Research Note

FIRST REPORT OF *LASIODIPLODIA THEOBROMAE* AND *COLLETOTRICHUM QUEENSLANDICUM,* FOLIAR PATHOGENS OF BREADFRUIT (*ARTOCARPUS ALTILIS)* IN PUERTO RICO[1,2]

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J. Agric. Univ. P.R. 106(1):123-136 (2022)

Breadfruit, *Artocarpus altilis* (Parkinson) Fosberg, Family Moraceae, is a perennial tropical fruit tree native to the Pacific Islands, where it has been a basic source of nutrition for more than 3000 years (Jones et al., 2011). The tree was introduced to the Caribbean during the 18th century and has since been of cultural importance (Jones et al., 2011; Ragone, 1997 and 2018). Worldwide, few diseases have been reported in *Artocarpus* spp., most caused by ascomycete fungi in Asia. Among them is anthracnose, caused by the *Colletotrichum gloeosporioides* complex, miscellaneous leaf spots, and fruit and collar rot. In addition, *Phytophthora palmivora*, an oomycete responsible for fruit, stem and root rot has been reported in Samoa (Jackson, 1988). However, economic losses caused by diseases in breadfruit are insignificant compared to other tropical fruits (Sangchote et al., 2003).

Botryosphaeriaceae is a large family of fungi belonging to the Filum Ascomycota, comprised of endophytes, saprobes and plant pathogens, across 23 genera and 187 species (Phillips et al., 2018). *Lasiodiplodia* species are commonly known as important plant pathogens causing leaf wilt, collar rot, branch dieback, regressive tree death, fruit mummification and inflorescence blight on a diverse group of plants (Kohler et al., 1997). Host trees include avocado (*Persea americana* Mill.), papaya (*Carica papaya* L.), coffee (*Coffea* spp.), mango (*Mangifera indica* L.), rambutan (*Nephelium lappaceum* L.), longan (*Dimocarpus longan* L.) and different species of *Citrus* (Picos-Muñoz et al., 2015; Khanzada et al., 2004; Serrato-Díaz et al., 2013 and 2020).

The fungal genus *Colletotrichum*, the causal agent of anthracnose, is known for infecting a wide range of tropical crops. In the Pacific Islands and Australia, anthracnose infections of breadfruit have been reported on leaves, fruits and twigs (Kohler et al., 1997; Lima et al., 2013; James et al., 2014). To our knowledge pathogens of breadfruit have not been studied in Puerto Rico and the Caribbean.

[1]Manuscript submitted to Editorial Board on 9 November 2021.

[2]This research was supported by projects: Encouraging Careers in Food Security and Safety: A multi-institutional approach for success in Puerto Rico (USDA NIFA-HIS Award No. 2016-38422-25541) and Plants: Friends and Foes (USDA AFRI Award No. 2016-67032-25007) in collaboration with Penn State University, and the Agricultural Experiment Station of the University of Puerto Rico-Mayagüez. Authors thank Lorena L. Simbaña, Víctor González and Luis Collazo for their collaboration on this research.

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In 2017, breadfruit leaf samples showing diffuse irregular necrotic lesions were collected from trees at the germplasm collections of the University of Puerto Rico. Initially mild symptoms were observed in the foliage (Figure 1). With time, necrotic tissue covered the leaf surface and defoliation occurred. Generally, mature lesions produced pycnidia, fungal asexual fruiting bodies that contained and exuded masses of conidia, typical of the genus *Lasiodiplodia* (Figure 1A). In addition, circular to oval brown lesions, humid sunken lesions and necrosis on leaf margins were observed. These lesions generally comprise acervulus, an asexual fruiting body containing masses of conidia, typical of the genus *Colletotrichum* (Figure 1B). These lesions were commonly observed throughout tree foliage at breadfruit germplasm collections of the University of Puerto Rico. Our objective was to identify the causal agents responsible for the unknown symptoms.

Symptomatic foliage was collected at breadfruit germplasm collections located at the Agricultural Experiment Substations of the University of Puerto Rico in Isabela and Lajas, Puerto Rico. Symptoms observed were limited only to the foliage of the tree. Advanced foliar symptoms caused their premature fall. This unknown symptomatology was widespread throughout both breadfruit germplasm collections sites. Disease incidence was estimated up to 70% at both location sites. Leaf tissue sections (4 mm^2) were superficially disinfected with 70% ethanol and 0.7% sodium hypochlorite and rinsed with sterile-distilled water (SDW). Tissue sections were transferred to acidified potato dextrose agar (APDA). Plates were incubated at room temperature (25° C) for four days. Fungal colonies were purified and characterized morphologically by examining reproductive structures under light microscopy. Fungal genera were identified using taxonomic keys (Barnett and Hunter, 1998; Phillips et al., 2013; Weir et al., 2012).

Pathogenicity tests were performed on six healthy detached leaves and on propagated six-month-old breadfruit trees planted in 7.57-L pots containing pro-mix soil and kept at plant nursery conditions. Before inoculation, leaves were superficially disinfected as described above. After inoculation, control and treated detached breadfruit leaves were placed in plastic containers (86.4-cm long x 40.6-cm wide), and leaves of nursery trees

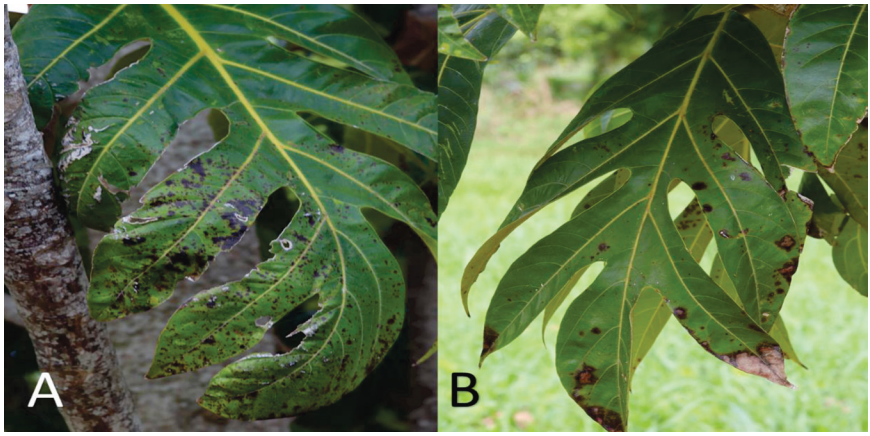

Figure 1. Lesions observed on leaves in the breadfruit germplasm collections of the Agricultural Experiment Substations of the University of Puerto Rico located in Isabela and Lajas, Puerto Rico. Early symptoms consisted of necrotic lesions caused by (A) *L. theobromae* and anthracnose caused by (B) *C. queenslandicum*.

were completely covered with plastic bags to create humid chambers. The experiment was repeated six times on detached leaves and twice on nursery plants. Inoculations were performed using mycelial disks (4 mm^2) of fungal isolates previously identified. Control treatments were inoculated with 4 mm^2 APDA disks. After pathogenicity tests, infected breadfruit leaf tissue was placed on a microscope glass slide containing a drop of SDW and examined using a light microscope. To complete Koch postulates, the pathogen was re-isolated from inoculated leaves and fungal cultures were examined for their morphological characteristics.

DNA analysis was used to confirm morphological identification of fungal isolates obtained from the field and pathogenicity tests. Briefly, genomic DNA was extracted using FastDNA™ SPIN Kit[6] for soil. For *Lasiodiplodia* spp. and *Colletotrichum* spp. isolates, genetic regions were amplified using Amplitaq Gold® polymerase (Applied Biosystems, Thermo Fisher Scientific, MA) with Polymerase Chain Reaction (PCR). Three genetic regions were amplified for *Lasiodiplodia* spp.: 1) the rDNA Internal Transcribed Region (ITS) using primers ITS5/ITS4 and ITS1/ITS4 (White et al., 1990); 2) elongation factor 1 alpha (Ef1-α) using primers EF728F/EF986R (Glass and Donaldson, 1995); and 3) β-tubulin using primers Bt-2a/Bt-2b (Carbone and Kohn, 1999; Alves et al., 2004 and 2008). Five genetic regions were amplified for *Colletotrichum* spp. isolates: 1) rDNA Internal Transcribed Spacer Region using primers ITS1/ITS4; 2) β- tubulin using primers T1/T2; 3) glyceraldehyde-3-phosphate dehydrogenase using primers GDF/GDR; 4) actin using primers ACT-512F/ACT-783R; and 5) chitin synthase using primers CHS79F/CHS345R (Weir et al., 2012). These genetic regions were sequenced at commercial laboratory facilities (Psomagen, Rockville, MD, USA) and compared to sequences available at GenBank of the National Center for Biotechnology Information using ex-type specimens (Phillips et al., 2013; Weir et al., 2012). A commercial bioinformatic software (Sequencher®, Gene Codes Corporation, Ann Arbor, MI, USA) was used for genetic sequence alignment and editing.

Phylogenetic trees were constructed using DNA nucleotide sequences from 37 Botryosphaeriaceae isolates obtained from GenBank as ingroup taxa and with ex-type specimens of *D. mutila* (CBS112553 and CBS23030) and *D. seriata* (CBS112555 and CBS119049) as outgroups for *Lasiodiplodia* spp. (Yang et al., 2017; Crous et al., 2006). For *Colletotrichum* spp., 71 DNA nucleotide ingroup taxa sequences were obtained from GenBank using *C. boninense* ex-type (MAFF306094 and MAFF305972) as outgroups (Weir et al., 2012; Serrato-Díaz et al., 2017). All selected nucleotide sequences were aligned using MAFFT (Multiple Alignment using Fast Fourier Transform), and the tree estimator was RAxML in SATé software. MAFFT with RAxML were used for phylogenetic tree estimations. The genetic regions studied were concatenated using Sequence Matrix. Maximum Likelihood was used in building phylogenetic trees to infer the species phylogeny using RAxML-HPC BlackBox, a tool for phylogenetic tree inference using maximum likelihood/rapid bootstrapping on XSEDE in CIPRES Science Gateway portal.

Based on morphology two fungal genera associated with foliage lesions were identified: *Lasiodiplodia* spp. and *Colletotrichum* spp. After six days of incubation, *Lasiodiplodia* spp. colonies were greenish gray with aerial mycelium that turned dark gray to black with age, from isolates obtained from both the field and pathogenicity tests (Figure 2A). Unilocular dark gray to black pycnidia contained conidiogenous cells producing masses of conidia (Figures 2B-D). Conidial size (n = 30) averaged 30.68 x 18.43 µm. Conidia were formed holoblastically at the tips of conidiogenous cells (Figure 2C). Immature conidia

[6]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the University of Puerto Rico.

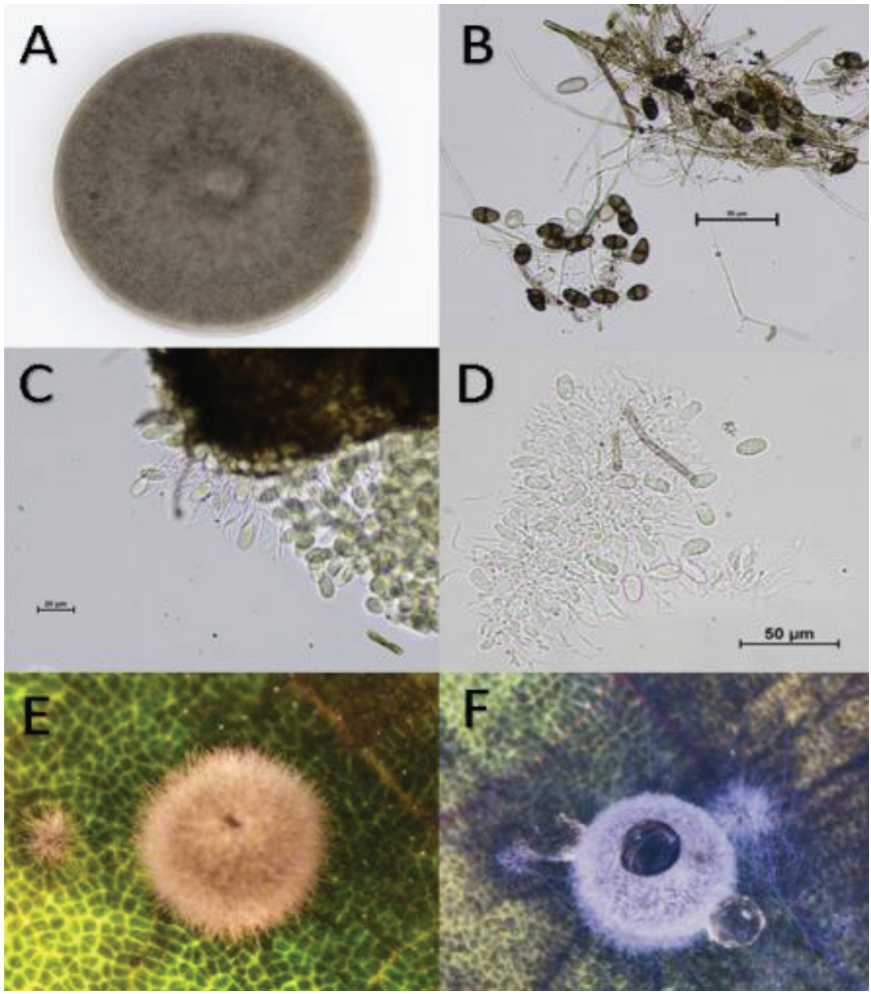

Figure 2. *Lasiodiplodia theobromae* (A) Colony grown on acidified PDA. (B) Hyaline one-cell immature conidia and dark brown two-cell mature conidia (20X). (C) Transverse section of a dark brown pycnidia showing conidiomata containing immature one-cell hyaline conidia. (D) Holoblastic immature conidia. (E and F) Mycelium developed throughout breadfruit leaf tissue during pathogenicity tests.

were thick walled, hyaline and one-celled; becoming dark brown and two-celled when mature, showing longitudinal striations.

After six days of incubation, *Colletotrichum* spp. colonies were gray to white, producing acervuli with profuse orange conidial ooze, from isolates obtained from both field and pathogenicity tests (Figure 3A). Unicellular conidia (n = 50) were hyaline, smooth walled, cylindrical shaped with round ends, measuring on average 14.87 x 4.02 µm. Simple melanized appressoria were globose to slightly elliptical, measuring 5.28 x 3.58 µm (Figure 3B).

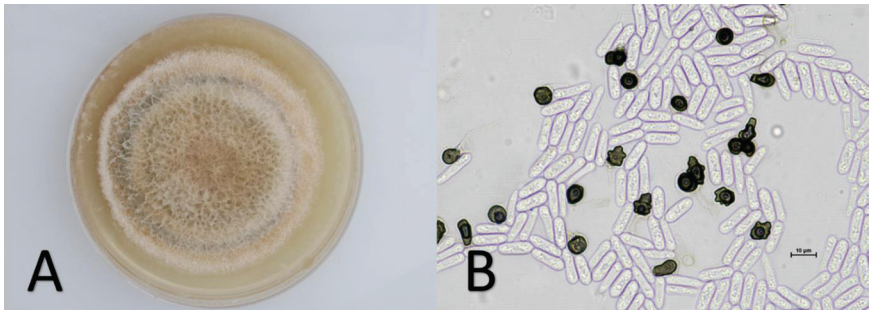

FIGURE 3. *Colletotrichum queenslandicum* (A) Colony grown in acidified PDA. (B) Hyaline conidia and melanized appressoria (60x).

During pathogenicity tests with *Lasiodiplodia* spp. conducted on detached leaves (Figure 4) four days post inoculation (DPI), necrosis and radial mycelial growth were observed at inoculation sites (Figure 4B and C). For *Colletotrichum* spp., four days post inoculation (DPI) (Figure 5), necrosis and thin aerial mycelium was observed around

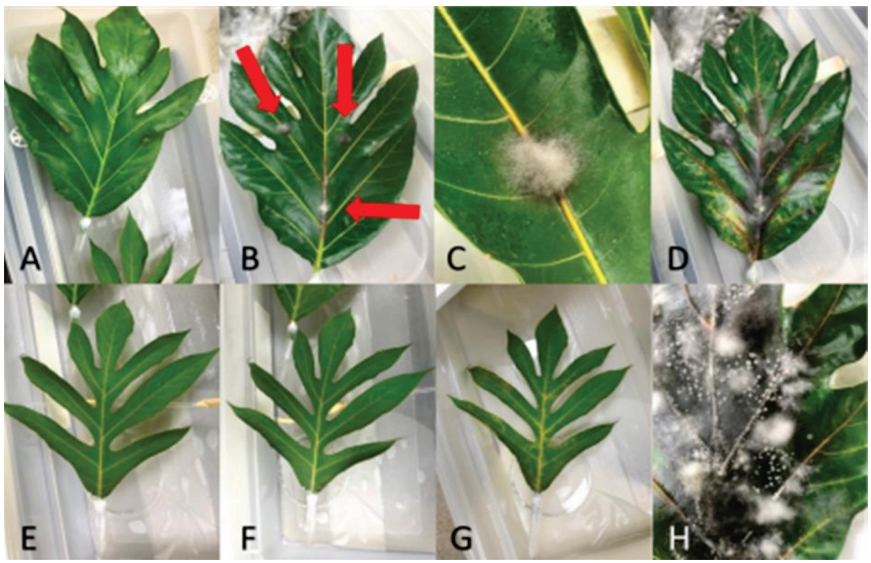

FIGURE 4. Pathogenicity tests of *L. theobromae* on detached breadfruit leaves: (A) Breadfruit leaf before inoculation. (B) Three inoculation sites (arrows) with mycelial disks (4 mm^2). (C) Four days post inoculation, slight aerial mycelium was observed on inoculation site. (D) Eight days post inoculation, central leaf veins developed profuse necrosis and mycelial growth. (H) Twelve days after inoculation, aerial mycelial growth was observed throughout leaf surface and complete necrosis of tissue seen. (E, F and G) Control treatments inoculated with acidified PDA disks at four, eight and twelve days after inoculation, respectively.

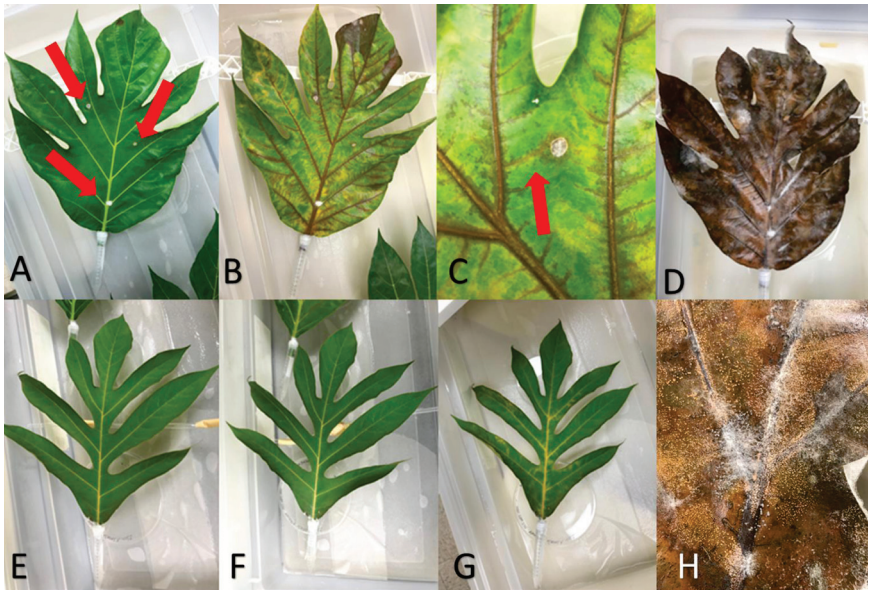

Figure 5. Pathogenicity tests of *C. queenslandicum* on detached breadfruit leaves: (A) Inoculation sites using mycelial disks (4 mm^2) (arrows). (B) Four days after inoculation, chlorosis, vein necrosis and thin aerial mycelia were observed on leaves. (C) Necrosis around inoculation site through leaf veins. (D and H) Twelve days after inoculation, masses of acervuli developed throughout leaf tissue. (E, F and G) Control treatments inoculated with acidified PDA disks at four, eight and twelve days post inoculation, respectively.

inoculation sites that extended to leaf vascular tissue (Figure 5B and C). Leaf tissue colonized by *Lasiodiplodia* spp. developed pycnidia covered with gray mycelium. Eight DPI, central veins necrotized and profuse mycelial growth was observed on inoculation sites. Twelve DPI, leaf tissue was completely necrotized (Figure 4D and H). Twelve DPI, detached leaves of *Colletotrichum* spp. isolates developed acervuli on lesions and necrosis was observed (Figure 5D and H). Control treatments did not manifest fungal growth or observable symptoms, but twelve DPI, leaves showed slight chlorosis and dehydration due to detachment (Figures 4G and 5G).

After pathogenicity tests conducted on propagated breadfruit trees twelve DPI, leaves covered with plastic bags fell from tree under high humidity conditions (Figures 6D and 7D). When examined under the light microscope, fallen leaves showed *Lasiodiplodia* sp. pycnidia or *Colletotrichum* sp. acervuli throughout the surface of each inoculation site (Figures 6E to H and 7E to H). When leaves were examined under the light microscope, infected breadfruit leaf tissue showed conidia typical of *Lasiodiplodia* spp. and *Colletotrichum* spp. Mycelium emerging through stomata and glandular trichomes was observed (Figures 8A and 9A) (Sá et al., 2019). After pathogenicity tests, *Lasiodiplodia* spp. and *Colletotrichum* spp. were re-isolated from detached leaves and from leaves of nursery trees, completing Koch's postulates.

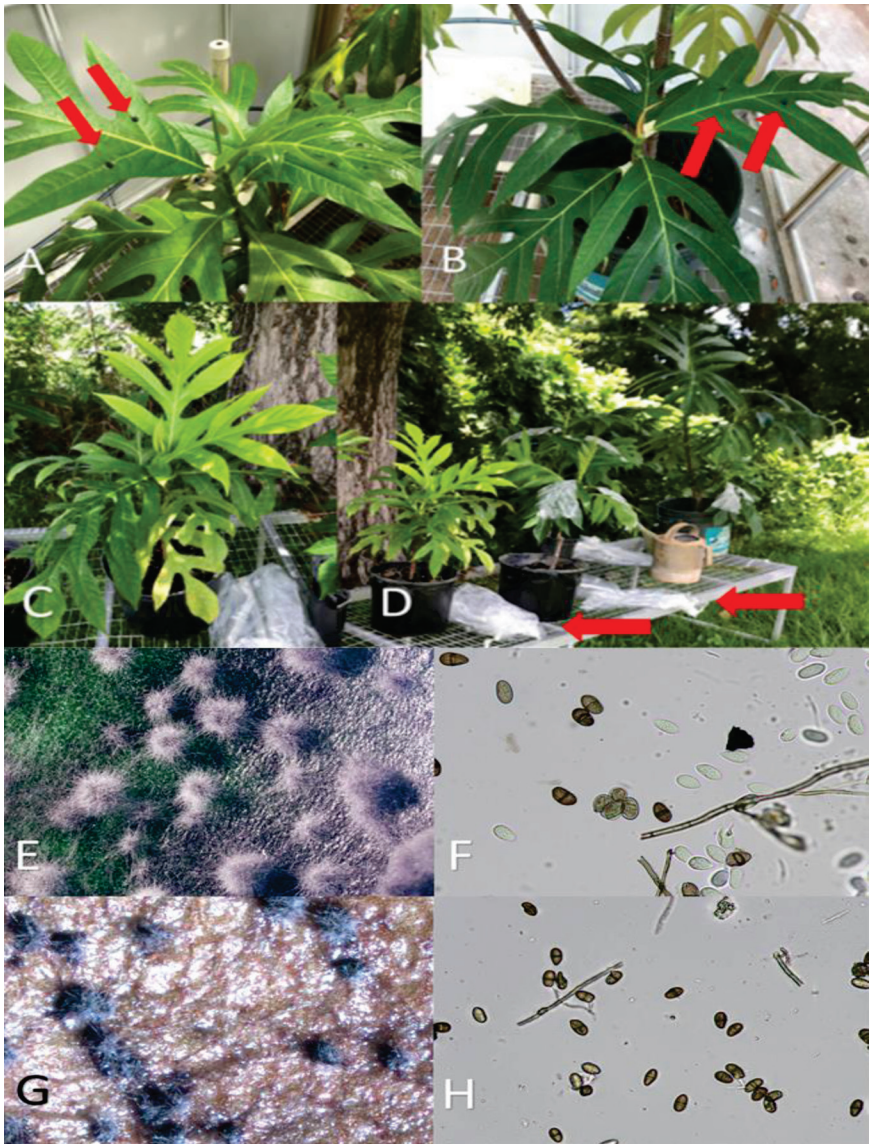

FIGURE 6. Pathogenicity test of *Lasiodiplodia theobromae* on propagated breadfruit trees: (A and B) Inoculation sites (arrows) with mycelial disks (4 mm^2). (C) Control breadfruit tree. (D) Inoculated trees with fallen leaves inside plastic bags 12 days after inoculation (arrows). (E and G) Mycelial development on leaves observed under the stereoscope. (F and H) Mature (melanized) and immature (hyaline) conidia observed with light microscope.

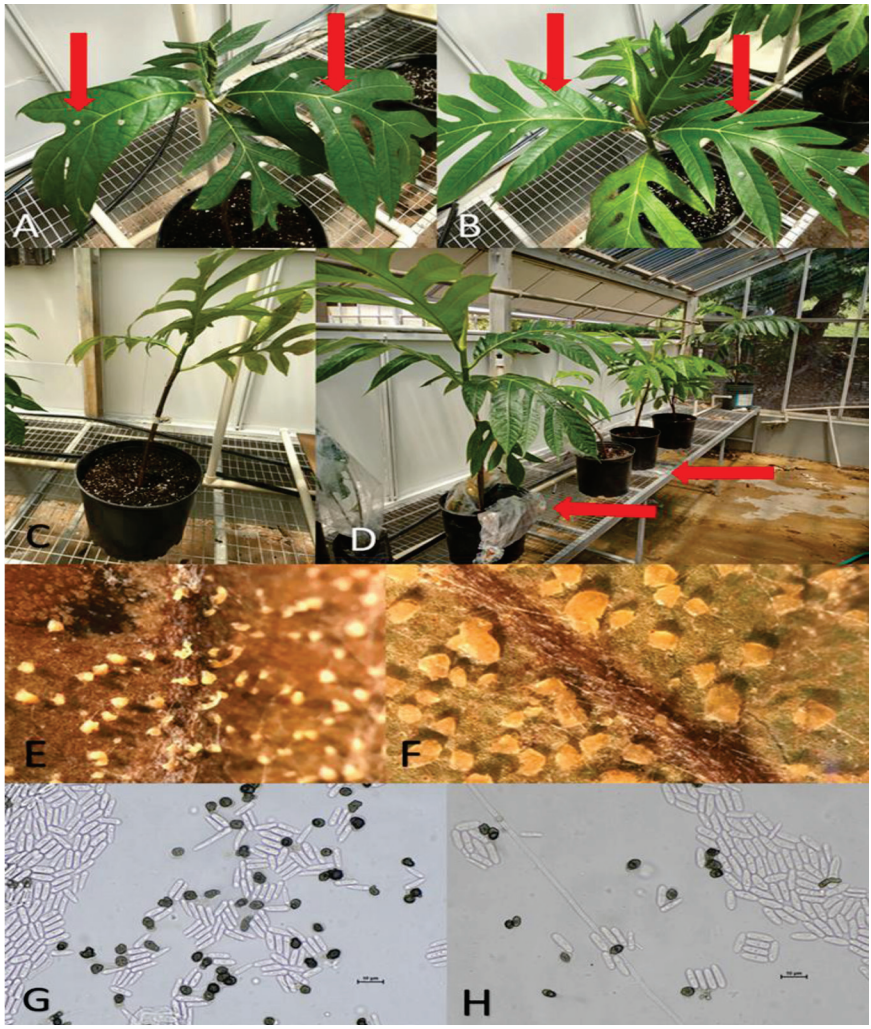

Figure 7. Pathogenicity tests of *Colletotrichum queenslandicum* on nursery breadfruit trees: (A and B) Inoculation sites (arrows) with mycelial disks (4 mm^2). (C) Control breadfruit tree. (D) Twelve days post inoculation, leaves fell from trees to inside plastic bags (arrows). (E and F) acervuli and conidial masses observed on fallen leaves under stereoscope. (G and H) Hyaline conidia and melanized appresoria.

Control treatments inoculated with pure APDA disks did not manifest fungal growth or observable symptoms (Figures 6C and 7C).

A DNA nucleotide sequence analysis of the three fungal genetic regions (i.e., ITS, Ef1-α and β-tubulin) of *Lasiodiplodia* spp. isolates obtained from the field and pathogenicity tests showed >90% homology with ex-type specimens of *L. theobromae* when

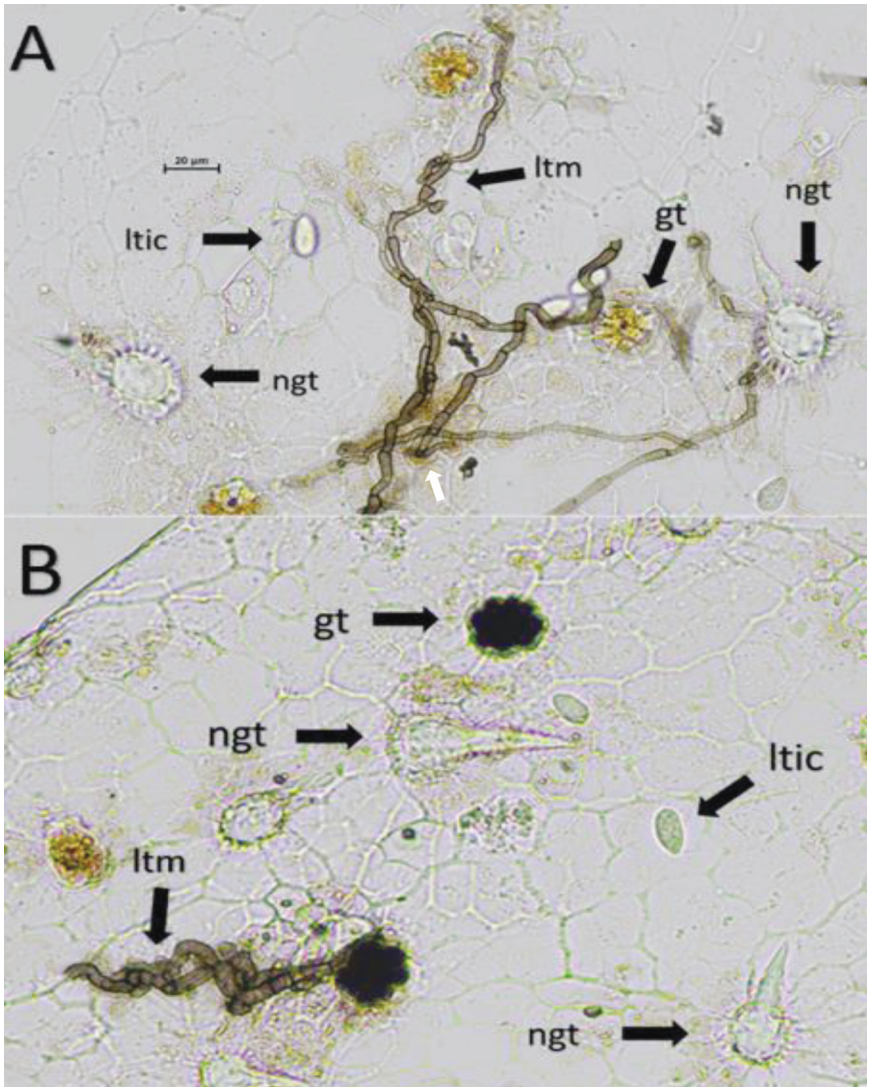

FIGURE 8. *Lasiodiplodia theobromae* reproductive structures observed on a thin layer of leaf epidermal tissue at 20X (A) and 60X (B), after Koch's postulates. Asexual reproductive structures were observed on leaf epidermis: immature conidia (ltic) and dark brown mycelium (ltm) emerging through stomata (white arrow). Breadfruit non-glandular (ngt) and glandular trichomes (gt).

compared to Genbank sequences of this species for all genes studied (Phillips et al., 2013 and 2018). A DNA nucleotide sequence analysis for *Colletotrichum* spp. using five fungal genetic regions (i.e., ITS, ACT, GADPH, CHS-1 and β-tubulin) of isolates, obtained from

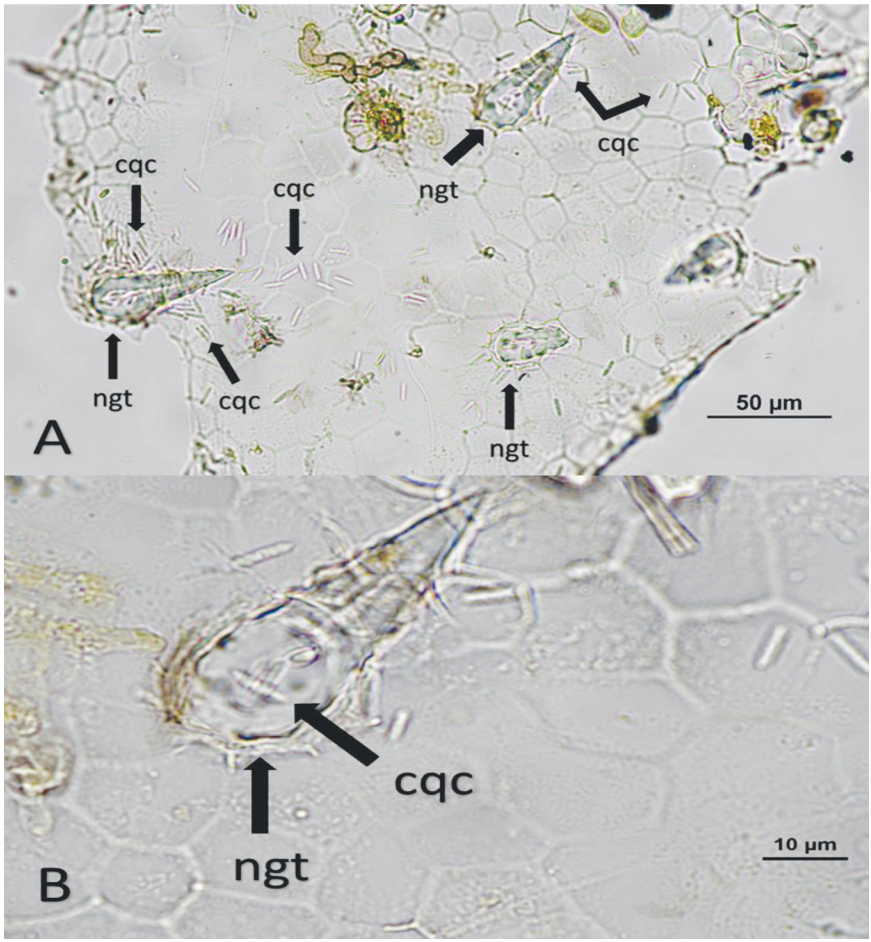

Figure 9. *Colletotrichum queenslandicum* conidia observed on a thin layer of leaf epidermal tissue at 20X (A) and 60X (B), after Koch's postulates. Unicellular hyaline conidia (cqc). Breadfruit non-glandular (ngt).

the field and after pathogenicity tests, showed >90% homology with ex-type specimens of *C. queenslandicum* when compared to GenBank sequences of this species for all genes studied (Weir et al., 2012). The phylogenetic tree built using concatenated sequences from the three genes studied for *Lasiodiplodia* spp. isolates grouped our isolates in the same clade of *L. theobromae* (Figure 10). *Lasiodiplodia theobromae* was identified using light microscopy, taxonomic keys and DNA sequences of the three nuclear genes previously mentioned (Phillips et al., 2013).

The *Colletotrichum* spp. phylogenetic tree, constructed using concatenated sequences from the five genes studied, grouped our isolates in the same clade of

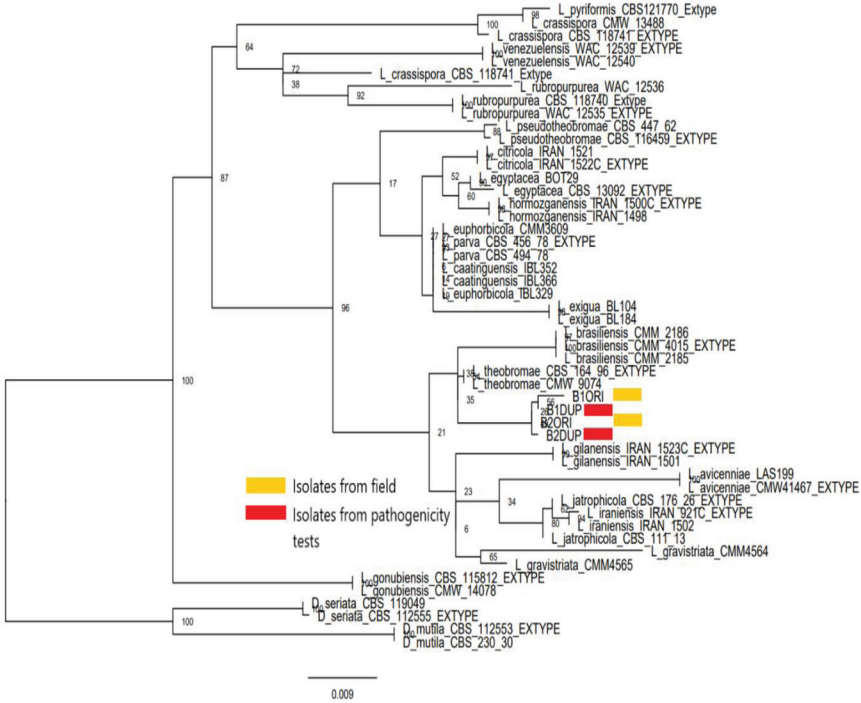

FIGURE 10. Maximum-Likelihood tree of different *Lasiodiplodia* species based on the concatenated sequences of internal transcribed spacer, beta tubulin and elongation factor genes. Bootstrap support values are shown at the nodes. Isolates from breadfruit leaves collected in the field and isolates recovered after pathogenicity tests are indicated by colored symbols. Phylogenetic tree was rooted with outgroups *Diplodia mutila* CBS 112553 Ex-type and CBS23030 and *Diplodia seriata* CBS112555 Ex-type and CBS119049.

C. queenslandicum (Figure 11). *Colletorichum queenslandicum* was identified using light microscopy, taxonomic keys and DNA sequences of the five nuclear genes mentioned (Weir et al., 2012).

Lasiodiplodia and *Colletotrichum* are fungal genera characterized by hosting a wide range of tropical crops. In Puerto Rico, *L. theobromae* has been reported causing inflorescence blight of mango and longan, and dieback of rambutan (Serrato Díaz et al., 2013 and 2020). In the Pacific Islands, *L. theobromae* has been reported causing dry rot of collar and trunk of breadfruit trees and has been studied due to the value of this crop (Kohler et al., 1997; Sangchote et al., 2003; Ragone, 1997). *Colletotrichum queenslandicum* was originally reported on papaya (*Carica papaya*) and avocado (*Persea americana*) in Queensland, Australia, and later, on *Coffea* sp. in Fiji (Simmonds, 1968; Phoulivong et al., 2012; Yang et al., 2009). It was reported on rambutan (*Nephelium lappaceum*) in Puerto Rico (Serrato-Diaz et al., 2015). To our knowledge, this is the first report of *L. theobromae* and *C. queenslandicum* affecting breadfruit foliage in Puerto Rico and the Caribbean.

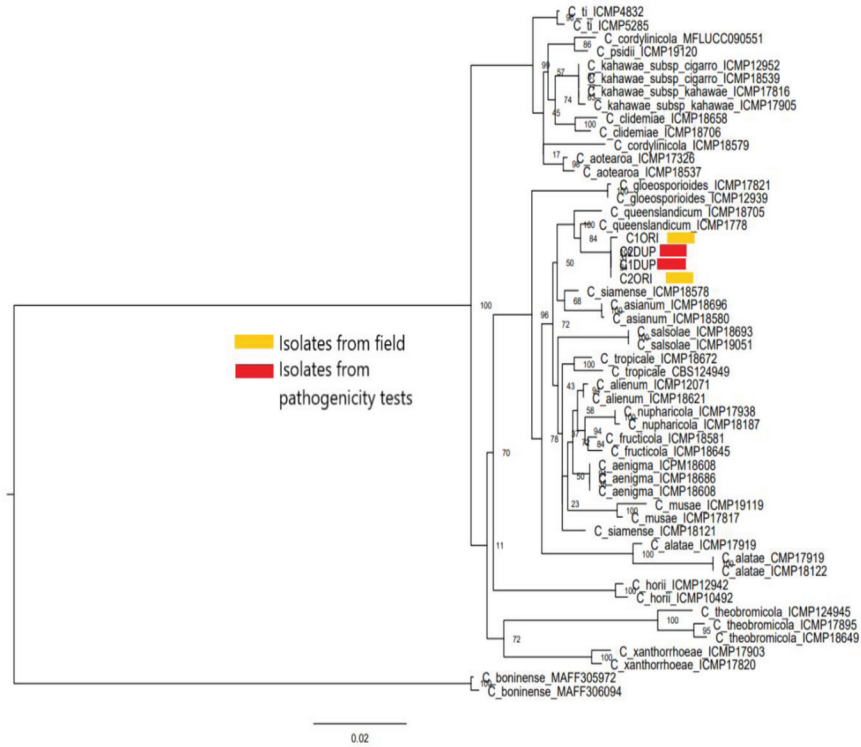

Figure 11. Maximum Likelihood tree of *Colletotrichum* species built with concatenated sequences of internal transcribed spacer, beta tubulin, chitin synthase, glyceraldehyde 3-phosphate dehydrogenase and actin genes. Bootstrap support values are shown at the nodes. Isolates from breadfruit collected in the field in Puerto Rico and isolates recovered from pathogenicity tests are indicated by colored symbols. Phylogenetic tree was rooted to the outgroups *C. boninense* ex-type MAFF306094 and MAFF305972.

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