Research Papers

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-a and ITS regions, Lasiodiplodia brasiliensis, L. crassispora, L. exigua, and L. gilanensis were identified. Lasiodiplodia 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.

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*, *Dothioremella*, *Lasiodiplodia*, *Neoscytalidium*, *Neofusicoccum*, *Sphaeropsis*, and *Spencermartinsia* (Úrbez-Torres, 2011; Rolshausen et al., 2013; Stempień et al., 2017; Gramaje et al., 2018). The main symptoms caused by these fungi are vascular discoloration 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 L⁻¹ glucose, 1.0 g L⁻¹ NH₄Cl, 0.5 g L⁻¹ NaCl, 2.5 g L⁻¹ K₂HPO₄, 2.5 g L⁻¹ KH₂PO₄) 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 (α < 0.05) were carried on these data using STATISTICA 8.0.
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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).
nucleotide primers EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-966R (5'-TACTTGAAGGACCCTTACC-3') were used to amplify part of the translation elongation factor-1a (tef-1α) gene (Carbone and Kohn, 1999); and ITS1 (5'-TCGGTAGGTGAACCTGCGG-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 MgCl2; 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 Lasiodiplodia 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 (Urbez-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:

\[ \text{GR} = \frac{R_f - R_i}{T_f - T_i} \]

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*) (Urbez-Torres et al., 2006; Obrador-Sánchez and Hernandez-Martinez, 2020) was used for comparisons.
| Species          | Isolate | Host     | Origin | GeneBank accession number |
|------------------|---------|----------|--------|----------------------------|
| Lasiodiplodia    | 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  | UCD1012BCa | Vitis vinifera | USA   | EU012372 EU012392         |
| L. brasiliensis  | UCD916SNa | Vitis vinifera | USA   | EU012366 EU012386         |
| L. brasiliensis  | UCD923SNa | Vitis vinifera | USA   | EU012371 EU012391         |
| L. brasiliensis  | MXBC28  | Vitis vinifera | Mexico | MT663281 MT719988         |
| L. brasiliensis  | MXVSCC1 | Vitis vinifera | Mexico | MT663282 MT719989         |
| L. brasiliensis  | MXVS15a | Vitis vinifera | Mexico | MT663283 MT719990         |
| L. brasiliensis  | MXVS16a | Vitis vinifera | Mexico | MT663284 MT719991         |
| L. brasiliensis  | MXVS18  | Vitis vinifera | Mexico | MT663285 MT719992         |
| 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 | EF622066 EF622066        |
| L. crassispora   | MXBCV5  | Vitis vinifera | Mexico | MT663286 MT719993         |
| L. crassispora   | MXVS1b  | Vitis vinifera | Mexico | MT663287 MT719994         |
| 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        | MXVS6b  | 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        | MXVS22  | Vitis vinifera | Mexico | MT663303 MT712010         |
| L. exigua        | MXVS2C1 | Vitis vinifera | Mexico | MT663297 MT712004         |
| L. exigua        | MXSVV1  | Vitis vinifera | Mexico | MT663298 MT712005         |
| L. gilanensis    | IRAN1523C | Unknown | Iran   | GU945351 GU945342         |
| L. gilanensis    | IRAN1501C | Unknown | Iran   | GU945352 GU945341         |
| L. gilanensis    | UCD256Ma | Vitis vinifera | USA   | DQ233594 GU294742         |
| L. gilanensis    | MXBC50  | Vitis vinifera | Mexico | MT663299 MT712006         |
| L. gilanensis    | MXBCS01 | Vitis vinifera | Mexico | MT663300 MT712007         |
| L. goniobifrons  | CMW 14077 | Syzygium cordatum | South Africa | AY639595 DQ103566       |

(Continued)
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 \textit{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 \textit{post hoc} Fisher LSD analyses, with $\alpha < 0.05$ for determination of significant differences in virulence between isolates using \textsc{Statistica} 8.0.

\textbf{RESULTS}

\textbf{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 \textit{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 isolates.

\begin{table}[h]
\centering
\tiny
\begin{tabular}{lllll}
\hline
Species & Isolate & Host & Origin & GeneBank accession number \\
\hline
\textit{L. gonubiensis} & CMW 14078 & \textit{Syzygium cordatum} & South Africa & Y63594, DQ103565 \\
\textit{L. iranensis} & IRAN1502C & \textit{Juglans} sp. & Iran & G945347, G945335 \\
\textit{L. iranensis} & IRAN921C & \textit{Mangifera indica} & Iran & G945346, G945334 \\
\textit{L. margaritacea} & CBS122519 & \textit{Adansonia gibbosa} & Australia & EU144050, EU144065 \\
\textit{L. margaritacea} & CBS122065 & \textit{Adansonia gibbosa} & Australia & EU144051, EU144066 \\
\textit{L. mediterranea} & BL101 & \textit{Vitis vinifera} & Italy & KJ638311, KJ638330 \\
\textit{L. mediterranea} & BL1 & \textit{Quercus ilex} & Italy & KJ638312, KJ638331 \\
\textit{L. missouriana} & UCD2193MO & \textit{Vitis} sp. & USA & HQ288225, HQ288267 \\
\textit{L. missouriana} & UCD2199MO & \textit{Vitis} sp. & USA & HQ288226, HQ288268 \\
\textit{L. parva} & CBS 456.78 & Cassava field-soil & Colombia & EF622083, EF622063 \\
\textit{L. parva} & CBS 494.78 & Cassava field-soil & Colombia & EF622084, EF622064 \\
\textit{L. pseudotheobromae} & CBS116459 & \textit{Gmelina arborea} & Costa Rica & EF622077, EF622057 \\
\textit{L. pseudotheobromae} & CBS447.62 & \textit{Citrus aurantium} & Suriname & EF622081, EF622060 \\
\textit{L. pyriformis} & CBS 121770 & \textit{Acacia mellifera} & Nambia & EU101307, EU101352 \\
\textit{L. pyriformis} & CBS 121771 & \textit{Acacia mellifera} & Nambia & EU101308, EU101353 \\
\textit{L. subglobosa} & CMM4046 & \textit{Jatropha curcas} & Brazil & CF234560, CF226723 \\
\textit{L. subglobosa} & CMM3872 & \textit{Jatropha curcas} & Brazil & CF234558, CF226721 \\
\textit{L. theobromae} & CBS 164.96 & Fruit along coral reef & Papua New Guinea & Y640255, YA40258 \\
\textit{L. theobromae} & CBS111530 & Unknown & Unknown & EF622074, EF622054 \\
\textit{L. venezuelensis} & WAC12539 & \textit{Acacia mangium} & Venezuela & DQ103547, DQ103568 \\
\textit{L. venezuelensis} & WAC12540 & \textit{Acacia mangium} & Venezuela & DQ103548, DQ103569 \\
\textit{Diplodia mutila} & CBS 136015 & \textit{Populus alba} & Portugal & KJ631838, KJ361830 \\
\textit{Diplodia seriata} & CBS 112555 & \textit{Vitis vinifera} & Portugal & Y259094, Y573220 \\
\hline
\end{tabular}
\caption{(Continued).}
\end{table}

Isolates from this study are highlighted in bold font.

*Isolates previously identified as \textit{L. theobromae}.
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. crassipora*, or *L. exigua*. *Lasiodiplodia brasiliensis* and *L. crassipora* 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 tef1 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, MXVS1, MXVS5a, MXVSSC1, MXVSS2, MXVSS2a, MXVS6a, MXVS16b, MXVS20, MXVS21a, and MXVS21b). Six isolates were *L. brasiliensis* (isolates MXBCL28, MXVSCC1, MXVSI5a, MXVS16a, MXVS18, and MXVS19a); two isolates were *L. gilanensis* (syn. *Lasiodiplodia missouriana*) (isolates MXBCCS01 and MXBC50); and two isolates were
L. crassipora (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 crassipora 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 MXBCCL28 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...
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length around the inoculation site, and were significantly different from the other inoculated isolates. L. exigua MXVS1b 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-

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

| Isolate          | Origin                | Conidium sizea | Mean ± SDb  |
|------------------|-----------------------|----------------|-------------|
| **Lasiodiplodia brasiliensis**<sup>b</sup> |                       |                |             |
| MXBCL28          | Valle de Guadalupe, B.C. | (21.9-)24.8-28.4 × (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**<sup>c</sup>

| Isolate | Origin | Conidium sizea | Mean ± SDb |
|---------|--------|----------------|------------|
| 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**<sup>a</sup>

| Isolate | Origin | Conidium sizea | Mean ± SDb |
|---------|--------|----------------|------------|
| 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±0.5 |
| MXVS7a  | 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**<sup>d</sup>

| Isolate | Origin | Conidium sizea | Mean ± SDb |
|---------|--------|----------------|------------|
| 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 |

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a Minimum size, most repetitive value and maximum size for length and width of 30 conidia selected.

b SD = standard deviation.

c,d Means accompanied by the same letters are not significantly different (α < 0.05).
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.
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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. crassipora 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

### 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 crassipora |      |      |      |      |      |      |      |
| 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 |      |      |      |      |      |      |      |
| MBC50            | 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.
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 x 14.2 ± 1.2 µm) (Phillips et al., 2013) had conidium dimensions similar to those for *L. brasiilesis* (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. brasiilesis* 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 brasiilesis* 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). *Lasiodiplodia 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-
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Lasiodiplodia missouriana has been reduced to synonymy with L. gilanesis (Zhang et al., 2021). Lasiodiplodia missouriana was isolated from grapevines in 2011, and was one of the most aggressive species to grapevine (Urbez-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. gilanesis isolates. Lasiodiplodia exigua was first isolated from broom bush (Retama raetam) in Tunisia (Linaldeddu et al., 2015), and was reported to cause brown discoloration and streaks in grapevine wood (Akgül et al., 2019). The L. crassisspora 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 GTD pathogens (Rolshausen et al., 2010; Agusti-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 (Urbez-Torres, 2011; Gisbert 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. gilanesis isolate MXBC50. These fungi probably entered a dormant state that recovers when temperatures decrease. This could explain why L. gilanesis 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. gilanesis. 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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