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Characterization of *Lasiodiplodia* species associated with grapevines in Mexico

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Summary. Botryosphaeria dieback is one of the most prevalent grapevine trunk diseases (GTDs), and is caused by fungi in the *Botryosphaeriaceae*. Fungi invade grapevine vascular systems mainly through pruning wounds, and cause cankers and necrotic lesions, which lead to grapevine decline and death. *Lasiodiplodia theobromae* has been reported as a highly virulent pathogen of grapevine, and was previously reported in Mexican vineyards. The taxonomy of *Lasiodiplodia* was recently revised, adding new species, and some were reduced to synonymy. This study aimed to characterize *Lasiodiplodia* producing grapevine dieback symptoms in Sonora and Baja California, Mexico. Using the phylogenetic markers *tef1*-α and ITS regions, *Lasiodiplodia brasiliensis, L. crassispora, L. exigua,* and *L. gilanensis* were identified. *Lasidiplodia exigua* was the most prevalent species. *Lasiodiplodia brasiliensis* and *L. gilanensis* were very virulent to 'Cabernet Sauvignon' plants, while *L. exigua* and *L. gilanensis* were less virulent, and *L. crassispora* did not produce lesions at 2 months post-inoculation. The optimum temperature of the *Lasiodiplodia* spp. was 28°C, but all four species grew up to 37°C, and the isolates of *L. exigua* grew slowly at 40°C. This is the first report of the four of *Lasiodiplodia* species in vineyards of Mexico.

Keywords. Grapevine Trunk Diseases (GTDs), Botryosphaeria dieback, *Botryosphaeriaceae*.

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INTRODUCTION

In Baja California and Sonora, Mexico, grapes are one of the most economically important fruit crops (García-Robles *et al*., 2007; González-Andrade, 2015). Baja California produces close to 90% of Mexico's wines, while Sonora produces approx. 95% of Mexican table grapes (SIAP, 2019).

Botryosphaeria dieback is a degenerative wood disease caused by *Botryosphaeriaceae* fungi, this disease has cosmopolitan distribution and predominates in warm climate regions (Úrbez-Torres, 2011; Gramaje *et al.,* 2018). Fungi in this family are known as opportunistic or latent plant pathogens, as they can remain endophytic for long periods in host tissues without causing symptoms (Slippers *et al.,* 2007).

More than 30 species in the *Botryosphaeriaceae* have been associated with Botryosphaeria grapevine dieback, and these are in *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neoscytalidium, Neofusicoccum*, *Sphaeropsis*, and *Spencermartinsia* (Úrbez-Torres, 2011; Rolshausen *et al.,* 2013; Stempien *et al.,* 2017; Gramaje *et al.,* 2018). The main symptoms caused by these fungi are vascular discolouration and perennial cankers in host plant vascular bundles, by occlusion of xylem and phloem, which leads to the death of branches and eventually of entire plants. This disease is distinguished from Eutypa dieback because it is not known to cause particular foliar symptoms (Úrbez-Torres, 2011; Bertsch *et al.,* 2013; Billones-Baaijens and Savocchia, 2019). Species in the *Botryosphaeriaceae* were commonly found in grapevines 7 to 10 years old and older, mainly in plants where large pruning wounds had been made in vines (Gubler *et al.,* 2005). However, incidence of symptoms caused by this group of fungi has greatly increased in recent years, especially in young vineyards (Gramaje and Armengol, 2011; Gispert *et al*., 2020).

Among the *Botryosphaeriaceae*, the *Lasiodiplodia* has been reported as highly virulent on grapevines (Úrbez-Torres and Gubler, 2009), and has also been identified on more than 500 host species (Punithalingam, 1976). Some of the main morphological characteristics of *Lasiodiplodia* include hyaline and smooth conidiogenous cells, with cylindrical to conical shapes, which produce conidia with subovoid to ellipsoid-ovoid shapes and which are hyaline without septa, or dark-brown with single septae (Phillips *et al.,* 2013). *Lasiodiplodia* are globally distributed, mainly in the tropics and subtropics, and are probably spread when plants are transported between regions due to the lack of restrictions on the movement of propagation material (Cruywagen *et al.,* 2017; Mehl *et al.,* 2017). *Lasiodiplodia theobromae* is the type species of the genus (Alves *et al.,* 2008), and this species is comprised of many cryptic species because of their morphological similarity (Alves *et al.,* 2008; Mehl *et al.,* 2017). As a result, the taxonomy of *Lasiodiplodia* has undergone revisions, and new species have been introduced (Dissanayake *et al*., 2016; Tibpromma *et al*., 2018). Several *Lasiodiplodia* species have been reduced to synonymy, particularly those with morphology similar to *Lasiodiplodia mahajangana*, *L. plurivora* and *L. theobromae*. There are currently 34 accepted *Lasiodiplodia* species (Zhang et al., 2021).

The only *Lasiodiplodia* species causing perennial cankers and dieback that has been reported in Mexican vineyards is *L. theobromae* (Úrbez-Torres *et al.,* 2008). However, given the recent taxonomical revision of *Lasiodiplodia*, we hypothesize that the species diversity within that group is broader than initially reported. Hence, the present study aimed to clarify and update the taxonomy of *Lasiodiplodia* present in vineyards from Baja California and Sonora, Mexico, and to evaluate the pathogenicity of these fungi to grapevine.

MATERIALS AND METHODS

Fungal isolation and morphological characterization of Lasiodiplodia *spp.*

This study encompassed ten vineyards in the main grape-growing areas of the States of Baja California and Sonora, from which 35 samples from grapevines exhibiting Botryosphaeria dieback symptoms were taken from trunks and branches (Figure 1). Small pieces of symptomatic plant tissue were obtained from each diseased plant, and these were immersed in 95% ethanol, quickly flamed, and then placed onto potato dextrose agar (PDA; Difco) supplemented with 25 mg mL^{-1} chloramphenicol in Petri plates. The plates were incubated at 30°C until fungal growth was observed. Smoke-gray fungal colonies with abundant aerial mycelium were sub-cultured onto PDA plates to obtain pure cultures, and were then preserved at 4°C in 20% glycerol.

Pure cultures were grown on PDA and incubated at 30°C for 7 d to determine morphological characteristics of fungal isolates, including their pigmentation and formation of aerial mycelium. Pycnidium production was induced using liquid Minimal Medium 9 (MM9) (10 $g{\cdot}L^{-1}$ glucose, 1.0 $g{\cdot}L^{-1}$ NH_4Cl, 0.5 $g{\cdot}L^{-1}$ NaCl, 2.5 $g{\cdot}L^{-1}$ K_2HPO_4, 2.5 $g{\cdot}L^{-1}$ KH_2PO_4) in flasks supplemented with sterile pine needles (5% w/v). The flasks were incubated at room temperature under an ultraviolet electromagnetic radiation lamp, using a 12 h light and 12 h darkness regime for 15 d. Formed pycnidia were suspended in 0.5% Tween 20 to obtain conidia, which were observed under a light microscope (Nikon Eclipse E200). Images of the conidia were captured with an Infinity 1 Lumenera camera, and analyzed using Infinity Analyze v 6.5.4 and ImageJ software. To compare conidium size across species, one-way ANOVA followed by a *post hoc* Fisher LSD analysis ($\alpha < 0.05$) were carried on these data using STATISTICA 8.0.

Figure 1. Locations of study sites and symptoms of Botryosphaeria dieback in *Vitis vinifera* associated with *Lasiodiplodia* spp. A) Field study sites in Baja California and Sonora regions. B-D) Grapevine plants showing vascular necroses, wedge-shape cankers and wood necroses. E) Pycnidia observed under a stereoscopic microscope found in some grapevine samples.

DNA extraction and PCR amplification from Lasiodiplodia *spp. isolates*

Total genomic DNA of each fungus isolate was extracted from mycelia recovered from cultures (3 d in PDB at 30°C), using the CTAB protocol (Wagner *et al.,* 1987). To characterize *Lasiodiplodia* spp., the ITS region and elongation factor *tef*-1α as phylogenetic markers were used, as recommend in TrunkDiseaseID.org (http://www.grapeipm.org/d.live/) (Lawrence *et al.,* 2017). The oligo-

nucleotide primers EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') were used to amplify part of the translation elongation factor-1a (*tef*-1α) gene (Carbone and Kohn, 1999); and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS region of the nuclear ribosomal DNA, including the 5.8S gene (White *et al.,* 1990). Each PCR reaction contained 2.5 µL of 10× PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatin), 0.5 µL of 20 mM dNTPs, 0.625 µL of 10 µM of each primer, 0.125 µL of Taq DNA polymerase (GoTaq® DNA polymerase, 5 units·µL^{-1}; Promega), and 1 µL of 30 ng·µL^{-1} template DNA, adjusted with purified water to a final volume of 25 µL. Amplification reactions were carried out in a Bio-Rad T-100 thermal cycler set to the following conditions: for *tef*-1α, an initial cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; for ITS region, an initial cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 90 s. Both programmes had a final cycle of 72°C for 10 min. Once observed in electrophoresis gels, PCR reactions were purified using the GeneJet PCR purification kit (Thermo Scientific), and purified products were sequenced by Eton Bioscience Inc.

Phylogenetic analyses

The sequences were analyzed using BioEdit v.7.0.5.3 (Hall, 1999) and a BLASTn analysis was carried out. Sequences with the greatest similarity were downloaded from the GenBank (Table 1) and aligned with ClustalW (pairwise alignment parameters: gap opening 10, gap extension 0.1, and multiple alignment parameters: gap opening 10, gap extension 0.2. Transition weight was set to 0.5, and delay divergent sequences to 25 %) (Thompson *et al.,* 1994). The alignment was adjusted manually where necessary. Alignment of ITS and *tef*-1α were imported in BioEdit v.7.0.5.3 to obtain the concatenated matrix. Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses were performed using MEGA-X (Kumar *et al.,* 2018), based on the concatenated sequence alignment. The best model of nucleotide substitution was selected according to the Akaike Information Criterion (AIC). The T3+G+I model was used for the ML analysis (Tamura, 1992). Parameters for Maximum Likelihood were set to Bootstrap method using 1000 replicates. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. Gaps were treated as missing data. The tree was visualized in MX: Tree Explorer. New sequences were deposited in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Table 1).

Determination of optimum growth temperature of selected Lasidioplodia *isolates*

The optimum growth temperature of identified *Lasiodiplodia* species was determined. Selected isolates of identified species were grown on PDA plates by inoculating each plate with a 3-mm diam. plug of a 2-d-old colony at the edge of the plate. Three replicates of each isolate for each temperature were included, and plates were then incubated at 20, 23, 25, 28, 30, 37, or 40°C. This temperature range was chosen based on previous reports (Úrbez-Torres *et al*., 2006; Paolinelli-Alfonso et al., 2016), and considering the prevalent summer temperatures of the zone from which the isolates were obtained. The colony radius was measured every 24 h for 3 d. The optimum growth temperature was determined as the temperature that produced the maximum mycelial growth rate (mm d^{-1}), which was calculated using the formula:

$$GR = \frac{R_f - R_i}{T_f - T_i}$$

where: GR = Growth rate, R_f = Final colony diam. (mm), R_i = Initial colony diam. (mm), T_f = Final time (d) when colony measured, and T_i = Initial time (day 1).

Production of aerial mycelium in Lasiodiplodia *spp.*

To evaluate aerial mycelium production as a phenotypic characteristic to differentiate among species, 2 d-old cultures of selected isolates were each used to inoculate a 3 mm diam. plug of each culture into a glass tube containing 5 mL of PDA medium. Tubes were incubated at 28°C for 5 d and the elevations of mycelia were measured.

Pathogenicity tests of selected Lasiodiplodia *isolates*

Based on the analyses of the morphological and genetic results, the isolates MXL28BC, MXCS01BC, MX50BC, MXV5BC, MXVSM1b, MXVSM6, MXVSM16a, MXVSM18, and MXVS21b were selected for pathogenicity tests. Grapevine plants of 'Cabernet Sauvignon' were used to evaluate the pathogenicity of these *Lasiodiplodia* isolates. Inoculation of each test plant was carried out through a mechanical wound in woody tissue made with a drill bit (2 mm diam.), and a mycelium plug of a selected isolate was placed inside the hole. An isolate of *L. gilanensis* UCD256Ma (formerly *L. theobromae*) (Úrbez-Torres *et al.,* 2006; Obrador-Sánchez and Hernandez-Martinez, 2020) was used for comparisons.

Table 1. List of GenBank and culture accession numbers of *Lasiodiplodia* spp. used in this study for phylogenetic analyses.

Species	Isolate	Host	Origin	GeneBank accession number	
				ITS	*tef*-1α
Lasiodiplodia brasiliensis	CMM2184	*Carica papaya*	Brazil	KC484801	KC481531
L. brasiliensis	CMM2185	*Carica papaya*	Brazil	KC484800	KC481530
L. brasiliensis	CMM2186	*Carica papaya*	Brazil	KC484812	KC481542
L. brasiliensis	CMM2188	*Carica papaya*	Brazil	KC484807	KC481537
L. brasiliensis	CMM2212	*Carica papaya*	Brazil	KC484806	KC481536
L. brasiliensis	UCD1012BC[a]	*Vitis vinifera*	USA	EU012372	EU012392
L. brasiliensis	UCD916SN[a]	*Vitis vinifera*	USA	EU012366	EU012386
L. brasiliensis	UCD923SN[a]	*Vitis vinifera*	USA	EU012371	EU012391
L. brasiliensis	**MXBCL28**	***Vitis vinifera***	**Mexico**	**MT663281**	**MT711988**
L. brasiliensis	**MXVSCC1**	***Vitis vinifera***	**Mexico**	**MT663282**	**MT711989**
L. brasiliensis	**MXVS15a**	***Vitis vinifera***	**Mexico**	**MT663283**	**MT711990**
L. brasiliensis	**MXVS16a**	***Vitis vinifera***	**Mexico**	**MT663284**	**MT711991**
L. brasiliensis	**MXVS18**	***Vitis vinifera***	**Mexico**	**MT663285**	**MT711992**
L. brasiliensis	**MXVS19a**	***Vitis vinifera***	**Mexico**	**MT663302**	**MT712009**
L. citricola	IRAN1522C	*Citrus* sp.	Iran	GU945354	GU945340
L. citricola	IRAN1521C	*Citrus* sp.	Iran	GU945353	GU945339
L. crassispora	WAC12533	*Santalum album*	Australia	DQ103550	DQ103557
L. crassispora	CBS110492	Unknown	Unknown	EF622086	EF622066
L. crassispora	**MXBCV5**	***Vitis vinifera***	**Mexico**	**MT663286**	**MT711993**
L. crassispora	**MXVS1b**	***Vitis vinifera***	**Mexico**	**MT663287**	**MT711994**
L. euphorbicola	CMM 4616	*Vitis vinifera*	Brazil	MG954348	MG979518
L. euphorbicola	CMM 4597	*Vitis vinifera*	Brazil	MG954347	MG979517
L. exigua	BL104	*Retama raetam*	Tunisia	KJ638317	KJ638336
L. exigua	BL184	*Retama raetam*	Tunisia	KJ638318	KJ638337
L. exigua	BL185	*Retama raetam*	Tunisia	KJ638319	KJ638338
L. exigua	BL186	*Retama raetam*	Tunisia	KJ638320	KJ638339
L. exigua	BL187	*Retama raetam*	Tunisia	KJ638321	KJ638340
L. exigua	PD161	*Pistacia vera*	USA	GU251122	GU251254
L. exigua	**MXBCV4**	***Vitis vinifera***	**Mexico**	**MT663288**	**MT711995**
L. exigua	**MXBCV6**	***Vitis vinifera***	**Mexico**	**MT663289**	**MT711996**
L. exigua	**MXBCV7**	***Vitis vinifera***	**Mexico**	**MT663290**	**MT711997**
L. exigua	**MXVS2Ta**	***Vitis vinifera***	**Mexico**	**MT663291**	**MT711998**
L. exigua	**MXVS5a**	***Vitis vinifera***	**Mexico**	**MT663301**	**MT712008**
L. exigua	**MXVS6a**	***Vitis vinifera***	**Mexico**	**MT663292**	**MT711999**
L. exigua	**MXVS16b**	***Vitis vinifera***	**Mexico**	**MT663293**	**MT712000**
L. exigua	**MXVS20**	***Vitis vinifera***	**Mexico**	**MT663294**	**MT712001**
L. exigua	**MXVS21a**	***Vitis vinifera***	**Mexico**	**MT663295**	**MT712002**
L. exigua	**MXVS21b**	***Vitis vinifera***	**Mexico**	**MT663296**	**MT712003**
L. exigua	**MXVSS2**	***Vitis vinifera***	**Mexico**	**MT663303**	**MT712010**
L. exigua	**MXVSSC1**	***Vitis vinifera***	**Mexico**	**MT663297**	**MT712004**
L. exigua	**MXVSV1**	***Vitis vinifera***	**Mexico**	**MT663298**	**MT712005**
L. gilanensis	IRAN1523C	Unknown	Iran	GU945351	GU945342
L. gilanensis	IRAN1501C	Unknown	Iran	GU945352	GU945341
L. gilanensis	UCD256Ma[a]	*Vitis vinifera*	USA	DQ233594	GU294742
L. gilanensis	**MXBC50**	***Vitis vinifera***	**Mexico**	**MT663299**	**MT712006**
L. gilanensis	**MXBCCS01**	***Vitis vinifera***	**Mexico**	**MT663300**	**MT712007**
L. gonubiensis	CMW 14077	*Syzygium cordatum*	South Africa	AY639595	DQ103566

(Continued)

Table 1. (Continued).

Species	Isolate	Host	Origin	GeneBank accession number	
				ITS	*tef*-1α
L. gonubiensis	CMW 14078	*Syzygium cordatum*	South Africa	AY639594	DQ103565
L. iraniensis	IRAN1502C	*Juglans* sp.	Iran	GU945347	GU945335
L. iraniensis	IRAN921C	*Mangifera indica*	Iran	GU945346	GU945334
L. margaritacea	CBS122519	*Adansonia gibbosa*	Australia	EU144050	EU144065
L. margaritacea	CBS122065	*Adansonia gibbosa*	Australia	EU144051	EU144066
L. mediterránea	BL101	*Vitis vinifera*	Italy	KJ638311	KJ638330
L. mediterranea	BL1	*Quercus ilex*	Italy	KJ638312	KJ638331
L. missouriana	UCD2193MO	*Vitis* sp.	USA	HQ288225	HQ288267
L. missouriana	UCD2199MO	*Vitis* sp.	USA	HQ288226	HQ288268
L. parva	CBS 456.78	Cassava field-soil	Colombia	EF622083	EF622063
L. parva	CBS 494.78	Cassava field-soil	Colombia	EF622084	EF622064
L. pseudotheobromae	CBS116459	*Gmelina arborea*	Costa Rica	EF622077	EF622057
L. pseudotheobromae	CBS447.62	*Citrus aurantium*	Suriname	EF622081	EF622060
L. pyriformis	CBS 121770	*Acacia mellifera*	Nambia	EU101307	EU101352
L. pyriformis	CBS 121771	*Acacia mellifera*	Nambia	EU101308	EU101353
L. subglobosa	CMM4046	*Jatropha curcas*	Brazil	KF234560	KF226723
L. subglobosa	CMM3872	*Jatropha curcas*	Brazil	KF234558	KF226721
L. theobromae	CBS 164.96	Fruit along coral reef	Papua New Guinea	AY640255	AY640258
L. theobromae	CBS111530	Unknown	Unknown	EF622074	EF622054
L. venezuelensis	WAC12539	*Acacia mangium*	Venezuela	DQ103547	DQ103568
L. venezuelensis	WAC12540	*Acacia mangium*	Venezuela	DQ103548	DQ103569
Diplodia mutila	CBS 136015	*Populus alba*	Portugal	KJ361838	KJ361830
Diplodia seriata	CBS 112555	*Vitis vinifera*	Portugal	AY259094	AY573220

Isolates from this study are highlighted in bold font.
[a]Isolates previously identified as *L. theobromae*.

Plugs of sterile PDA were used in control plants, and all drill wounds were covered with Parafilm®. The grapevine plants were left in greenhouse conditions for 2 months. Samples were then taken to measure the length of the necrotic lesion caused by *Lasiodiplodia* isolates, and attempts were made to recover the inoculated fungus onto PDA. The experiments in plants were conducted twice. Statistical analyses were carried out using one-way ANOVA followed by *post hoc* Fisher LSD analyses, with $\alpha < 0.05$ for determination of significant differences in virulence between isolates using STATISTICA 8.0.

RESULTS

Host symptoms, and morphological characteristics of fungal isolates

Botryosphaeria dieback symptoms observed on sampled grapevine plants were mainly dead spurs, cordons, and arms, and shorter shoot internodes. The collected wood exhibited wedge-shaped cankers and necrotic lesions in the vascular bundles.

From necrotic tissue placed in PDA, rapid fungus growth was observed after 2 d. From these colonies, 23 fungal isolates with a similar phenotype were recovered, seven from Baja California and sixteen from Sonora. According to their morphological characteristics, these isolates were identified as *Lasiodiplodia*. Morphological characteristics included initially white colonies with abundant aerial mycelium, which became smoke-gray and produced pycnidia in PDA as they aged (Figure 2). Pycnidium induction allowed observation of hyaline and pigmented conidia in all the isolates (Figure 3). Inside pycnidia, only hyaline aseptate conidia, with granular contents, were observed, while one-septate pigmented conidia with longitudinal striations were mainly found in cirri (Figure 3). The dimensions (length and width) of 30 conidia per isolate were measured, and minimum, maximum, mean, and standard deviations were calculated (Table 2). Statistically significant differences in conidium dimensions were observed among the four analyzed

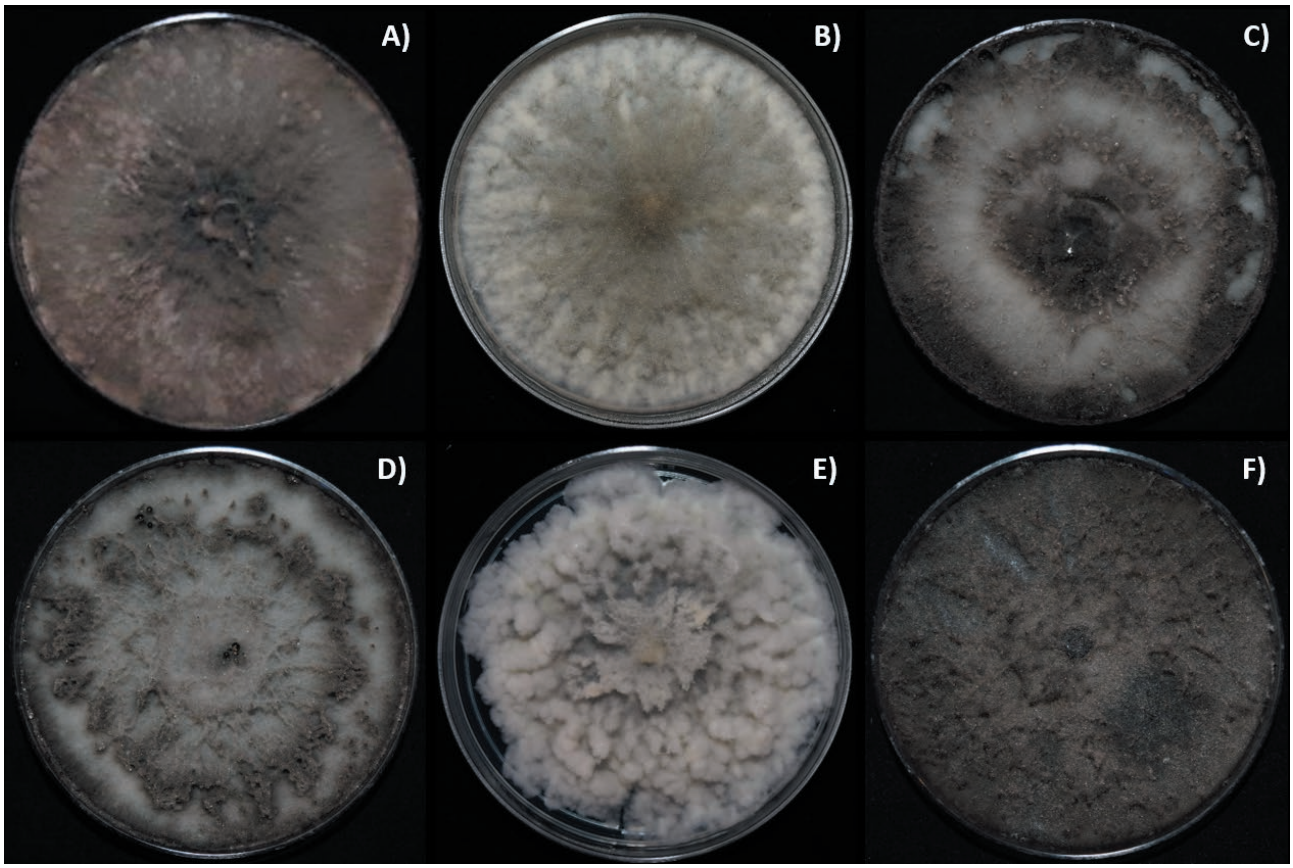

Figure 2. *Lasiodiplodia* spp. isolates grown on PDA at 30°C for 7 d. A) *L. brasiliensis* MXBCL28, B) *L. brasiliensis* MXVS18, C) *L. exigua* MXVS21b, D) *L. exigua* MXVS5a, E) *L. gilanensis* MXBCCS01, F) *L. crassispora* MXVS1b.

Lasiodiplodia species. Isolates characterized as *L. gilanensis*, MX50 (av. = 28.5 × 16.6 mm), and MXCS01 (av. = 30.2 × 15.6 mm), produced larger and wider conidia than *L. brasiliensis*, *L. crassispora*, or *L. exigua*. *Lasiodiplodia brasiliensis* and *L. crassispora* isolates had similar sized conidia (respective mean lengths = 24.0 and 25.6. mm. The *L. exigua* isolates had shorter conidia (av. = 21.2 × 12.2 mm).

Molecular identification of Lasiodiplodia *isolates*

The ITS region and *tef*-1α loci sequences obtained were, respectively, approx. 500 and 263 bp. The combined dataset comprised 832 characters including gaps after alignment (541 corresponded to the ITS gene and 291 corresponded to the *tef*1 gene), and 72 taxa. *Diplodia mutila* (CBS 136015) and *Diplodia seriata* (CBS 112555) were used as the outgroup taxa. Maximum parsimony analysis yielded one most parsimonious tree [(length = 151, CI = 0.711864 (0.677885), RI = 0.922197, RC = 0.714550 (0.656479)] for all sites and parsimony-informative sites. Maximum likelihood analysis using the Tamura 3-parameter model resulted in a tree with the log likelihood value of -2252.61. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 41.41% sites). Estimated base frequencies were: A = 0.21487, C = 0.28764, G = 0.25966, and T = 0.23783; and a discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.5665)].

The phylogenetic analysis of the ITS region and *tef*-1α revealed that the isolates were of four different *Lasiodiplodia* spp. (Figure 4). Most of the isolates were *L. exigua* (syn. *Lasiodiplodia mahajangana*) (isolates MXBCV4, MXBCV7, MXBCV6, MXVSV1, MXVS5a, MXVSSC1, MXVSS2, MXVS2Ta, MXVS6a, MXVS16b, MXVS20, MXVS21a, and MXVS21b). Six isolates were *L. brasiliensis* (isolates MXBCL28, MXVSCC1, MXVS15a, MXVS16a, MXVS18, and MXVS19a); two isolates were *L. gilanensis* (syn. *Lasiodiplodia missouriana*) (isolates MXBCCS01 and MXBC50); and two isolates were

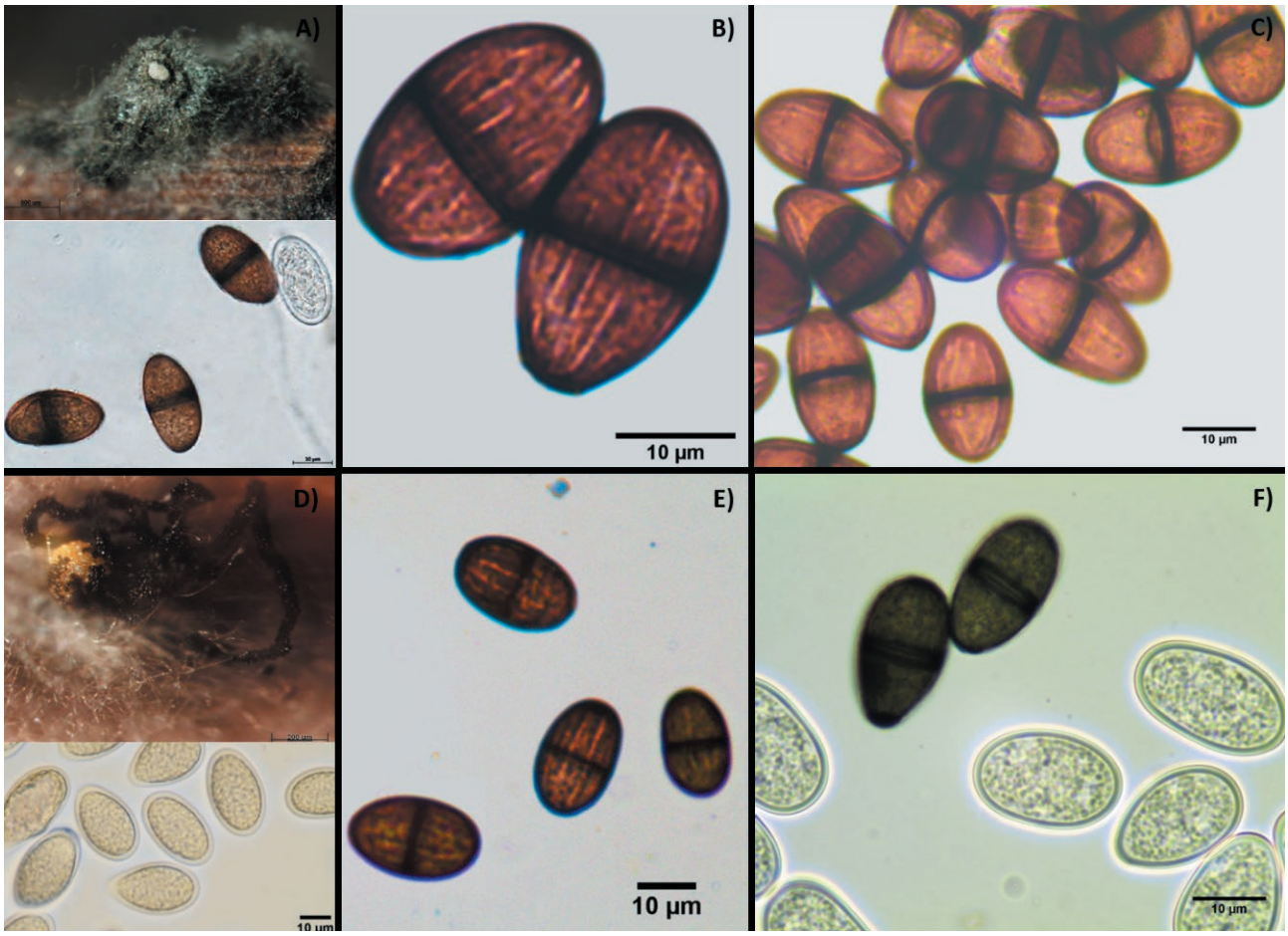

Figure 3. Conidia of *Lasiodiplodia* spp. isolates. A) *L. brasiliensis* MXBCL28, B) *L. brasiliensis* MXVS18, C) *L. exigua* MXVS21b, D) *L. gilanensis* MXBCCS01, E) *L. crassispora* MXVS1b, F) *L. exigua* MXVS5a.

L. crassispora (syn. *Lasiodiplodia pyriformis*) (isolates MXBCV5 and MXVS1b). Previously, only *L. theobromae* had been described in Baja California and Sonora (Úrbez-Torres *et al.,* 2008). Nonetheless, the three *L. theobromae sensu stricto* isolates used as references were clustered separately, and the isolates from the 2008 study of Baja California and Sonora were clustered within the clade of *L. brasiliensis* (Figure 4, Figure S1).

Optimum growth temperature and aerial mycelium production of Lasiodiplodia *spp.*

The *Lasiodiplodia* isolates selected had optimum growth temperatures of 28°C. Most of the isolates grew at greater than 20 mm d^{-1} at 30°C (Table 3). *Lasiodiplodia exigua* grew at up to a mean of 24.6 mm d^{-1} at 37°C, and this was the only species that grew at 40°C. *Lasiodiplodia gilanensis* had the least mycelium growth rate, with a maximum mean growth rate of 19.8 mm d^{-1} at 28°C.

All the *Lasiodiplodia* isolates produced aerial mycelium, but in *L. gilanensis* this was less (mean = 0.8 ± 0.4 mm) than for the other species. The most abundant and longest aerial mycelium was observed in *L. exigua* isolate MXVS5a (16 ± 4.8 mm), followed by *L. brasiliensis* (9.0 ± 2.56 mm). The species *Lasiodiplodia crassispora* produced less abundant aerial mycelium (5.4 ± 2.3 mm) than the other species, and this species melanized more rapidly than the other species (Figure 5).

Evaluation of the pathogenicity of selected isolates of Lasiodiplodia *spp.*

Pathogenicity assays on grapevine plants showed that two-months post inoculation *L. brasiliensis* MXBCL28 and MXVS18, and *L. gilanensis* MXCS01 were the most virulent isolates (Figure 5, C, D, and F), in the woody shoots induced necrotic lesions up to 6 cm in

Table 2. Conidium dimensions of the *Lasiodiplodia* spp. isolates from this study.

Isolate	Origin	Conidium size[a]	Mean ± SD[b]
Lasiodiplodia brasiliensis[b]			
MXBCL28	Valle de Guadalupe, B.C.	(21.9-)24-28.4 × (12.8-)13.6-14.7	24.3±1.4 × 13.7±0.7
MXVSCC1	Hermosillo, Sonora	(20.4-)24.6-27.1 × (11.3-)12.5-14.8	23.7±1.7 × 12.8±0.8
MXVS15a	Hermosillo, Sonora	(20.3-)22.3-24.6 × (11.5-)12.5-14.4	22.8±1 × 12.5±0.7
MXVS16a	Hermosillo, Sonora	(22.1-)26.8-27.6 × (10.6-)11.7-13.1	24.7±1.6 × 11.9±0.5
MXVS18	Hermosillo, Sonora	(21.3-)24.8-29.4 × (11.3-)13.5-15.2	24.7±2 × 13.3±0.8
MXVS19a	Hermosillo, Sonora	(20.1-)23.3-26.4 × (11.4)13.4-16.8	23.2±1.7 × 13.3±1.3
Lasiodiplodia crassispora[c]			
MXBCV5	Valle de Guadalupe, B.C.	(23.0-)24.4-29.9 × (13.3-)16.7-20.2	26.1±2.2 × 17.5±1.7
MXVS1b	Hermosillo, Sonora	(23.7-)24.6-27.1 × (13-)14.7-16.7	25.0±0.9 × 14.7±1.1
Lasiodiplodia exigua[a]			
MXBCV4	Valle de Guadalupe, B.C.	(18.6-)21.1-24.8 × (11-)12-13.9	21.5±1.6 × 12.2±0.8
MXBCV6	Valle de Guadalupe, B.C.	(18.4-)19.2-22.5 × (10.5-)11.4-12.7	20.2±1.1 × 11.2±0.7
MXBCV7	Valle de Guadalupe, B.C.	(19.1-)20.1-21.7 × (12.0-)12.9-14.2	20.3±0.7 × 12.9±0.5
MXVS5a	Hermosillo, Sonora	(21.1-)22.5-25.6 × (11.7-)13.2-16	22.7±1.1 ×13.9±1.0
MXVS6a	Hermosillo, Sonora	(21.0-)23.4-24.6 × (11.9-)12.9-13.9	22.8±1.0 × 13±0.5
MXVS2Ta	Hermosillo, Sonora	(19.7-)21.3-22.8 × (11.3-)12.3-12.9	21.3±0.9 × 12.2±0.5
MXVS16b	Hermosillo, Sonora	(19.6-)23-26.9 × (11.1)13-14.9	22.5±2.0 × 12.9±0.9
MXVS20	Hermosillo, Sonora	(20.2-)21.9-23.7 × (11.2-)12.7-13.9	22.2±0.9 × 12.8±0.7
MXVS21a	Hermosillo, Sonora	(18.4-)19.6-23.8 × (10.1-)12.5-13.9	20.6±1.5 × 12.5±0.9
MXVS21b	Hermosillo, Sonora	(19.3-)20.3-23.2 × (10.7-)11.9-13.4	21±1.0 × 12±0.7
MXVSV1	Hermosillo, Sonora	(19.1-)20.8-23.4 × (10.2)12-12.8	20.6±1.0 × 11.6±0.7
MXVSSC1	Hermosillo, Sonora	(18.2-)19.8-24.1 × (10.5-)11.5-13.5	20.8±1.9 × 11.7±0.6
MXVSS2	Hermosillo, Sonora	(18.3-)20-23 × (11.4-)11.9-14.2	20.5±1.2 × 12.5±0.7
Lasiodiplodia gilanensis[d]			
MXBC50	Valle de Guadalupe, B.C.	(25.6-)28-33.8 × (15-)17.1-18.1	28.5±1.7 × 16.6±0.6
MXNCCS01	Valle de Guadalupe, B.C.	(25.4-)28.9-33 × (13.8-)15.4-18.7	30.2±1.8 × 15.6±1.2

[a] Minimum size, most repetitive value and maximum size for length and width of 30 conidia selected.
[b] SD = standard deviation.
a,b,c,d Means accompanied by the same letters are not significantly different ($\alpha < 0.05$).

length around the inoculation site, and were significantly different from the other inoculated isolates. *L. exigua* MXVS21b caused necrotic lesions in length, similar to *L. gilanensis* UCD256Ma (Figure 5 and 6). *L. crassispora* MXBCV5 and MXVS1b caused lesion below 1 cm in length (Figure 5 and 6) and showed a non-significant difference in comparison to control plants. All isolates were recovered from the inoculate site at three days after incubation at 30°C on PDA plates, which confirmed Koch's postulates. Non-necrotic lesions were observed in the control plants, only the wound effect; instead, green tissue was found, which indicated tissue regeneration of the caused wound.

DISCUSSION

In this study, four *Lasiodiplodia* species causing Botryosphaeria dieback symptoms were identified from Mexican vineyards. *Lasiodiplodia theobromae,* the type species of *Lasiodiplodia*, is one of the most common species associated with Botryosphaeria dieback in grapevine (Úrbez-Torres, 2011; Fontaine *et al.,* 2016), and for several years, it was the only known species within the genus. Later, *L. theobromae* was shown to be a complex of cryptic species (Alves *et al.,* 2008), which led to taxonomic revision of *Lasiodiplodia*. As a result, fungal isolates previously reported as *L. theobromae* have been re-

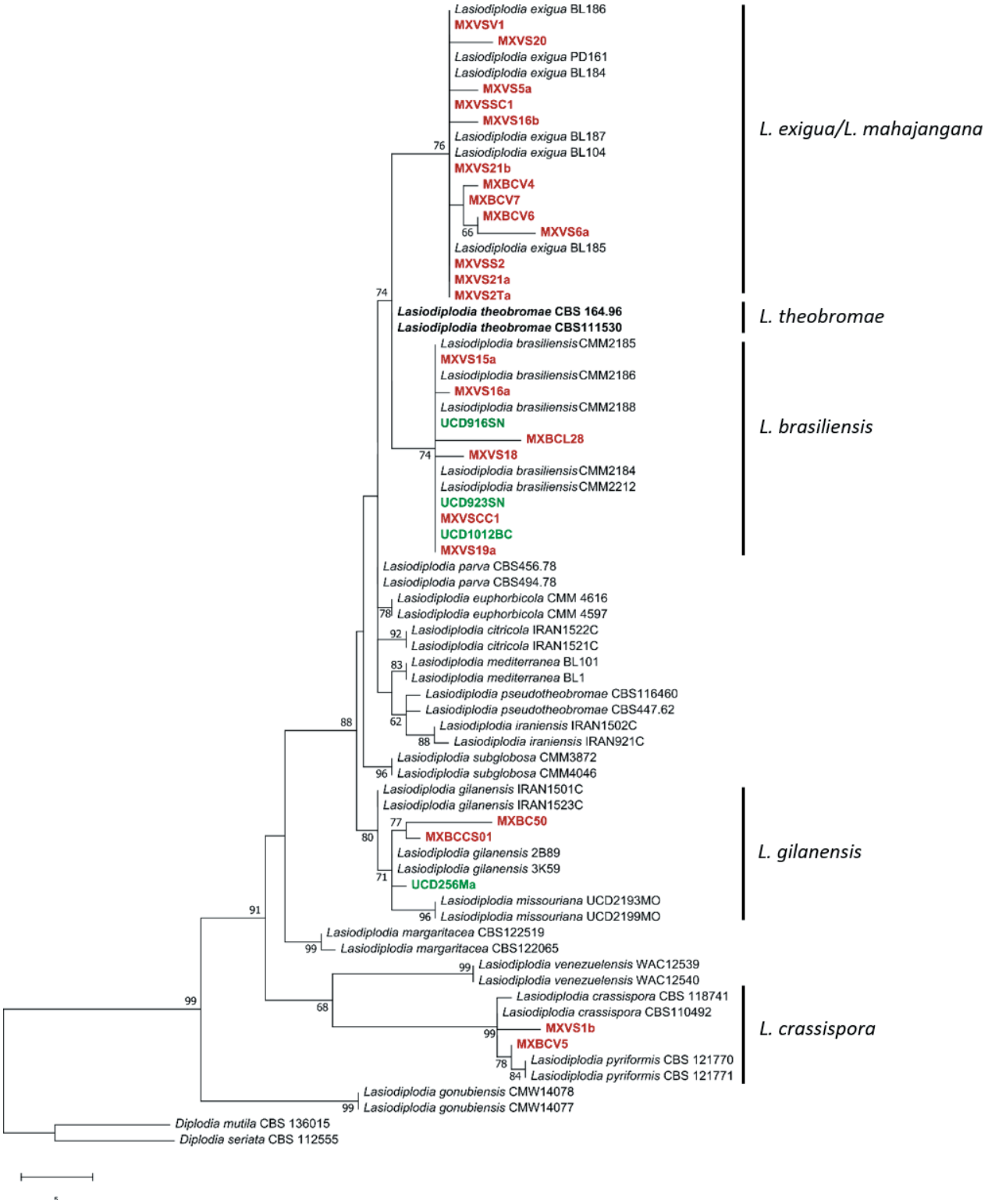

Figure 4. Phylogenetic analysis**.** Most-parsimonious tree (length = 151) obtained from analysis of ITS and *tef1* concatenated datasets. Bootstrap values from 1000 replicates greater than 50 are indicated at the nodes. The tree is rooted with *Diplodia mutila* (CBS 136015) and *Diplodia seriata* (CBS 112555). The isolates from the present study are indicated in bold red font, isolates previously identified as *L. theobromae* are indicated in bold green font, and the *L. theobromae sensu stricto* isolates are indicated in bold black font.

Table 3. Mean colony diameters at different temperatures for Mexican *Lasiodiplodia* isolates grown in PDA cultures.

Isolate	Temperature						
	20°C	23°C	25°C	28°C	30°C	37°C	40°C
Lasiodiplodia brasiliensis							
MXBCL28	19.1 ± 0.7	21.6 ± 2.4	20 ± 1.3	28.1 ± 0.2	20.6 ± 3.6	6.8 ± 0.57	0
MXVS18	15 ± 0	20 ± 0.8	23.1 ± 1.0	27.3 ± 1.7	22.0 ± 1.0	20.0 ± 1.8	0
Lasiodiplodia crassispora							
MXBCV5	12.6 ± 0.2	17.3 ± 0.2	19.1 ± 1.5	23.1 ± 0.2	20.1 ± 1	3.8 ± 0.7	0
Lasiodiplodia exigua							
MXVS5a	15 ± 1.3	21.3 ± 2	19.8 ± 0.7	28.1 ± 1.5	20.5 ± 2.2	21.6 ± 1	0.5 ± 0
MXVS21b	17.16 ± 0.2	19.6 ± 0.5	20.6 ± 1.5	23 ± 2.1	22.3 ± 0.7	24.6 ± 0.7	0.5 ± 0
Lasiodiplodia gilanensis							
MXBC50	11 ± 2.4	8.1 ± 0.7	5.6 ± 1.6	6.1 ± 1.2	11.3 ± 7.2	5.8 ± 1.6	0
MXBCCS01	16.3 ± 0.35	17.1 ± 2.46	17.5 ± 3.6	19.8 ± 5.0	18.1 ± 1.89	9.5 ± 0.5	0

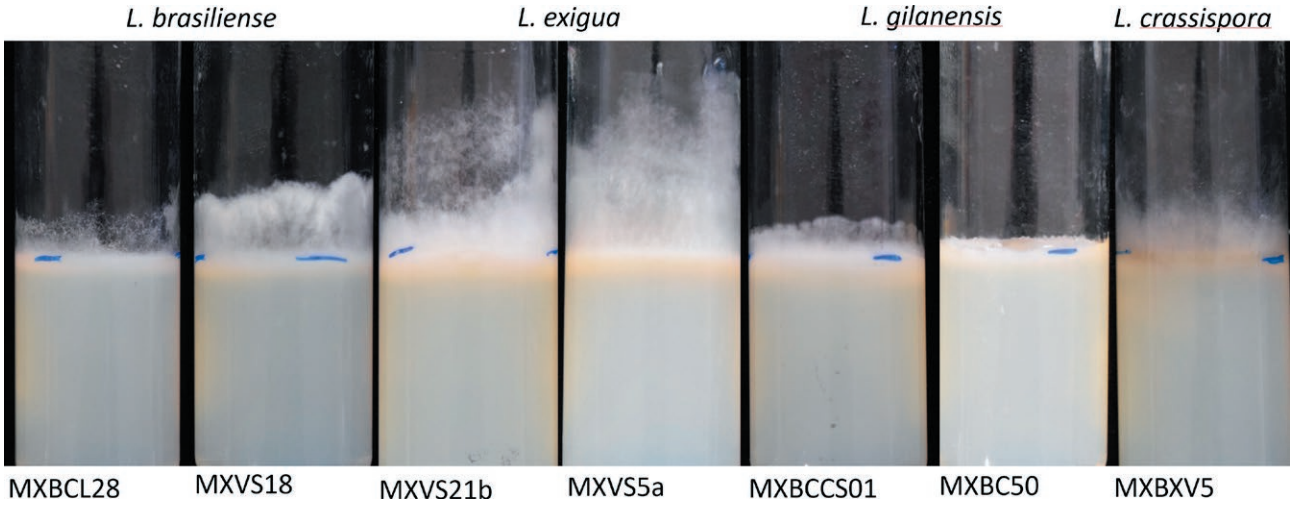

Figure 5. Aerial mycelium growth of different *Lasiodiplodia* spp. isolated from grapevines in Mexico. The isolates were grown in glass tubes containing PDA medium for 5 d at 28°C.

classified as new species (Dissanayake *et al.,* 2016; Cruywagen *et al.,* 2017; Mehl *et al.,* 2017; Tibpromma *et al.,* 2018). Some species were subsequently reduced to synonymy (Zhang *et al*., 2021). The fungal rDNA internal transcribed spacer region (ITS) is the primary barcode used to identify fungal species, but in *Lasiodiplodia* spp., this region has low interspecific variation. The translation elongation factor 1-α (*tef*-1α) is more variable than ITS, and has been recommended as a secondary barcode region to estimate species identity for *Botryosphaeriaceae* (Lawrence *et al.,* 2017), and this locus allowed us to segregate *L. brasiliensis* from *L. theobromae.*

Pathogens associated with wood dieback diseases are generally found in vineyards that are at least 10 years old (Gubler *et al.,* 2005), but we have isolated these fungi in younger vineyards in Mexico. *Lasiodiplodia exigua*, *L. brasiliensis,* and *L. crassispora* were recovered from the two Mexican viticulture areas (Baja California and Sonora), whereas *L. gilanensis* was only found in Baja California. *Lasiodiplodia exigua* was the most prevalent species. Previously, only *L. theobromae* was reported in Mexico in grapevine (Úrbez-Torres *et al.,* 2008), but our phylogenetic analyses indicated that those isolates clustered with *L. brasiliensis*, suggesting that *L. brasiliensis* has been in Mexico for a long time.

Production of reddish-pink pigment by the isolates of *L. brasiliensis* and *L. gilanensis* was observed. This characteristic has been reported in other species

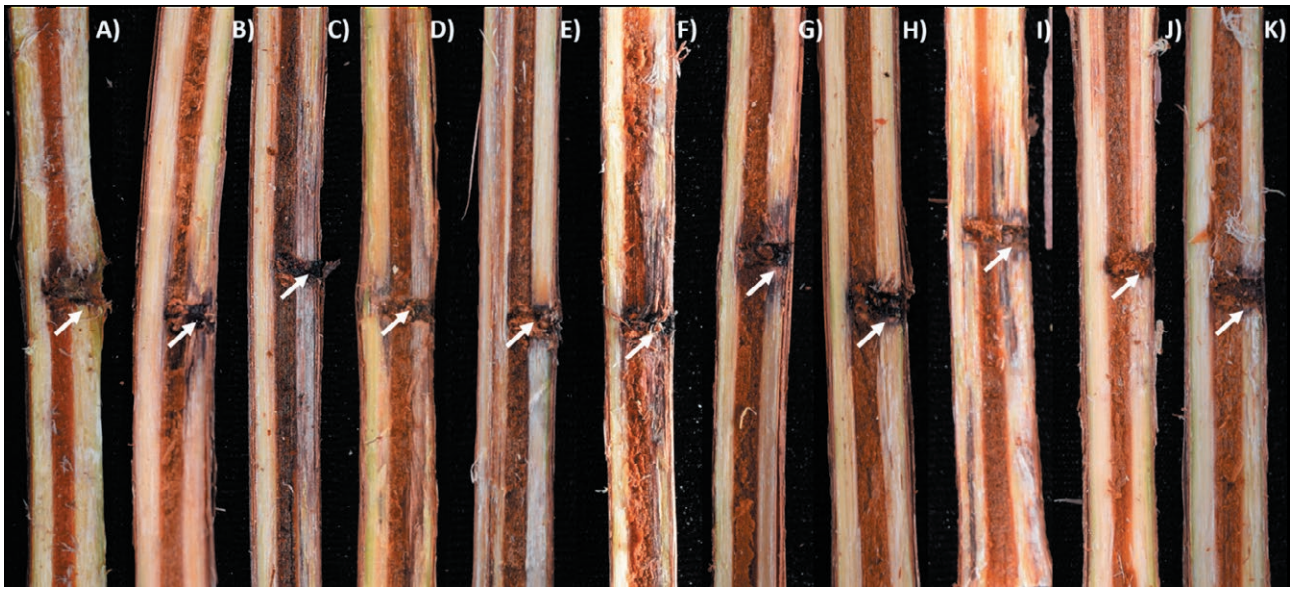

Figure 6. Grapevine woody shoots showing dark-brown lesions at 2-months post inoculation with *Lasiodiplodia* isolates**.** A) Control plant (PDA), B) *L. gilanensis* UCD256Ma, C) *L. brasiliensis* MXBCL28, D) *L. brasiliensis* MXVS18, E) *L. brasiliensis* MXVS16a F) *L. gilanensis* MXBCCS01, G) *L. gilanensis* MXBC50, H) *L. exigua* MXVS6a, I) *L. exigua* MXVS21b J) *L. crassispora* MXVS1b, and K) *L. crassispora* MXBCV5. White arrows indicate the point of inoculation.

including *L. pseudotheobromae*, *L. parva*, and *L. theobromae* (Alves *et al.,* 2008; Abdollahzadeh *et al.,* 2010). Although *L. missouriana* has been reduced to synonymy with *L. gilanensis* (Zhang *et al*., 2021), conidium dimensions of the Mexican isolates of *L. gilanensis* (isolates MX50 and MXSC01) and one from California, USA (isolate UCD256Ma) were larger (av. = 29.6 x 15.6 µm) than those for *L. missouriana* (av. = 18.5 x 9.8 µm) from Missouri, USA (Phillips *et al.,* 2013). On the other hand, *L. theobromae* (av. = ± SD = 26.2 ± 2.6 × 14.2 ± 1.2 µm) (Phillips *et al.,* 2013) had conidium dimensions similar to those for *L. brasiliensis* (av. ± SD = 26.01 ±1.36 x 14.64 ±1.16 µm) (Netto *et al.,* 2014), making these species difficult to distinguish based solely on morphological traits. In the present study, aerial mycelium height was another morphological characteristic evaluated, and the observed differences suggested that this trait could help with the differentiation of *Lasiodiplodia* species.

The pathogenicity tests showed that the *L. brasiliensis* isolates MXBCL28 and MXVS18, and *L. gilanensis* isolate MXCS01 were the most virulent to grapevine plants 'Cabernet Sauvignon'. These isolates caused necrotic lesions to the host vascular systems at 2 months post-inoculation. *Lasiodiplodia brasiliensis* was also reported for the first time on grapevine in Brazil, and this was the most virulent species on green shoots, followed by *L. theobromae* (Correia *et al.,* 2016). *Lasidiplodia gilanensis* was described for the first time from Iran, from an unknown tree showing branch dieback, cankers, and fruit rot (Abdollahzadeh *et al.,* 2010). Considering isolate UCD256Ma, formerly identified as *L. theo-*

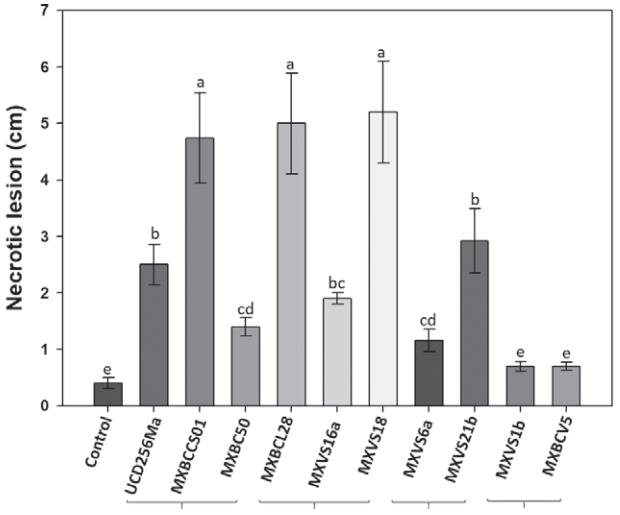

Figure 7. Mean lesion length caused by *Lasiodiplodia* isolates in grapevine plants 2-months post inoculation under greenhouse conditions. Bars indicate the standard deviation of each treatment. Significance letters were grouped based on Fisher's analysis (P<0.05); Bars indicate standard deviations. Means accompanied by the same letters are not significantly different (α < 0.05)

bromae (Úrbez-Torres *et al.,* 2006) belongs to *L. gilanensis*, the present study data supports taxonomic reassignment. *Lasiodiplodia missouriana* has been reduced to synonymy with *L. gilanensis* (Zhang *et al*., 2021). *Lasiodiplodia missouriana* was isolated from grapevines in 2011, and was one of the most aggressive species to grapevine (Úrbez-Torres *et al*., 2012), confirming results from the present study.

Lasiodiplodia exigua isolates MXVS6a and MXVS21b were of different virulence than *L. brasiliensis* and *L. gilanensis* isolates. *Lasiodiplodia exigua* was first isolated from broom bush (*Retama raetam*) in Tunisia (Linaldeddu *et al.,* 2015), and was reported to cause brown discolouration and streaks in grapevine wood (Akgül *et al.,* 2019). The *L. crassispora* isolates MXBCV5 and MXVS1b from the present study were the least virulent, which is similar to the results from previous studies (Correia *et al.,* 2016).

Grapevine plants are susceptible to several different wood pathogens during the pruning period, so it is important to consider factors such as climatic conditions and life cycles of GTDs pathogens (Rolshausen *et al.,* 2010; Agustí-Brisach *et al.,* 2015; Gramaje *et al.,* 2018; Waite *et al.,* 2018). Spread of fungus pathogens involved in Botryosphaeria dieback within vineyards is linked with rainfall and associated wind dispersal of inocula (Mehl *et al.,* 2017). *Lasiodiplodia* has been reported to be prevalent in regions with high temperatures and low precipitation (Úrbez-Torres, 2011; Gispert *et al*., 2020). The isolates examined in the present study had optimum growth temperatures of 28°C, but all grew at 37°C, and the isolates of *L. exigua* grew at 40°C. This could be an adaptation of *L. exigua* to extreme hot weather conditions. This species is the most commonly found in the Baja California and Sonora grape-growing regions. Even when the other isolates did not grow at 40 °C, they recovered their average growth once they were transferred to room temperature, except for *L. gilanensis* isolate MXBC50. These fungi probably entered a dormant state that recovers when temperatures decrease. This could explain why *L. gilanensis* is the most common species in Baja California and Sonora, where prevalent climate conditions are annual precipitation of 280 mm and temperatures greater than 40°C during the summer, conditions which favour growth of *L. gilanensis*. More studies are required of these fungi under extreme growing conditions. However, the present study has contributed to recognizing GTD pathogen species present in Mexico's most economically important viticulture region, representing the first step for epidemiological studies to assist controlling the spread of these pathogens.

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