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Descending death in soursop (*Annona muricata* L.) caused by *Lasiodiplodia theobromae* in Nayarit, Mexico

Lasiodiplodia theobromae agente causal de muerte descendente en guanábana (*Annona muricata* L.) en Nayarit, México

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ABSTRACT

The study consisted of identifying the causal agent of branch dieback in soursop in the municipalities of Compostela and San Blas, Nayarit, Mexico. *Lasiodiplodia* is a poorly studied pathogen regarding its pathogenicity and hosts in Mexico, reaffirming the need for studies involving morphological and genetic characteristics of Lasiodiplodia, especially due to the association with a complex of cryptic species. From the tissues of soursop showing apparent symptoms of dieback, 9 isolates were obtained with morphological macro and microscopic characteristics typical of *Lasiodiplodia*. Universal primers ITS4 and ITS5 allowed the molecular identification of *Lasiodiplodia* species, including *L. theobromae* (6 isolates), *L. pseudotheobromae* (1 isolate), *L. venezuelensis* (1 isolate), and *L. iranensis* (1 isolate). The pathogenicity of the L. theobromae isolate (4To2) was confirmed, marking the first report in Mexico for soursop cultivation, confirmed through molecular techniques. Its identity was corroborated by sequencing TEF1a using primers EF-728F and EF-986R. The isolated strain induced leaf wilting, branch death, and necrotic lesions in vegetative apices.

KEW WORDS: Pathogen, apices, branches, necrotic tissue, regressive.

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RESUMEN

El estudio consistió en identificar el agente causal de la muerte descendente de ramas en guanábana en los municipios de Compostela y San Blas, Nayarit, México. *Lasiodiplodia* es un patógeno poco estudiado respecto a su patogenicidad y hospederos en México; lo cual reafirma la necesidad de conducir estudios que involucren caracteres morfológicos y genéticos de *Lasiodiplodia*, especialmente por la asociación de un complejo de especies crípticas. De los tejidos de guanábana con síntomas aparentes de muerte descendente se obtuvieron 9 aislamientos con caracteres morfológicos macro y microscópicos típicos de Lasiodiplodia. Los cebadores universales ITS4 e ITS5 permitieron la identidad molecular de especies de *Lasiodiplodia*, *L. theobromae* (6 aislamientos), *L. pseudotheobromae* (1 aislamiento), *L. venezuelensis* (1 aislamiento) y *L. iranensis* (1 aislamiento). Se corroboró la patogenicidad del aislamiento de *L. theobromae* (4To2), primer reporte para México en el cultivo de guanábana, confirmado mediante técnicas moleculares, del cual se corroboró su identidad con la secuenciación de TEF1a utilizando los cebadores EF-728F y EF-986R, el aislado indujo marchitamiento de hojas, muerte de ramas y lesiones necróticas en los ápices vegetativos.

PALABRAS CLAVE: Patógeno, ápices, ramas, tejido necrótico, regresiva.

Introduction

Soursop (*Annona muricata* L.) (Magnoliales: Annonaceae), is the most extensively cultivated Annonaceae species in Mexico, with a current cultivation area of 3,378.5 ha with an average yield of 10.4 ton ha^{-1} (SIAP, 2023). In Nayarit, 72.9 % of the national production is concentrated, amounting to a production close to 35,000 tons (SIAP, 2023). Soursop faces significant phytosanitary issues, particularly fungal diseases, which are economically important. In Mexico, anthracnose caused by *Colletotrichum gloeosporioides* and soft fruit rot by *Lasiodiplodia pseudotheobromae* have been reported (Cambero-Ayón *et al*., 2019). In the case of downy dieback on soursop, particularly in Nayarit (Mexico), the fungus reported to be associated is *L. theobromae* in conjuntion with xylophagous insects, although there is no evidence of pathogenicity (Hernández *et al*., 2013; Hernández *et al*., 2018). Species belonging to the *Lasiodiplodia* genus affect over 500 plant species, especially economically important tropical and subtropical fruit trees (Punithalingam, 1980; Alves *et al*., 2008; Picos-Muñoz *et al*., 2015), Reported damage includes different symptoms such as rots, cankers and branch death. However, these pathogens are considered weak, as they require the host to be stressed (e.g., nutritional deficiency) and have wounds caused by pests (insects or mites) or mechanical factors (Gonçalves *et al.,* 2016).

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The initial symptoms of downward dieback in branches occur in the apices, which begin to wilt; the leaves turn brown and die. Subsequently, the leaves fall, and the branch takes on a dry appearance. In the most severe cases, the branches throughout the entire tree dry up successively. *L. theobromae* is considered the most virulent species (Úrbez & Gubler, 2009) and its severity ranges from 8 to 100 % (Nam *et al.,* 2016). Some symptoms associated with *L. theobromae are the* presence of dark brown to black exudates on the bark and necrotic tissue inside trunks and branches, especially the vascular system, where a discoloration resembling a dark brown streak is evident, descending in nature (Hernández *et al*., 2013).

In avocado (*Persea americana*) in Peru, *L. theobromae* was reported causing necrosis in the tree bark (Leon and Mattos, 2016). In macadamia (*Macadamia integrifolia* Maiden & Betche) in Brazil, *L. theobromae* causes apex necrosis, peduncle rot and downward dieback (Fischer *et al*., 2017). In Nayarit, Mexico *L. theobromae* causes soft fruit rot in jackfruit (*Artocarpus heterophyllus*) (Medina *et al*., 2018). In mango (*Mangifera indica*), mamey (*Pouteria sapota*), grapevine (*Vitis vinifera*), and 'Persian' lime (*Citrus* × *latifolia*) in Mexico, *L. citricola* and *L. pseudotheobromae* have been reported as causal agents of peduncle rot, stem cankers and downward dieback (Úrbez & Gubler, 2009; Sandoval-Sánchez *et al*., 2013; Tovar *et al*., 2013; Valle de la Paz *et al*., 2019). Cambero-Ayón *et al*. (2019) reported fruit rot in soursop caused by *L. pseudotheobromae*. *Lasiodiplodia* is a little studied pathogen in Mexico regarding its pathogenicity, virulence, and host range. Hence, the objective of this study was to identify morphologically and molecularly the causal agent of downward dieback in soursop.

Material and Methods

Sample collection

Biweekly samplings were conducted during the dry periods (March, April, and May) and rainy periods (August, September, and October) of 2020 in nine orchards with 4- to 7-year-old free-standing trees in the productive areas of Nayarit, Mexico (Table 1). Tissue samples showing symptoms of descending death were collected from five trees in each orchard. Tissues were placed in Kraft paper bags and transferred to the Agricultural Parasitology laboratory at CEMIC 03, Universidad Autónoma de Nayarit, where they were processed.

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Table 1. Location of soursop orchards sampled for the *Lasiodiplodia* spp. isolation in Nayarit, Mexico.

Municipality	Location	Coordinates	Altitude (masl)
Compostela	El Tonino	21° 04' 05" N-105° 12' 51" W	74
Compostela	Divisadero	21° 7' 23.5 " N-105° 11' 14.2" W	36
Compostela	Capomo G	21° 6' 51.7" N-105° 9' 2.9" W	38
Compostela	Divisadero2	21° 7' 33.9" N-105° 12' 9.3" W	39
Compostela	Chacala	21° 9' 26.9" N-105° 12' 9.6" W	43
San Blas	Tecuitata 1	21° 27' 35.6 N-105° 9' 21.4" W	367
San Blas	La bajada	21° 32' 0" N-105° 10' 26.2" W	222
San Blas	Infiernillo	21° 32' 13.2" N-105° 11' 4.1" W	188
San Blas	Tecuitata 2	21° 27' 39.8" N-105° 9' 11.3" W	379
San Blas	La palma	21° 31' 0.9" N-105° 11' 5.3" W	16

masl=meters above sea level

Isolation and purification

The symptomatic tissue (branches) was washed with sterile distilled water (SDW) for 1 min Starting from the advancing (transition) zone of the disease, tissue sections of 0.5 cm² were cut, immersed for 3 min in 3 % sodium hypochlorite, then rinsed three consecutive times with SDW for 3 min and dried with sterile paper towels. Formerly, pieces of diseased tissue were placed directly on the Potato Dextrose Agar (PDA) culture medium contained in Petri dishes; they were seeded in duplicate with five portions distributed in each dish. Subsequently, they were sealed with plastic wrap and incubated at 26 ± 2 °C, in darkness. Fungal growth was systematically checked every 24 h (Saldarriaga *et al*., 2008; Abdollahzadeh *et al*., 2010). From the fungal isolates of 7 d of growth, they were purified by hyphal tip technique, then expanded and preserved on PDA under the previously described incubation conditions.

Pathogenicity tests

To determine the pathogenicity and host range of the fungal isolates, approximately 1-year-old healthy soursop, mango, jackfruit, avocado, and 'Persian' lime trees grown under 50 % shade mesh were inoculated. Due to the similarity in macroscopic and microscopic characteristics of the 9 *Lasiodiplodia* isolates obtained, one was randomly selected. This isolate was inoculated into branches with diameters of approximately 8 mm and an approximate length of 30 cm, in both primary and secondary branches about 30 cm from the apex. The inoculum consisted of explants (5 mm diameter PDA discs) of the fungus (mycelium and unquantified conidia), grown for 7 days. The fungus was inoculated directly onto the xylem, for which part of the bark was removed from the branches. The control consisted of a PDA disc of the same dimensions without the fungus.

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Subsequently, the inoculated xylem zone was covered with sterile damp cotton and plastic wrap to prevent external contamination. The branches that showed symptoms were separated from the plant and taken to the Agricultural Parasitology laboratory at CEMIC 03 of the Universidad Autónoma de Nayarit for processing, where the causal agent was re-isolated and corroborated as corresponding to the inoculated fungus, through the observation of gray-black aerial mycelium typical of *Lasiodiplodia* (Gonçalves *et al*., 2016). A randomized complete block design was used, with each plant considered as an experimental unit. The experiment consisted of three repetitions, and control trees were without fungal inoculations.

DNA extraction

Genomic DNA (gDNA) was extracted from the isolated fungi for identity analysis using the CTAB II extraction method. For this purpose, fungal mycelium grown for 7 days was collected. One gram of the sample was macerated with 700 µL of CTAB I and placed in a sterile 2 mL microtube, processed according to the Allers & Lichten protocol (2000). The gDNA pellet was re-suspended in 30 µL of MiliQ water and stored at -20 °C until use. DNA concentration and purity were quantified in a Nanodrop (JENWAY, Bibby Scientific Ltd, UK®). The obtained gDNA was visualized by electrophoresis on a 1 % agarose gel and subsequently, used for reaction with primers ITS5F (GGAAGTAAAAGTCGTAACAAGG) / ITS4R (TCCTCCGCTTATTGATATATGC) and EF-728F (GGAAGTAAAAGTCGTAACAAGG) / EF-986R (TCCTCCGCTTATTATTGATATATGC) (White *et al*., 1990; Casarrubias-Carrillo *et al*., 2003; Abdollahzadeh *et al*., 2010).

Morphological and molecular identification

Preliminary identification of *Lasiodiplodia* was based on its microscopic morphological characteristics and growth on Potato Dextrose Agar (PDA) culture medium (macroscopic), through the taxonomic keys of Abdollahzadeh *et al*. (2010) and Shah *et al*. (2010). Subsequently, molecular identification was performed using the extracted genomic DNA (gDNA) with the primers ITS4/ITS5 and EF1-728F/EF1-986R. The quantities, final concentration, and reagents used to prepare the reaction mixture for a PCR volume of 26 µL were as follows: 0.9 µL primers, 13.5 µL RedTaq polymerase, 1.5 µL gDNA, and 9.2 µL SDW. PCR amplification conditions included an initial denaturation step at 94 °C for 5 min, 38 cycles of denaturation at 94 °C for 1 min, alignment at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension stage at 72 °C for 4 min. PCR products were visualized by electrophoresis of 1.5 % agarose gels in 1 X TBE buffer 1 X at 80 V for 1 h (White *et al*., 1990; Casarrubias-Carrillo *et al*., 2003; Shah *et al*., 2010). The PCR product was sequenced by Macrogen Genome Center (Seoul, Korea®). The obtained sequence was compared with the NCBI databases using the BLAST algorithm (Altschul *et al*., 1990) to verify the percentage of identity corresponding to the species.

Analysis of obtained band patterns

The sequences obtained with each primer were edited and assembled with MEGA X software. Consensus sequences were compared with the GenBank database using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, with representative sequences of pre-

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identified genera and species in the BLAST analysis, alignment was performed in MEGA X. The obtained matrix was used to build a phylogenetic analysis based on the Neighbor-Joining (NJ) method. Internal topology support of the phylogenetic tree was achieved through bootstrap analysis with 1000 repetitions (Kumar *et al*., 2018). The consensus bootstrap tree inferred from 1000 repetitions was taken to represent the evolutionary history of the analyzed taxa. The percentage of tree replication in which associated taxa grouped in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Evolutionary distances were calculated using the composite maximum likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. This analysis involved 19 nucleotide sequences. The codon positions included were 1st + 2nd + 3rd + No coding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 528 positions in the final dataset. Evolutionary analyses were performed in MEGA X (Kumar *et al*., 2018).

Results and Discussion

Pathogenicity tests

Twenty-two days after inoculating the plants with the fungus, only the soursop trees showed apparent symptoms of downward death of branches and rotting of apices (Figure 1A and 1B), developed chlorosis, leaf wilting, and subsequent branch death, as well as, necrotic lesions in the apices of the inoculated branches. These necrotic lesions developed with a downward trend. The control plants did not exhibit these symptoms. Polanco *et al*. (2019) reported symptoms of necrosis, wilting, and death of branches in a downward direction in sweet orange (*C. sinensis*), as well as, external necrotic lesions of 2 cm, 32 d after being inoculated with *L. theobromae*. Valle de la Paz *et al*. (2019) reported leaf chlorosis, downward death of primary and secondary branches, cancer formation and gummy exudates on ‘Persian’ lime plants 22 d after being inoculated with strains of *Lasiodiplodia* species (*L. citricola*, *L. pseudotheobromae* and *L. theobromae*). In mamey, the pathogenicity of *L. theobromae* was confirmed 30 d after inoculation through wounds in branches, evidenced by the presence of necrotic lesions, and two years later, the downward death of vegetative shoots was observed (Vásquez-López *et al*., 2009).

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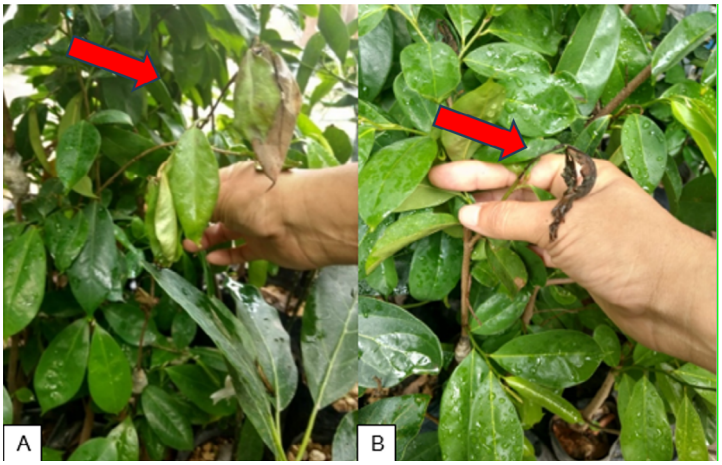

Figure 1. Appearance of branch death in soursop plants 22 days after inoculation with *Lasiodiplodia* fungus (A). Apex rot in soursop plants 22 days after inoculation with *Lasiodiplodia* (B).

Morphology of *Lasiodiplodia* isolates

The nine *Lasiodiplodia* isolates produced abundant aerial mycelium, resulting in two types: one of cottony appearance and the other smooth. This suggests the possibility of two species. The mycelium of both types is septate, initially white when young, turning black over time (Figure 2A), approximately 30 d of mycelial growth showed the presence of pycnidia with conidia characteristic of *Lasiodiplodia* (Figure 2B). The conidia are ovoid to ellipsoidal, with rounded apices that narrow to a truncated base, hyaline and coenocytic in young cultures, turning dark brown as they age, with one septum, and showing longitudinal striations. It is noteworthy that, according to their macroscopic characteristics, no considerable differences were detected. Gómez *et al*. (2009) identified *L. theobromae* as a causal agent of postharvest rot in mamey fruits, describing the fungus as having abundant, septate, branched, dark gray to black mycelium; the conidia are initially unicellular, hyaline, granular, subovoid to ellipsoid-oblong with a thin wall and truncated base; upon maturation, they developed a thick, septate, half-dark brown wall, often longitudinally striated (Figure 2 C and D). Similarly, Tovar-Pedraza *et al*. (2012) confirmed *L. theobromae* as the fungus responsible for the death of grafted mamey vines in Guerrero, Mexico, and mentioned that the fungal colony showed rapid and abundant aerial mycelial growth, initially gray, becoming olive-gray and denser in the center of the disc, pycnidia in stroma, simple or compound, scattered and often aggregated. Immature conidia were hyaline, ellipsoidal to subovoid, and mature conidia were dark brown, ellipsoidal to ovoid, with irregular longitudinal striations.

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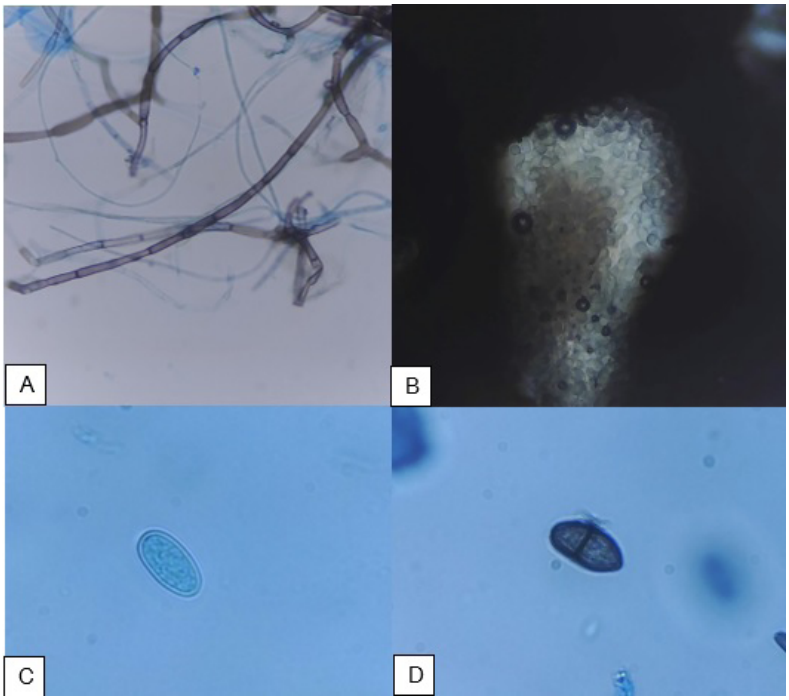

Figure 2. Distinctive microscopic features of *Lasiodiplodia*.

(A) Septate hyphae at 10 d of age viewed at 40X. (B) Agglomerated conidia developing into conidiogenous cells within the pycnidium (10X). (C) Young, hyaline, and cenocitic conidia viewed at 40X. (D) Mature, dark conidia with a septum viewed at 40X.

Valle de la Paz *et al.* (2019) reported three species of the genus *Lasiodiplodia* (*L. theobromae, L. citricola,* and *L. pseudotheobromae*), causing gummosis, branch dieback and death of 'Persian' lime trees in Morelos, Mexico, observed colonies with smoky gray mycelial growth, then olive-gray to greenish-gray and the presence of pycnidia at 30 d of incubation. In the states of Nuevo León and Tamaulipas, Mexico, the fungi *L. theobromae*, *Fomitopsis meliae and Eutypella citricola* were identified by cultural, morphological, molecular and pathogenic characters in sweet orange trees with dieback symptoms. The colony of *L. theobromae* initially showed a whitish coloration, turning gray and darkening over days, the conidia initially oval-shaped, hyaline and without septa, but as the culture aged they became septate and acquired a brown coloration (Polanco *et al*., 2019). In Nayarit, Medina *et al*. (2018) reported *L. theobromae* as a causal agent of postharvest soft rot in jackfruit (*Artocarpus heterophyllus*). The fungus exhibited uniform and continuous growth, with colonies developing abundant, cottony, white-grayish aerial mycelium that turned olive-gray and eventually black. Pycnidia were observed at 20 days.

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Molecular identification

30 ng μL^{-1} of DNA were obtained from each isolate, with absorbance ratios 260/280 between 1.8 and 2. DNA amplification from the 9 isolates resulted in 540 bp and 300 bp amplicons for the ITS-4/ITS-5 and EF-728/EF-986 regions of rDNA, respectively. Consensus sequences of PCR products were obtained with lengths of 530 bp and 290 bp. Amplification of rDNA regions in Lasiodiplodia isolates showed a variation of 99.4 to 100 % in the ITS rDNA sequences. Similar results were obtained by Al-Sadi *et al*. (2013) where the range was 99.4 to 100 %. When entering the consensus DNA sequences in BLAST of the 9 isolates and comparing them with GenBank sequences, all matched the genus *Lasiodiplodia* (Table 2). For the pathogenic isolate, with primers EF1-728F and EF1-986R, the nucleotide similarity was 99.6 % with *L. theobromae* sequences. The phylogenetic tree (Figure 3) was constructed with the nine sequences obtained in the present study; nine sequences of *Lasiodiplodia* species deposited in GenBank by other authors and selected for comparative purposes for presenting high levels of identity with our isolates. The fungus *Rhizopus oryzae* was used as an external group of analysis and selected for not being phylogenetically related to *Lasiodiplodia*.

The obtained isolates are presented in 8 clades, where LP2 and LP3 are in the same clade and the others, although close, are in different clades, the isolates Ch2 and Go are quite close to each other, but they are quite distant from the first six and the most distant is Dv2 because it presents less similarities with the other isolates due to a possible selection pressure. *Lasiodiplodia* is a pathogen little studied regarding its genetic variability, pathogenicity, virulence and host range in Mexico. This underscores the need for studies involving morphological and molecular or genetic characters of *Lasiodiplodia*, especially due to the potential association of a complex of cryptic species in fruit trees affected by *Lasiodiplodia* in Nayarit. Nowadays, the application of molecular tools, such as DNA markers, allows for greater reliability in evolutionary studies of phytopathogenic fungal populations (Manzo *et al*., 2005). In another study with 64 isolates, three clades were obtained, the first with 25 isolates, the second with 11 isolates and the third with the remaining 28 isolates (Al-Sadi, *et al*., 2013). Similarly Burgess *et al*. (2006), grouped 45 *Lasiodiplodia* isolates into five clades corresponding to *L. theobromae*, *L.gonubiensis,* and three undescribed taxa named clade III, clade IV, and clade V.

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Table 2. Molecular identity of *Lasiodiplodia* isolates isolated from soursop tissues with dieback symptoms in Nayarit, Mexico.

Isolated	ITS	Size bp	Identity (%)	EF	Size bp	Identity (%)
To1	**L. iranensis**	530	99.81	**L. jatrophicola**	286	99.65
To2	**L. theobromae**	532	100	**L. theobromae**	290	99.65
Cha1	**L. theobromae**	529	100	**L. theobromae**	295	100
Cha2	**L. venezuelensis**	532	99.81	L. laosensis	289	99.65
Div	**L. theobromae**	538	99.81	**L. theobromae**	284	100
Go	**L. pseudotheobromae**	535	99.44	**L. theobromae**	289	100
LP1	**L. theobromae**	529	100	**L. theobromae**	285	100
LP2	**L. theobromae**	533	100	**L. theobromae**	282	100
LP3	**L. theobromae**	531	100	L. brasiliensis	260	99.59

EF=elongation factor; bp=base pairs

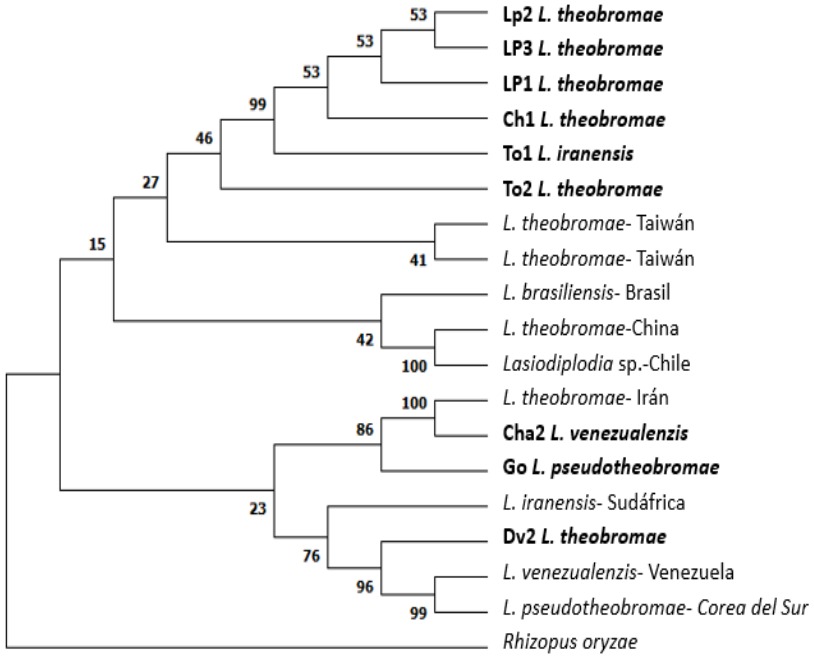

Figure 3. Phylogenetic tree constructed with ITS 4/5 rDNA sequences of *Lasiodiplodia* isolates (highlighted in bold) obtained from soursop from Compostela and San Blas, Nayarit, Mexico and reference sequences from GenBank.

Bootstrap values are presented in branches.

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Conclusions

Lasiodiplodia theobromae is the causal agent of branch dieback on soursop trees in Nayarit, Mexico. It induces leaf wilting and subsequent death of the branch, along with necrotic lesions on the branch tips 22 days after inoculation. *L. theobromae*, *L. pseudotheobromae L. venezuelensis,* and *L. iranensis* were identified as putative pathogens on soursop from Compostela and San Blas, Nayarit, Mexico. The isolates show characteristics of the genus *Lasiodiplodia*, abundant aerial mycelium, white and cenocitic hyphae when young and dark septate at 5 d, pycnidia with conidia at 30 d. Ovoid to ellipsoidal conidia, with rounded apices, hyaline and cenocitic in young cultures, turning dark brown as they age, with one septum, showing longitudinal striations.

Authors contribution

Conceptualization of the work, author 1, author 2; methodology development, author 1, author 2; software management, author 1, author 4; experimental validation, author 2, author 3, author 4; analysis of results, author 2, author 3, author 4; data management, author 1, author 4.; manuscript writing and preparation, author 1, author 2, author 3, author 4, author 5, author 6; drafting, revising and editing, author 1, author 2, author 3, author 4, author 5, author 6; project management, author 1, author 2; fund acquisition, author 2.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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