propagules, which partially explains the predominance of species within the same complex as the causal agents of anthracnose in commercial fruit trees (Vieira et al., 2018; Chung et al., 2020).

Similar results were also found by Álvarez et al. (2014) in a study carried out in Colombia with anthracnose in soursop plants. In their study, *Colletotrichum* species were distributed in three complexes: *gloeosporioides* (*C. theobromicola, C. tropicale, C. siamense and C. gloeosporioides stricto sensu*), *boninense* (*C. karstii*) and *acutatum* (an unresolved isolate). In Mexico, Villanueva-Arce et al. (2005) reported *C. gloeosporioides* as the causal agent of anthracnose in *Annonaceae*, with incidences above 50% of this species in cherimoya fruits. All these results reinforce the importance of this disease in *Annonaceae* plants and demonstrate the wide geographic distribution of *Colletotrichum* species.

All *Colletotrichum* species identified in our study induced circular brownish lesions, typical anthracnose symptoms observed in atemoya leaves as described by Junqueira & Junqueira (2014). Pathogenicity tests are essential for epidemiological studies, identifying whether a given *Colletotrichum* species is specific to a given host or many others (Cai et al. 2009).

The *Colletotrichum* isolates identified showed wide variation in colony color as observed in other reports (Kamei et al. 2014; Damm et al. 2014; Balendres et

al. 2020; Silva-Cabral et al. 2020). Temperature, storage, luminosity and growth medium can influence the stability and aspects of colonies (Vieira et al. 2018; Khodadadi et al. 2020). The colony characteristics observed in our study corroborate with previous studies. The isolates of the *gloeosporioides* complex showed similar characteristics to those described by Cannon et al. (2008), Weir et al. (2012) and Costa et al. (2019). The isolates colonies of the *boninense* complex were similar to those described by Costa et al. (2019) on isolates obtained from *Annonaceae* plants.

Anthracnose caused by different species of *Colletotrichum* is the most important disease of atemoya and other *Annonaceae* members in Brazil. The correct identification of the etiological agent is essential in understanding the pathogen life cycle and for effective management strategies aimed at reducing the damages caused by this disease in the field and post-harvest.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Supplementary table** 1- *Colletotrichum* species and GenBank accession number used for phylogenetic analysis in this study.

| ESPÉCIES | ISOLADOS | Números de acesso do GenBank | | | |
|---|---|---|---|---|---|
| | | GAPDH | ITS | TUB2 | APMaT |
| *C. aenigma* | ICMP18686 | JX009913 | JX010243 | JX010390 | - |
| *C. aenigma** | ICMP18608 | JX010044 | JX010244 | JX010389 | KM360143 |
| *C. aeschynomenes** | ICMP17673 | JX009930 | JX010176 | JX010392 | KM360145 |
| *C. alatae* | ICMP17919 | JX009990 | JX010190 | JX010383 | KC888932 |
| *C. alatae** | CBS 304.67* | JX010011 | JX010191 | JX010449 | - |
| *C. alienum* | ICMP18621 | JX009959 | JX010246 | JX010386 | - |
| *C. alienum** | ICMP12071 | JX010028 | JX010251 | JX010411 | - |
| C. annellatum* | CBS129826 | JQ005309 | JQ005222 | JQ005656 | - |
| *C. asianum* | ICMP18696 | JX009915 | JX010192 | JX010384 | - |
| *C. asianum** | ICMP18580 | JX010053 | FJ972612 | JX010406 | FR718814 |
| *C. aotearoa** | ICMP18537 | JX010005 | JX010205 | JX010420 | KC888930 |
| *C. aotearoa* | ICMP17326 | JX010049 | JX010202 | JX010422 | - |
| *C. beeveri** | CBS128527 | JQ005258 | JQ005171 | JQ005605 | - |
| *C. boninense* | CBS 128547 | JQ005246 | JQ005159 | JQ005593 | - |
| *C. boninense** | CBS 123755 | JQ005240 | JQ005153 | JQ005588 | - |
| *C. brasiliense* | CBS128528 | JQ005321 | JQ005234 | JQ005668 | - |
| *C. brasiliense** | CBS128501 | JQ005322 | JQ005235 | JQ005669 | - |
| *C. brassicicola** | CBS101059 | JQ005259 | JQ005172 | JQ005606 | - |
| *C. clidemiae* | ICMP18706 | JX009909 | JX010274 | JX010439 | - |
| *C. clidemiae** | ICMP18658 | JX010265 | JX010265 | JX010438 | KC888929 |
| *C. colombiense* | CBS129817 | JQ005260 | JQ005173 | JQ005607 | - |
| *C. colombiense** | CBS129818 | JQ005261 | JQ005174 | JQ005608 | - |
| *C. conoides** | CAUG17 | - | KP890162 | KP890168 | KP890174 |
| *C. cordylinicola** | ICMP18579 | JX009975 | JX010226 | JX010440 | JQ899274 |
| *C. cynbibiicola* | CBS128543 | JQ005254 | JQ005167 | JQ005601 | - |
| *C. cynbibiicola** | IMI347923 | JQ005253 | JQ005166 | JQ005600 | - |
| *C. fructicola* | CBS 125395 | JX009992 | JX010172 | JX010408 | - |
| C. fructicola | **COUFAL0332** | ON479100 | ON564677 | ON508044 | - |
| ***C. fructicola*** | **COUFAL0333** | ON479101 | - | - | - |
| ***C. fructicola*** | **COUFAL0334** | ON479102 | ON564678 | ON508045 | - |
| *C. fructicola** | ICMP18581 | JX010033 | JX010165 | JX010405 | JQ807838 |
| *C. gloeosporioides** | ICMP17821 | JX010056 | JX010152 | JX010445 | JQ807843 |
| *C. gloeosporioides* | DAR76936 | JX009976 | JX010151 | - | - |
| *C. hebeiense** | MFLUCC130726 | KF377495 | KF156863 | KF288975 | - |
| *C. hippeastri* | CBS125377 | JQ005317 | JQ005230 | JQ005664 | - |
| *C. hippeastri** | CBS125376 | JQ005318 | JQ005231 | JQ005665 | - |
| *C. horii* | C1180 | GQ329681 | GQ329690 | JX010450 | - |
| *C. horii** | C1069 | GQ329685 | GQ329687 | JX10375 | - |
| *C. karstii* | CBS128500 | JQ005289 | JQ005202 | JQ005636 | - |
| *C. karstii* | CBS128550 | JQ005306 | JQ005219 | JQ005653 | - |
| ***C. karstii*** | **COUFAL0335** | ON479117 | ON564686 | ON508052 | - |
| ***C. karstii*** | **COUFAL0336** | ON479118 | ON564687 | ON508053 | - |
| *C. musae* | ICMP 17817 | JX010015 | JX010142 | JX010395 | - |
| *C. musae** | CBS116870 | JX010050 | JX010146 | HQ596280 | KC888926 |
| *C. novae zelandiae* | CBS130240 | JQ005316 | JQ005229 | JQ005663 | - |
| *C. novae zelandiae** | CBS128505 | JQ005315 | JQ005228 | JQ005662 | - |
| *C. nupharicola* | CBS472.96 | JX010031 | JX010188 | JX010399 | - |
| C. nupharicola* | ICMP18187 | JX009972 | JX010187 | JX010398 | JX145319 |
| *C. oncidii* | CBS130242 | JQ005257 | JQ005170 | JQ005604 | - |
| *C. oncidii** | CBS129828 | JQ005256 | JQ005169 | JQ005603 | - |
| *C. parsonsiae** | CBS128525 | JQ005320 | JQ005233 | JQ005667 | - |
| *C. perseae** | GA100 | KX620242 | KX620308 | KX620341 | KX620177 |
| *C. petchii* | CBS118774 | JQ005312 | JQ005225 | JQ005659 | - |
| *C. petchii** | CBS378.94 | JQ005310 | JQ005223 | JQ005657 | - |
| *C. phyllanthi** | CBS175.67 | JQ005308 | JQ005221 | JQ005655 | - |
| *C. proteae* | CBS134301 | KC842379 | KC842385 | KC842387 | - |
| *C. proteae** | CBS132882 | KC297009 | KC297079 | KC297101 | - |
| *C. psidii** | CBS 145.29 | JX009967 | JX010219 | JX010443 | KC888931 |
| *C. queenslandicum* | ICMP 1780 | JX010010 | JX010186 | - | JX009693 |
| *C. queenslandicum** | ICMP1778 | JX009934 | JX010276 | JX010414 | - |
| *C. salsolae* | CBS119296 | JX009917 | JX010241 | - | - |
| *C. salsolae** | ICMP19051 | JX009916 | JX010242 | - | KC888925 |
| *C. siamense* | ICMP17795 | JX010051 | JX010162 | JX010393 | - |
| ***C. siamense*** | **COUFAL0337** | ON479103 | ON564679 | - | ON532813 |
| ***C. siamense*** | **COUFAL0338** | ON479104 | ON564680 | ON508046 | ON532814 |
| *C. siamense** | ICMP18578 | JX009924 | JX010171 | JX010404 | JQ899289 |
| *C. tainanense* | UOM1119 | MH728819 | MH728805 | MH846570 | MH728824 |
| *C. tainanense** | CBS143666 | MH728823 | MH728818 | MH846558 | MH728836 |
| *C. theobromicola* | ICMP 17895 | JX010057 | JX010284 | JX010382 | - |
| *C. theobromicola** | CBS124945 | JX010006 | JX010294 | JX010447 | KC790726 |
| ***C. theobromicola*** | **COUFAL0339** | ON479105 | ON564681 | ON508047 | ON532815 |
| ***C. theobromicola*** | **COUFAL0340** | ON479106 | - | - | - |
| ***C. theobromicola*** | **COUFAL0341** | ON479107 | ON564682 | ON508048 | - |
| ***C. theobromicola*** | **COUFAL0342** | ON479108 | ON564683 | ON508049 | - |
| ***C. theobromicola*** | **COUFAL0343** | ON479109 | - | - | - |
| ***C. theobromicola*** | **COUFAL0344** | ON479110 | - | - | - |
| ***C. theobromicola*** | **COUFAL0345** | ON479111 | ON564684 | ON508050 | ON532816 |
| ***C. theobromicola*** | **COUFAL0346** | ON479112 | - | - | - |
| ***C. theobromicola*** | **COUFAL0347** | ON479113 | - | - | - |
| ***C. theobromicola*** | **COUFAL0348** | ON479114 | - | - | - |
| ***C. theobromicola*** | **COUFAL0349** | ON479115 | ON564685 | ON508051 | - |
| ***C. theobromicola*** | **COUFAL0350** | ON479116 | - | - | - |
| *C. ti* | ICMP5285 | JX009910 | JX010267 | JX010441 | - |
| *C. ti** | ICMP4832 | JX009952 | JX010269 | JX010442 | KM360146 |
| *C. torulosun* | CBS102667 | JQ005252 | JQ005165 | JQ005599 | - |
| *C. torulosun** | CBS128544 | JQ005251 | JQ005164 | JQ005598 | - |
| *C. tropicale* | ICMP18672 | JX010020 | JX010275 | JX010396 | - |
| *C. tropicale** | CBS124949 | JX010007 | JX010264 | JX010407 | KC790728 |
| *C. viniferum* | gg4 | JN412800 | JN412802 | JN412811 | - |
| *C. viniferum** | yg1 | JN412798 | JN412804 | JN412813 | - |
| *C. xanthorrhoeae* | ICMP17820 | JX010008 | JX010260 | - | - |
| *C. xanthorrhoeae** | BRIP45094 | JX009927 | JX010261 | JX010448 | KC790689<br>- |

*Culturas ex-tipo. **COUFAL** - Coleção de Fitopatógenos da Universidade Federal de Alagoas.