Report of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. in citrus trees in Tamaulipas

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Abstract

This study determined the presence of *Lasiodiplodia theobromae* in citrus trees with deterioration and descending death in the central area of Tamaulipas, Mexico in 2017 and 2018. In the study area, plant samples were collected in the trees of valencia orange, grapefruit, mandarin and Italian lemon. In the laboratory, the samples were processed and the fungi were isolated and identified with taxonomic keys and by genetic analysis of the transcribed internal spacer and elongation factor 1 alpha TEF1. 33 strains of fungi were isolated in the 19 commercial citrus orchards, 26 belonged to the genus *Lasiodiplodia* sp., 3 of *Botryosphaeria* sp., 1 of *Colletotrichum* sp., 1 of *Cyphellophora* sp., 1 of *Fusarium* sp., and 1 of *Nigrospora* sp. Of the strains of the genus *Lasiodiplodia* sp., these were identified as *L. theobromae* in citrus trees with gummosis, rot, descending death in branches and fruit mummification.

Keywords: fungi, genetic analysis, rot.

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Introduction

The fungus *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. it is classified within the Ascomycetes, in the order Botryosphaeriales and family Botryosphaeriaceae (Schoch *et al.*, 2006; Slippers *et al*., 2013). It is a saprophyte and endophyte fungus and is considered a latent pathogen. However, it is pathogenic when the host is weakened or stressed (Rubini *et al*., 2005; Mohali *et al*., 2005).

The fungus causes regressive death of the branches, lesions on the stems, generates rubber and post-harvest fruit rots (Sánchez *et al*., 1989; Herrera *et al*., 1993). In the field, in the crop of valencia orange and ruby red grapefruits, damage by *L. theobromae* consists of defoliation and presence of rubber in the secondary branches, necrosis of the phloem and xylem.

On the other hand, it also causes damage to the crop of mamey [*Pouteria sapota* (Jacq.) H. E. Moore and Stearn], grape (*Vitis vinifera* L.), avocado (*Persea americana* Mill), kumquat [*Fortunella margarita* (Lour.) Swingle] and mango (*Mangifera indica* Lin.) (Urbez y Gubler, 2011). In these trees, *L. theobromae* can occur alone or in interaction with *Colletotrichum* sp., *Fomitoporia maxonii* and *Fusarium* sp. Such interaction causes chlorosis, necrosis, screened, cankers, blights, wet or dry rots, mummifications, wounds, scabs and withering (Kimati *et al*., 1995).

Also, the interaction of *L. theobromae* with *Fomitiporia maxonii* Murrill has been demonstrated; *Alternaria citri* Ellis and Pierce; *Colletotrichum gloeosporioides* (Penz.) Sacc; *Fusarium solanii* (Mart.), Appel and Wollen, *Fusarium* sp., *Dothiorella* sp., *Phytophthora* (Oomycetes), *Cephaleuros virescens*, Kunze) and *C. Liberibacter asiaticus* (Cabrera *et al*., 2012, Cabrera *et al*., 2017). Therefore, in this work the presence of *Lasiodiplodia theobromae* was determined in citrus trees with deterioration and dieback in the central area of Tamaulipas, Mexico in 2017 and 2018.

Materials and methods

Study area

The research work was carried out in 2017 and 2018 in 19 commercial citrus orchards located in the municipality of Güémez, Llera de canales, Padilla and Victoria Tamaulipas, Mexico (Table 1).

Table 1. Location of the sites where the study was carried out.

Localization		Coordinates	
Locality	Orchard	Latitude	Longitude
Güémez	The Lomas	23.920595	-99.050012
Güémez	The Cascabeles	23.91841	-99.153615
Güémez	Macabeos III	23.923206	-99.041665
Güémez	Providencia	23.927547	-99.078619
Güémez	Three Sabinos	23.896745	-99.051205
Llera	The Angelicas	23.249581	-98.839627

Localization		Coordinates	
Locality	Orchard	Latitude	Longitude
Llera	The Cecilia	23.225730	-98.826750
Padilla	San Juan	24.047400	-99.031250
Padilla	The Tejon	24.043433	-98.891232
Padilla	Macarena	24.101300	-99.019300
Padilla	Caluche	24.101393	-99.018491
Victoria	San Francisco	23.928958	-99.232792
Victoria	Real del 14	23.759185	-99.072300
Victoria	The Huichol	23.877121	-99.238929
Victoria	San Jose	23.903197	-99.163759
Victoria	The 12	23.827501	-99.082200
Victoria	Casa Graciela	23.896745	-99.051205
Victoria	The Encino	23.784240	-99.063962
Victoria	The Anhelo	23.773975	-99.074120

Collect plant material

The plant material was collected in 27 Italian lemon trees (*Citrus limon* Burm), 10 valence orange (*Citrus sinensis* L. Osbeck), 1 red double grapefruit (*Citrus paradisi* Macfad) and 1 mandarin (*Citrus reticulata* Blanco*).* The trees had the following symptoms: rot of the wood, dried branches, rotten fruits with black mycelium, rot and cankers in the bark of the branch, dried branches with rubbers and leaves with black and white mycelium. In each structure (wood, branch, fruit and leaf), 200 g of material was collected. The samples were poured into polyethylene bags labeled and transported to the laboratory of Molecular Biology and Biotechnology of the postgraduate degree in biology of the Institute of Technology of Victoria, Tamaulipas.

Isolation and taxonomic identification of phytopathogenic fungi

Five 0.5 cm tissue sections were cut from each sample. These were disinfected with sodium hypochlorite at 1% by 3 min, washed with sterile distilled water, dried and sown separately in potato-dextrose-agar (Cabrera *et al*., 2012). The crops were incubated at 25 °C with white light for 3 days. Of the insulation obtained, monospiric cultures were made in agar water (18 g agar dissolved in one liter of distilled water). Afterwards, fungi were identified at the genus and species level based on taxonomic characteristics published by Punithalingam, (1976); Burgess *et al*. (2006); Barnett and Hunter (2006).

DNA extraction and PCR development of *L. theobromae*

DNA was extracted using the technique of Ahrens and Seemüller (1992). In the extracted DNA, the ITS genomic region (ITS1, 5.8 S and ITS2) and the alpha elongation factor gene (EF-1α) were amplified. The ITS1 region was amplified with the initiators ITS1 (5'-TCCGTAGGTGACTCTGCGG-3') and ITS4 (3'-TCCTCCGCTTATTGATATGC-5') and ITS4 (3'-TCCTCCGCTTATTGATATGC-5') and ITS2 with ITS5 initiators (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (3'-TCCTCCGCTTATTGATATGC-5')

(White *et al*., 1990). While, the EF-1α was amplified with the initiators EF1F (5'-TGTTGCTGTTAAGGATTTGAAGCG-3') and EF1R (3'-AACAGTTTGACGCA TGTCCCTAAC-5') (Rehner and Buckley, 2005).

The mixture of PCR to amplify both regions consisted of: ultrapure water (13.22 µl), buffer solution TBE 1X (2.5 µl), MgCl at 2.5 mM (2.08 µl), dNTPs to 0.2 mM (2 µl), initiators at 20 µmol (2 µl of each), DNA polymerase (Biogenica®) to 1U (0.2 µl) and 1 ml of DNA (80 ng).

The parameters for PCR were: 94 °C for 5 min, 35 cycles of 94 °C for 5 min, 60 °C for 1 min, 72 °C for 15 min and a final cycle of 72 °C for 5 min. The amplified regions were purified with a commercial kit (Promega) and the fragments obtained were sent to sequence to the Faculty of Sciences of the National Autonomous University of Mexico (UNAM).

Genetic analysis

The sequences obtained from the ITS region and EF-1α were edited to build consensus, lined up, with the Clustal W algorithm included in The BioEdit v7.0.9 software and compared to the sequences deposited at the National Center for Biotechnological Information (NCBI). The generated matrices were used to perform phylogenetic analysis with the 'nearest neighbor' method based on the Neighbor-Joining method (Saitou and Nei, 1987). Selecting the sequences with the greatest similarity and equality in the size of the fragment in order to calculate the evolutionary distance (Tajima and Nei, 1984) in the MEGA7 software (Kumar *et al*., 2016).

Results

The presence of the fungus was determined at prospective sites characterizing tree damage and symptoms (Figure 1). 33 strains of fungi were isolated in the 19 commercial orchards (Table 2). Of which, 20 strains were collected in Italian lemon, 11 in valence orange, 1 in mandarin and 1 in grapefruit crop. In the trees, 13 strains were collected in the trunk, 13 in the branches, 3 in fruits, 3 in the root and one in the leaves.

Figure 1. Symptoms of citrus damage: gummosis in branches (a), damage to bark and wood (b) and (c); rot in fruit with mycelium growth (d).

Table 2. Record of symptoms in citrus trees sampled in the citrus zona of Tamaulipas, Mexico.

Municipality	Orchard	Variety	Structure	Symptoms
Güémez	The Lomas	Lemon I	Branch	Rot and dried branches
	The Cascabeles		Branch	Rot and dried branches
			Trunk	Wood rot and xylem
		V. orange	Trunk	Bark and wood rot
			Branch	Immature conidia
			Fruit	Rotten fruits
		Lemon I	Branch	Black mycelium in xylem
			Root	Dry, rotten root
	Macabeos III		Branch	Rot in the branch
			Root	Damaged bark and xylem
	Providencia		Trunk	Rot in wood
	Three Sabinos		Branch	Dry branch
Llera	The Angelicas	V. orange	Trunk	Xylem rot in branches
	La Cecilia	Lemon I	Branch	Xylem rot on branches
Padilla	San Juan	Mandarin	Trunk	Dry wood with affectations
		V. orange	Trunk	Log rot
		Lemon I	Branch	Dried branches with rubber
			Branch	Dried branches with rubbers
	The Tejon	Grapefruit	Trunk	Xylem damage
	Macarena	V. orange	Trunk	Rot and necrosis of the wood
		Lemon I	Fruit	Rotten fruits
	Caluche		Trunk	Xylem rot and bark
Victoria	San Francisco	Orange	Branch	Dry branches with rubber
	Real 14		Fruit	Rotten fruit
		Lemon I	Root	Dry roots
	The Huichol		Trunk	Damage to wood and xylem
			Trunk	Xylem and bark damage
			Trunk	Xylem and wood damage
	San Jose	V. orange	Branch	Dry branches
	The 12		Branch	Dry branches
	Casa Graciela		Branch	Dry branch
	The Encino	Lemon I	Trunk	Xylem damaged
	The Anhelo		Leaves	Leaves with white mycelium

Of the total strains collected, 26 belonged to the genus *Lasiodiplodia* sp., 3 of *Botryosphaeria* sp., 1 of *Colletotrichum* sp., 1 of *Cyphellophora* sp., 1 of *Fusarium* sp., and 1 of *Nigrospora* sp.

Of the strains of the genus *Lasiodiplodia* sp., 17 strains were isolated from Italian lemon crop, 7 in valencia orange, 1 in mandarina and 1 in grapefruit. In tree structures, 13 strains were collected on the trunk, 10 on the branches and 3 on the fruits. While the three strains of the genus *Botryosphaeria* sp. were collected in the branches of the valencia orange crop. In contrast, the strain *Colletotrichum* sp., *Cyphellophora* sp. and *Fusarium* sp., was collected from the root of the Italian lemon trees and the strain of the genus *Nigrospora* sp., was collected on the leaf of this same citrus species (Table 3).

Table 3. Distribution of isolated fungi in the sampled structures of the tree.

Municipality	Orchard	Variety	Structure	Fungus
Güémez	The Lomas	Italian lemon	Branch	*Lasiodiplodia* sp.
	The Cascabeles		Branch	*Lasiodiplodia* sp.
			Trunk	*Lasiodiplodia* sp.
		Valencia orange	Trunk	*Lasiodiplodia* sp.
			Branch	*Botryosphaeria* sp.
			Fruit	*Lasiodiplodia* sp.
		Italian lemon	Branch	*Lasiodiplodia* sp.
			Root	*Colletotrichum* sp.
	Macabeos III		Branch	*Lasiodiplodia* sp.
			Root	*Cyphellophora* sp.
	Providencia		Trunk	*Lasiodiplodia* sp.
	Three Sabinos		Branch	*Lasiodiplodia* sp.
Llera	The Angelicas	Valencia orange	Trunk	*Lasiodiplodia* sp.
	The Cecilia	Italian lemon	Branch	*Lasiodiplodia* sp.
Padilla	San Juan	Mandarina	Trunk	*Lasiodiplodia* sp.
		Valencia orange	Trunk	*Lasiodiplodia* sp.
		Italian lemon	Branch	*Lasiodiplodia* sp.
			Branch	*Lasiodiplodia* sp.
	The Tejon	Grapefruit	Trunk	*Lasiodiplodia* sp.
	Macarena	Valencia orange	Trunk	*Lasiodiplodia* sp.
		Italian lemon	Fruit	*Lasiodiplodia* sp.
	Caluche		Trunk	*Lasiodiplodia* sp.
Victoria	San Francisco	Valencia orange	Branch	*Lasiodiplodia* sp.
	Real 14		Fruit	*Lasiodiplodia* sp.
		Italian lemon	Root	*Fusarium* sp.
	The Huichol		Trunk	*Lasiodiplodia* sp.

Municipality	Orchard	Variety	Structure	Fungus
			Trunk	*Lasiodiplodia* sp.
			Trunk	*Lasiodiplodia* sp.
	San Jose	Valencia orange	Branch	*Botryosphaeria* sp.
	The 12		Branch	*Botryosphaeria* sp.
	Casa Graciela		Branch	*Lasiodiplodia* sp.
	The Encino	Italian lemon	Trunk	*Lasiodiplodia* sp.
	The Anhelo		Leaves	*Nigrospora* sp.

On the other hand, in the case of strains of the genus *Lasiodiplodia* sp., the colonies in the culture medium at 10 days developed a cottony mycelium white and abundant, after 16 days it changed to a dark gray tone (Figure 2) from there, these presented paraphyses hyalines, pycnidia alone or added in the stromatic tissue, immature hyalin conidia, ellipsoid, granulose and truncated-based. As well, dark brown mature conidia, ellipsoid, with longitudinal stretch marks and truncated base (Figure 3).

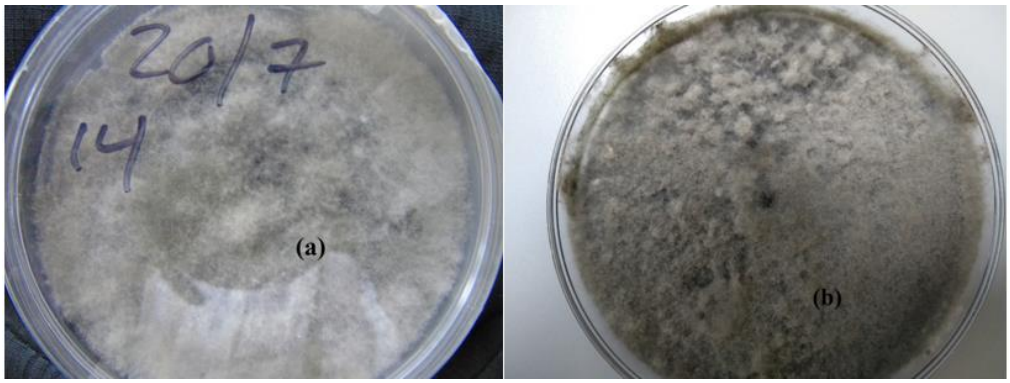

Figure 2. Mycelium of *Lasiodiplodia* sp. (a), at 10 days the color of the mycelium is white and covers the whole box, at 16 days it turned to a gray color with black center (b).

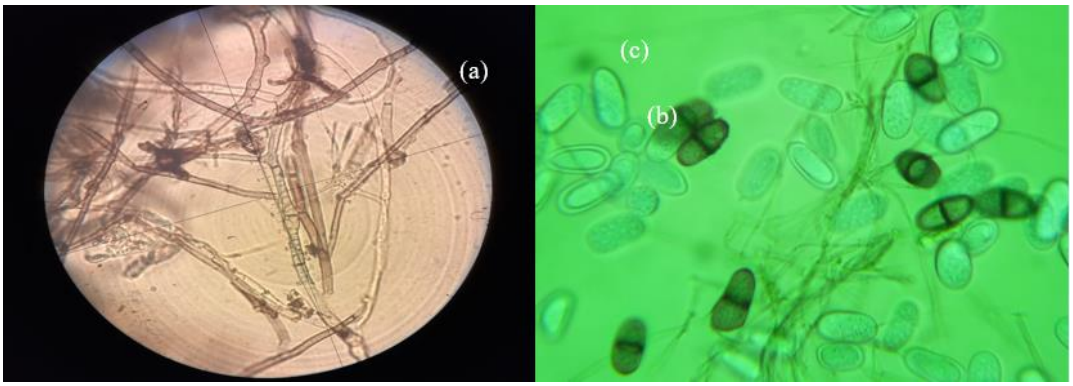

Figure 3. Mycelium (a) and mature conidia (b) and young (c) of *Lasiodipodia* sp.

Genetic characterization of isolated strains of *L. theobromae*

Of the isolated strains, 17 consensus sequences were obtained from 542 base pairs from the ITS1/5.8S rDNA/ITS2 region and 17 sequences of 314 base pairs of the elongation factor EF-1α. The seventeen sequences of the region ITS1/5.8S rDNA/ITS2 showed homology of 100% with the species *L. theobromae* (HM466958). These sequences showed no genetic differences between them. Therefore, they were grouped into a single consensus sequence. This sequence was then recorded at the National Center for Biotechnology Information (NCBI) under access number MK886711.

On the other hand, the 17 sequences of the EF-1α elongation factor showed no genetic differences between them. For this, they were grouped into a single consensus sequence. These were then compared with sequences of *L. theobromae* downloaded from the NCBI. The sequence show homology of 100% with the species *L. theobromae*. Finally, the analyzed sequence was deposited in the NCBI with access number MK531139.

The construction of the phylogenetic tree, allowed to group the 17 ITS sequences found in eight groups or clades according to the homology in the sequences (Figure 4). While in the phylogenetic tree of the TEF1 alpha sequences, they were grouped into three main clades (Figure 5). Both constructions show a close genetic relationship between the isolates, although the samples came from different municipalities and tissue type. Evolutionary history was inferred using neighbor-joining method. The tree is drawn at scale, with the length of the branches in the same units as the evolutionary distances used to infer the phylogenetic tree.

Evolutionary distances were calculated using the composite maximum probability method. The analysis involved 17 nucleotide sequences. The codon positions included were $1^{st} + 2^{nd} + 3^{rd}$. All positions containing missing data were deleted. There was a total of 507 positions in the final dataset. Evolutionary analyses were performed in Mega 7.

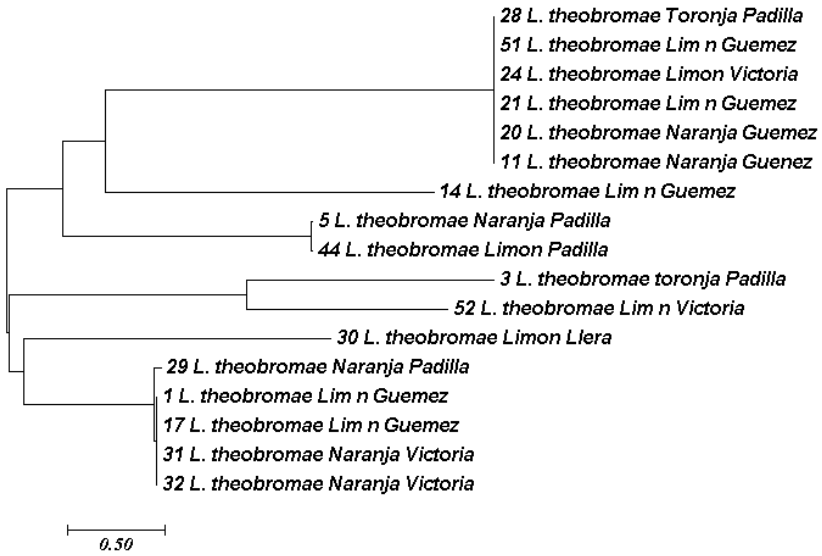

Figure 4. Phylogenetic analysis generated from the sequences of the ribosomal ITS region of the isolated trains of *L. theobromae*. The dendogram was obtained from the analysis by the 'nearest neighbor' method based on the Neighbor-Joining method using the Mega 7 program.

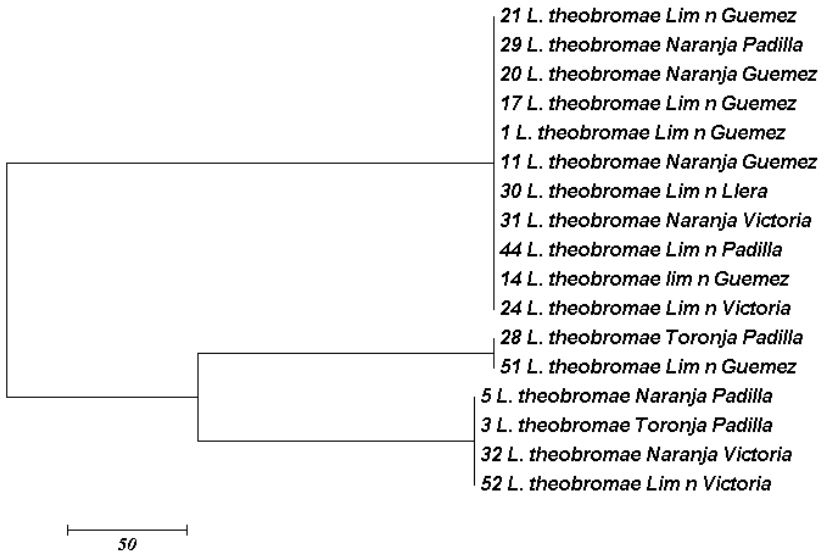

Figure 5. Phylogenetic analysis generated from the EF-1α elongation factor sequences of the fungus *L. theobromae*. The dendogram was obtained from the analysis by the 'nearest neighbor' method based on the Neighbor-Joining method using the Mega 7 program.

Discussion

In different parts of the world the fungus *L. theobromae* is reported in citrus trees (Al-Sadi *et al*., 2014; Adesemoye *et al*., 2014; Rodríguez *et al*., 2016). In Mexico, *L. theobromae* has been reported causing different diseases in several mainly fruit crops. In this work, the fungus was isolated from the crop of valencia orange, Italian lemon, mandarin and grapefruit in the municipality of Güémez, Llera de Canales, Padilla and Victoria Tamaulipas.

In the municipality of Llera de Canales, Polanco *et al*. (2019) reported this fungus in the trees of valencia orange. While, in the present work, the fungus was reported in the Italian lemon crop and valence orange in the commercial orchard 'The Angelicas' and 'The Cecilia'. These authors also reported the fungus in the valencia orange crop in General Terán and Montemorelos, Nuevo León.

In sites where the fungus has been recorded, it has commonly been associated with dieback and has been constantly isolated from the branches, bark, vascular tissue and fruits of affected plants (Mullen *et al*., 1991; Moghal *et al*., 1993; Mohali *et al*., 2005). For example, in Venezuela, *L. theobromae* was isolated from citrus trees with symptoms of dieback and gummosis (Ferrari *et al*., 1996). In China, *L. theobromae* generated gummosis in *Jatropha podagrica* plants (Fu *et al*., 2007).

While, in India this fungus was the causal agent of root rot and collar rot disease in *J. curcas* (Latha *et al*., 2009). In this work, en the trees of both citrus species, the fungus was collected in branches and trunks with xylem rot, in bark and dried branches with the presence of rubber. While, Polanco *et al*. (2019) reported in trees with symptoms of dieback and necrosis in the trunk and branches. Cedeño y Palacios (1992), mention that *L. theobromae* produces gummosis and lesions in citrus plants, symptoms similar to those observed in the field.

Of the total strains collected, 26 belonged to the genus *Lasiodiplodia* sp., 3 of *Botryosphaeria* sp., 1 of *Colletotrichum* sp., 1 of *Cyphellophora* sp., 1 of *Fusarium* sp. and 1 of *Nigrospora* sp. Of the strains of the genus *Lasiodiplodia* sp., 17 strains were isolated from Italian lemon crop, 7 in valencia orange, 1 in mandarin and 1 in grapefruit. Cabrera *et al*. (2012) in their study describes that citrus plantations in a state of stress and deterioration are important sources of phytopathogenic fungal inoculums. Tropical fruit trees are hosting a large number of these agents that cause serious damage to the different organs of these plants, reduce their productive life, yield and can cause the death of these plants.

In the case of the dieback of citrus branches, this is not an exclusive disease of this fungus, as the species of *L. theobromae* are presented in conjunction with *N. mangiferum and N. parvum* of the family Botryosphaeriaceae and cause tree decline disorders and peduncle rots of mango crop (Sakalidis *et al*., 2011).

In addition to interaction with other phytopathogenic fungi, *L. theobromae* interacts with HLB, Cabrera *et al*. (2017) suggests that *C. liberibacter* may in some way affect the plant's resistance or immunity mechanisms to certain pathogens such as fungi and algae among others and cause the disease. In this sense, the results infer that the bacteria could cause immunodeficiency in citrus plants. The degree of incidence of dry branches appears to be based on the progression of the disease and the degree of weakening of the plant, mainly those that manifest HLB symptoms.

Positive interaction and accelerated deterioration until death have been demonstrated, which citrus trees suffer when affected at the same time by HLB and *L. theobromae.* It was found, through inoculation trials of these fungi in healthy and sick plants with HLB, that plants with the fungus and HLB were the most affected and exhibited a more severe dieback (Cabrera *et al*., 2012). In this study, *L. theobromae* was collected in trees with the presence of the bacterium *C. liberibacter* asiaticus in psyllid; this may accelerate the final deterioration of the sick tree.

Nariani and Singh (1971) attributed to fungi *C. gloeosporioides; L. theobromae* and *Fusarium* sp., accelerated deterioration and dieback of plants following defoliation caused by HLB. In this sense, these pathogens, most present in citrus fruits, could also be considered to play an important role in initial defoliation. Considering that HLB-affected plants show considerable fruit abscission, with a premature drop of 60% to 70%. This would allow inferring that other fungi, such as those mentioned above and not only the bacterium *C. liberibacter asiaticus*, could be the main responsible for both the fall of fruits and other symptoms in plants with a complex pathogenic situation (Gottwald *et al*., 2012; Cabrera *et al*., 2017).

Conclusions

The results of symptomatologic, morphological, ITS sequence analysis and elongation factor 1α, determined that *L. theobromae* is present and it is associated with the symptoms of mummification and rot of fruits, gummosis, rot and dieback in branches and trees of different citrus varieties, in addition were detected *C. gloesporoides*, *C. eucalipti* and *F. keratoplasticum* in rotten roots of trees with the presence of *L. theobromae* where a possible association with the deterioration of the trees is evident. This document is the first report on *L. theobromae* in the citrícola production center region in Tamaulipas.

Acknowledgments

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