Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

Doctorado en Ciencias
Ciencias de la Vida
con orientación en Microbiología Celular y Molecular

Characterization, evaluation of the colonization process and search for biological control strategies of fungi of the Botryosphaeriaceae family associated with grapevines in Mexico

Tesis
para cubrir parcialmente los requisitos necesarios para obtener el grado de Doctor en Ciencias

Presenta:

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Soy de las que piensan que la ciencia tiene una gran belleza. Un científico en su laboratorio no es sólo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas.

-Marie Curie

Voy pisando fósiles, no me dejarán caer, un mundo microscópico me sostiene los pies

Fuerza Natural

-Gustavo Cerati

Resumen de la tesis que presenta **Edelweiss Airam Rangel Montoya** como requisito parcial para la obtención del grado de Doctor en Ciencias en Ciencias de la Vida con orientación en Microbiología Celular y Molecular.

Caracterización, evaluación del proceso de colonización y búsqueda de estrategias de control biológico de hongos de la familia Botryosphaeriaceae asociados a la vid en México

Resumen aprobado por:

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Dra. Rufina Hernández Martínez
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Las enfermedades de la madera de la vid (GTDs) afectan la productividad y provocan importantes pérdidas económicas. La muerte regresiva por Botryosphaeria es una de las GTDs más destructivas causada por hongos de la familia Botryosphaeriaceae. En México, el cultivo de la vid es de gran importancia, siendo los estados de Sonora y Baja California los principales productores de uva de mesa y vino, respectivamente. Las Botryosphaeriaceae spp. penetran las plantas principalmente por heridas de poda y provocan lesiones necróticas y cancros en forma de cuña en el sistema vascular. Sin embargo, los mecanismos por los cuales estos hongos colonizan la planta han sido poco estudiados y las prácticas para el manejo de las GTDs no son eficientes. Este estudio tuvo como objetivo contribuir al conocimiento epidemiológico de Botryosphaeriaceae spp. en regiones vitivinícolas de México y estudiar alternativas para su control. Primero, se tomaron muestras de plantas de vid con síntomas de muerte regresiva por Botryosphaeria en los estados de Baja California, Chihuahua, Coahuila y Sonora. En total se obtuvieron 47 aislados fúngicos del tejido sintomático y utilizando los marcadores filogenéticos *tef1-α* e ITS, se identificaron nueve especies de Botryosphaeriaceae: *Lasiodiplodia brasiliensis, Lasiodiplodia crassispora, Lasiodiplodia exigua, Lasiodiplodia gilanensis*, *Neofusicoccum parvum*, *Neofusicoccum* australe*, Neofusicoccum vitifusiforme*, *Botryosphaeria dothidea*, y *Diplodia seriata*. *Lasiodiplodia exigua* fue la especie más frecuente aislada en Baja California y Sonora, mientras que *L. brasiliensis*, *L. gilanensis* y *N. parvum* fueron las más virulentas en los ensayos de patogenicidad. Para estudiar el proceso de colonización de *L. brasiliensis*, se inocularon plantas de cv. Cabernet Sauvignon con *L. brasiliensis* MXBCL28 mediante una herida mecánica, y se mantuvieron en invernadero por 2 meses. Se realizaron cortes transversales y longitudinales de 70 µm de espesor utilizando un micrótomo manual de muestras del sitio de inoculación fijadas en FAA. Mediante microscopía se observó que las plantas infectadas carecían de almidón en el parénquima radial y de celulosa, hemicelulosa y lignina en el área de la lesión. Se observó la inducción de compuestos fenólicos y suberina en el cambium vascular, el corcho del cambium, los haces vasculares y la médula como respuesta a la infección. El hongo colonizó el cambium vascular, los haces vasculares, las oclusiones y la médula. Finalmente, se caracterizaron y evaluaron los aislados de *Bacillus amyloliquefaciens* BsMXA3 y BsMXC11 para el biocontrol de *Lasiodiplodia* spp. Estos aislados presentaron características de promoción de crecimiento vegetal, como la producción de sideróforos y ácido indolacético, inhibieron el crecimiento de *Lasiodiplodia* spp. por la producción de compuestos volátiles y no volátiles y afectaron la germinación de conidios de *L. gilanensis*. Los ensayos de biocontrol *in planta* mostraron que *Lasiodiplodia* causó lesiones necróticas menores en las plantas inoculadas con los aislados de *B. amyloliquefaciens* y las bacterias promovieron el crecimiento de la planta. Este estudio amplía el conocimiento de los hongos causantes de muerte regresiva por Botryosphaeria presentes en México, la forma en que estos hongos colonizan la planta y propone el uso de dos agentes de biocontrol para el manejo de las especies de *Lasiodiplodia*.

Palabras clave: Muerte regresiva por Botryosphaeria, *Lasiodiplodia*, sistema vascular vegetal, hongos de la madera de vid, agentes de control biológico, promoción del crecimiento vegetal.

Abstract of the thesis presented by **Edelweiss Airam Rangel Montoya** as a partial requirement to obtain the Doctor of Science degree in Science Life with orientation in Cellular and Molecular Microbiology

Characterization, evaluation of the colonization process and search for biological control strategies of fungi of the Botryosphaeriaceae family associated with grapevines in Mexico

Abstract approved by:

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Grapevine trunk diseases (GTDs) affect productivity and cause significant economic losses. Botryosphaeria dieback is one of the most destructive GTDs caused by fungi from the Botryosphaeriaceae family. In Mexico, grapevine cultivation is of great importance, being the states of Sonora and Baja California the leading producers of table grapes and wine. Botryosphaeriaceae spp. penetrate the plants mainly through pruning wounds and cause necrotic lesions and wedge-shaped cankers in the vascular system. The mechanisms by which these fungi colonize the plant are poorly studied, and the practices for the management of GTDs are not efficient. This work aimed to contribute to the epidemiological knowledge of Botryosphaeriaceae species in Mexico, their process of colonization in grapevine, and to look for biocontrol alternatives. First, samples of grapevine plants exhibiting Botryosphaeria dieback symptoms were obtained from vineyards of Baja California, Chihuahua, Coahuila, and Sonora. From a total of 47 fungal isolates, using the phylogenetic markers tef1-α and ITS, nine Botryosphaeriaceae species were identified, *Lasiodiplodia brasiliensis, L. crassispora, L. exigua, L. gilanensis*, *Neofusicoccum parvum*, *N.* australe*, N. vitifusiforme*, *Botryosphaeria dothidea*, and *Diplodia seriata. Lasiodiplodia exigua* was the most frequently isolated species in Baja California and Sonora; meanwhile, *L. brasiliensis*, *L. gilanensis*, and *N. parvum* were the most virulent. To study the colonization process of Botryosphaeriaceae, *L. brasiliensis* MXBCL28 was inoculated in plants of cv. Cabernet Sauvignon. After two months, samples close to the inoculation point were obtained and fixed. Transverse and longitudinal sections 70 µm thick were stained and analyzed using microscopy. In infected grapevines, depletion of starch in ray parenchyma, lack of cellulose, hemicellulose, and lignin in the lesion site, and induction of phenolic compounds and suberin in the vascular cambium, cambium cork, vascular bundles, and pith were observed. The fungus was found colonizing the vascular cambium, vascular bundles, vessel occlusions, and the pith. Finally, bacterial isolates of *Bacillus amyloliquefaciens* BsMXA3 and BsMXC11 were evaluated for the biocontrol of *Lasiodiplodia* spp. Both strains produced siderophores, indoleacetic acid, and volatile and non-volatile compounds that inhibited the growth of *Lasiodiplodia* spp. They also affected the germination of conidia of *L. gilanensis*. Biocontrol assays *in planta* showed that *Lasiodiplodia* caused less necrotic lesions in plants inoculated with *B. amyloliquefaciens* isolates and bacteria promoted plant growth. This study expands the knowledge of the status of Botryosphaeriaceae in Mexico, examines how these fungi colonize grapevine plants, and propose the use of *B. amyloliquefaciens* as biocontrol agent to manage *Lasiodiplodia* species.

Key words: Botryosphaeria dieback, *Lasiodiplodia*, plant vascular system, fungi colonization, biocontrol agents, plant growth promotion.

Dedicatory

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Chapter 1. Introduction

Fungi species belonging to Botryosphaeriaceae family are considered pathogen agents of plants, which could have an endophytic life phase (Slippers and Wingfield 2007). These fungi are the main causal agents of degenerative diseases known as trunk diseases, which affected a wide range of global important woody plants, among them the grapevine (Damm et al., 2007; Bertsch et al., 2013; Phillips et al., 2013; Fontaine et al., 2016). The main symptoms caused by these fungi in grapevine are foliar spots, fruit rot, dieback, shoot necrosis, vascular discoloration, and perennial cankers (Úrbez-Torres, 2011).

The genus *Lasiodiplodia* are the most aggressive pathogens affecting a wide range of plants, and *L. theobromae* has been reported in more than 500 host species (Punithalingam, 1976). Currently, there are no efficient treatments for trunk diseases, mainly due to the diversity of fungal species associated. Control techniques are based on preventive strategies through cultural practices such as: using disease-free propagation material, sterilization of pruning tools, applying protective paint on pruning cuts, and removing infected plants from the vineyards to avoid damage (Mondello et al., 2018).

In Mexico, in the states of Baja California and Sonora, grapevine cultivation is of great economic importance. The region of Baja California produces close to 90% of the country's wines, while Sonora produces around 95% of the Mexican table grape (González-Andrade, 2015). Previously, in Mexico, only the presence of *L. theobromae* and *Diplodia seriata* has been reported (Úrbez-Torres et al., 2008).

On the other hand, despite the importance of these pathogens, the mechanisms of pathogenicity during the infection process with the plants are not yet clear since the mode of action of Botryosphaeriaceae species is poorly documented (Claverie et al., 2020). Pathogens must overcome the defense barrier of the plant to establish a successful infection (Massonnet et al., 2018). Botryosphaeriaceae fungi colonize the xylem and phloem of grapevine plants, penetrating mainly through the tracheas and tracheids (Obrador-Sánchez and Hernandez-Martinez, 2020). However, it remains unknown how fungi establish themselves in the plant and carry out the colonization

process. In recent years, by analyzing the genome and transcriptome of different species of Botryosphaeriaceae, the importance of cell wall-degrading enzymes (CWDE) has been highlighted as possible virulence mechanisms (Paolinelli et al., 2016; Massonnet et al., 2018; Yan et al., 2018; Félix et al., 2019; Gonçalves et al., 2019; Garcia et al., 2021; Nagel et al., 2021). Therefore, this work aimed to contribute to the epidemiological knowledge of Botryosphaeriaceae species in the wine-growing region in Mexico and study alternatives for their control.

In chapter two, species of *Lasiodiplodia* associated with grapevines in Mexico were characterized. Finding the presence of *Lasiodiplodia exigua*, *L. brasiliensis, L. gilanensis*, and *L. crassispora*, being *L. exigua* the most frequently isolated, and *L. brasiliensis* and *L. gilanensis* the most virulent. In chapter three, Botryosphaeriaceae species present in vineyards of Mexico were isolated, identified and their pathogenicity in grapevine was compared. It was found *B. dothidea D. seriata*, *N. australe*, *N. vitifusiforme*, and *N. parvum*; being the *N.* parvum isolates, which showed the highest virulence in grapevine plants. In chapter four, the colonization process of *L. brasiliensis* in grapevine plants was studied through microscopic observations, ray parenchyma showed total starch depletion in the lesion area, and the fungus was found colonizing the vascular cambium, occluded vessels, and the pith of the plant, as well was able to use components of plant cell wall and starch as carbon sources. Finally, in the chapter five, two biological control agents, BsA3MX and BsC11MX, were evaluated against *Lasiodiplodia* spp., these BSAs were identified as *B. amyloliquefaciens* and presented characteristics of plant growth promotion and inhibited the growth of *Lasiodiplodia* spp. *in vitro* above 60%, and *in planta* reduced the lesion length caused by *Lasiodiplodia*, nonetheless, field evaluations are necessary.

1.1. Justification

Botryosphaeria dieback is one of the most destructive grapevine trunk diseases (GTDs), causing great economic losses worldwide. In Mexico, grapevine crop is of great importance, but studies on the impact and incidence of GTDs are lacking. The knowledge of the species of Botryosphaeriaceae present in the Mexican vineyards and their pathogenicity will help to design control strategies to avoid the spread of these pathogens. On another hand, the mechanisms by which Botryosphaeriaceae fungi infect grapevine plants have been little studied. To have a better

understanding of the interaction of the fungus with its hosts, it is important to investigate the colonization process of these fungi. Finally, given that the management and control measures for Botryosphaeria dieback are limited and not very efficient, the use of biological control microorganisms rises as an alternative to mitigate the effect of this diseases in the field.

1.2. Objectives

1.2.1. General objective

To contribute to the epidemiological knowledge of Botryosphaeriaceae species in the wine-growing region in Mexico and study alternatives for their control

1.2.2. Specific objectives

1. To characterize Botryosphaeriaceae species associated with grapevine in Mexico.

2. To evaluate the colonization process of Botryosphaeriaceae fungi in the grapevine by microscopy.

3. To characterize and evaluate biocontrol agents against *Lasiodiplodia* spp.

Chapter 2. Characterization of *Lasiodiplodia* species associated with grapevines in Baja California and Sonora, Mexico

2.1. 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 the country's wines, while Sonora contributes to about 95% of the Mexican market of table grape (SIAP, 2019).

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

More than 30 species belonging to the Botryosphaeriaceae have been associated with Botryosphaeria dieback grapevine, including the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neoscytalidium*, *Neofussicoccum*, *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 discoloration and perennial cankers in the vascular bundles resulting from the occlusion of the host xylem and phloem, which leads to the death of branches and eventually of the entire plant. This disease is distinguished from Eutypa dieback known to cause particular foliar symptoms (Úrbez-Torres, 2011; Bertsch et al., 2013; Billones-Baaijens and Savocchia, 2019). Species withsin the family Botryosphaeriaceae were commonly found in grapevines seven to ten years old and older, mainly in plants where large pruning wounds have been made by retraining vines (Gubler et al., 2005). However, symptoms incidence caused by this group of fungi has dramatically increased in recent years, especially in young vineyards (Gramaje and Armengol, 2011; Gispert et al., 2020).

Among the Botryosphaeriaceae, the genus *Lasiodiplodia* has been reported as highly virulent on grapevine (Úrbez-Torres and Gubler, 2009) and has been identified on more than 500 host species (Punithalingam, 1976). Some of the main morphological characteristics of the genus *Lasiodiplodia* include hyaline and smooth conidiogenous cells, with a cylindrical to conical shape that gives rise to conidia with subovoid to ellipsoidovoid shape, which can be hyaline without septa, or dark-brown pigmented with one-septa

(Phillips et al., 2013). This genus is globally distributed, mainly in the tropics and subtropics, and 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). The species *L. theobromae* comprises many cryptic species because of their morphological similarity (Alves et al., 2008; Mehl et al., 2017). As a result, the taxonomy of the genus *Lasiodiplodia* has undergone revisions in recent years, and new species were introduced (Dissanayake et al., 2016; Tibpromma et al., 2018). Recently, several *Lasiodiplodia* species were reduced to synonymy, particularly those with morphology similar to *Lasiodiplodia mahajangana*, *L. plurivora* and *L. theobromae*; thus, there are currently 34 accepted 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 this *Lasiodiplodia* group, we hypothesized that the species diversity within that group is broader than initially reported. Hence, this study aims to clarify and update the taxonomic identity of *Lasiodiplodia* species present in vineyards from Baja California and Sonora, Mexico, and to evaluate their pathogenicity to grapevine.

2.2. Materials and methods

2.2.1. Fungal isolation and morphological characterization of *Lasiodiplodia* spp.

The study encompassed ten vineyards in the main growing areas of the States of Baja California (Valle de Guadalupe) and Sonora (Hermosillo). Thirty-five samples of grapevine exhibiting Botryosphaeria dieback symptoms were taken from trunks and branches (Figure 1). Small pieces of symptomatic plant tissue were obtained from the diseased plant, immersed in 95% ethanol, quickly flamed and then placed onto potato dextrose agar (PDA Difco) supplemented with 25 $mg \cdot mL^{-1}$ chloramphenicol. Plates were incubated at 30 °C until fungal growth was observed. Fungal colonies that showed smoke-gray growth with abundant aerial mycelium were sub-cultured on PDA plates to obtain pure cultures and then preserved at 4°C in 20% glycerol.

Pure cultures were grown on PDA and incubated at 30°C for seven days to determine morphological characteristics of fungal isolates as pigmentation and aerial mycelium formation. Pycnidia production was

induced using liquid Minimal Medium 9 (MM9) ($g \cdot L^{-1}$: 10.0 glucose, 1.0 NH_4Cl, 0.5 NaCl 2.5 K_2HPO_4, 2.5 KH_2PO_4) supplemented with sterile pine needles (5% w/v). Flasks were incubated at room temperature under a nearby ultraviolet electromagnetic radiation lamp, using 12 h in light irradiation and 12h darkness for 15 days. Pycnidia were picked and suspended in 0.5% Tween 20 to obtain conidiospores, which were observed under a light microscope (Nikon Eclipse E200). Pictures of the conidiospores were taken with a camera Infinity 1 Lumenera and analyzed using Infinity Analyze v 6.5.4 and ImageJ software. To compare spore size across species, one-way ANOVA followed by a post hoc Fisher LSD analysis with an $\alpha<0.05$ was done using STATISTICA 8.0.

Figure 1. 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 necrosis, wedge-shape canker and wood necrosis. E) Pycnidia formed on wound grapevines canes found in some samples observed under a stereoscopic microscope.

2.2.2. DNA extraction and PCR amplification of *Lasiodiplodia* spp.

Total genomic DNA of each strain was extracted from mycelia recovered from cultures of three days 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 recommended in TrunkDiseaseID.org (http://www.grapeipm.org/d.live/) (Lawrence et al., 2017). The oligonucleotide primers EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'- TACTTGAAGGAACCCTTA CC -3') were used to amplify part of the translation elongation factor-1α (tef-1α) gene (Carbone and Kohn, 1999); and ITS1 (5´-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') 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 10X PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatin), 0.5µL 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 25µL. 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 1:30 min. Both programs had a final cycle of 72°C for 10 min. Once observed by gel electrophoresis, amplicons were purified using the GeneJet PCR purification kit (Thermo Scientific), and purified products were sequenced by Eton Bioscience Inc.

2.2.3. Phylogenetic analysis

The sequences were analyzed using BioEdit v.7.0.5.3 (Hall, 1999) and a BLASTn analysis was carried out. Sequences with the highest 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), and 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) analysis and Maximum Parsimony (MP) analysis 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/). The heuristic search was obtained automatically by applying the Maximum Parsimony method. Gaps were treated as missing data. The tree was visualized in MX: Tree Explorer.

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

Species	Isolate	Host	Origin	GeneBank accession number	
				ITS	*tef*1-α
L. 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*	*Vitis vinifera*	USA	EU012372	EU012392
L. brasiliensis	UCD916SN*	*Vitis vinifera*	USA	EU012366	EU012386
L. brasiliensis	UCD923SN*	*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*	*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
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*	PNG	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

PNG: Papua New Guinea
Isolates from this study are highlighted using text in bold.
* Isolates previously identified as *L. theobromae*.

2.2.4. Determination of optimal growth temperature of selected *Lasiodiplodia i*solates

The optimal growth temperature of the identified *Lasiodiplodia* species was determined as follows. Selected isolates of the identified species were grown on PDA plates by inoculating a 3-mm diameter plug of a 2-day-old colony on the edge of the plate. Three replicates of each isolate for each temperature were done and plates were incubated at 20, 23, 25, 28, 30, 37, and 40°C. The range of temperatures 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 where isolates were obtained. The colony radio was measured every 24h for 3 days. The optimal growth temperature was determined as the temperature that produced the maximum mycelial growth rate (mm/day), which was calculated according to equation (1)

$$GR = \frac{R_f - R_i}{T_f - T_i} \quad (1)$$

Where: GR= Growth rate, Rf= Final radial growth in mm, Ri= Initial radial growth in mm, Tf= Final time in which fungal growth is measure, and Ti= Initial time (day 1).

2.2.5. Production of aerial mycelium in *Lasiodiplodia* spp.

To evaluate aerial mycelium production as a phenotypic characteristic to differentiate among species, a 2 days-old culture of selected isolates was used to inoculate a plug of 3 mm in a glass tube containing 5 ml of PDA media. Tubes were incubated at 28°C for five days and the height of aerial mycelia was measured.

2.2.6. 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 to perform pathogenicity tests. Grapevine plants of cv. Cabernet Sauvignon were used to evaluate the pathogenesis of different *Lasiodiplodia* isolates. Inoculation of the plants was carried out through a mechanical wound in woody tissue made with a drill bit of 2 mm diameter and a mycelial plug of each 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 comparison. Plugs of sterile PDA were used in control plants, and all wounds were covered with Parafilm®. The grapevine plants were left in greenhouse conditions for 2 months. After this time, samples were taken to measure the length of the necrotic lesion caused by *Lasiodiplodia* isolates. In order to accomplish Koch's postulates, part of the sample was placed in PDA to recover the inoculated fungus. The experiments in plants were conducted in duplicates. Statistical analysis was carried out using one-way ANOVA followed by a post hoc Fisher LSD analysis with an $\alpha<0.05$ for determination of significant differences in virulence using STATISTICA 8.0.

2.3. Results

2.3.1. 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 the necrotic tissue placed in PDA, fast fungal growth was observed on the second day. From these samples, 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 belonging to the genus *Lasiodiplodia*. Some of the morphological characteristics included colonies that were initially white with abundant aerial mycelium, which became smoke-gray and produced pycnidia even in PDA with age (Figure 2). Pycnidia induction allowed observation of both hyaline and pigmented conidia in all the isolates (Figure 3). Inside the pycnidia, only hyaline conidia, aseptate with granular content were observed, while pigmented conidia, single septa and longitudinal striations were mainly found in form of cirrus (Figure 3). The dimensions (length and width) of 30 conidia per isolate were measured and minimum, and maximum mean standard deviation were calculated (Table 2). Significant differences in spore size were observed among the four analyzed *Lasiodiplodia* species. Isolates characterized as *L. gilanensis*, MX50 (av. = 28.5 x 16.6 µm), and MXCS01 (av. = 30.2 x 15.6 µm), presented larger and wider conidia in comparison to *L. brasiliensis*, *L. crassispora*, and *L. exigua*. *Lasiodiplodia brasiliensis* and *L.* crassispora isolates showed similar size at 24.02 and 25.57µm for length, respectively. Whereas, *L. exigua* isolates presented shorter conidia (av. = 21.2 x12.2 µm).

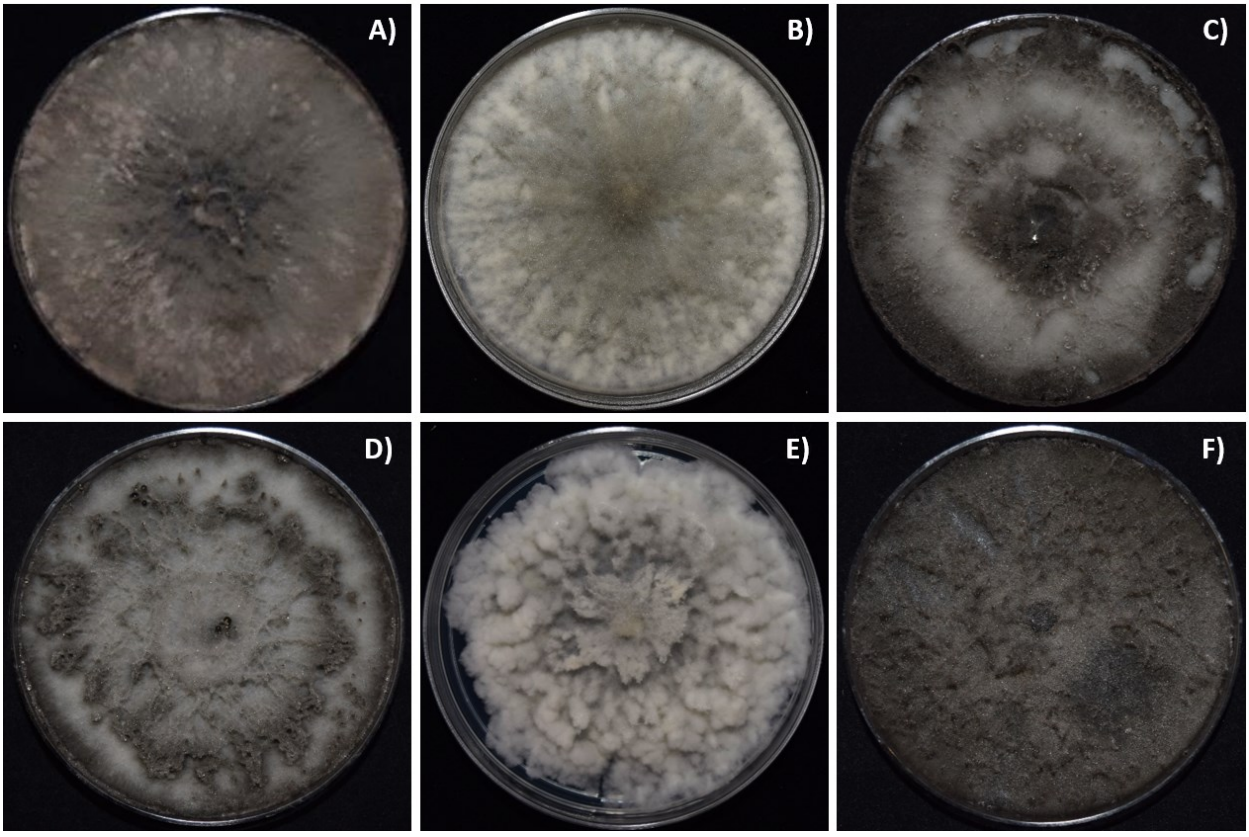

Figure 2. *Lasiodiplodia* spp. isolates grown on PDA at 30°C for 7 days. A) *L. brasiliensis* MXBCL28, B) *L. brasiliensis* MXVS18, C) *L. exigua* MXVS21b, D) *L. exigua* MXVS5a E) *L. gilanensis* MXBCCS01 F) *L. crassispora* MXVS1b.

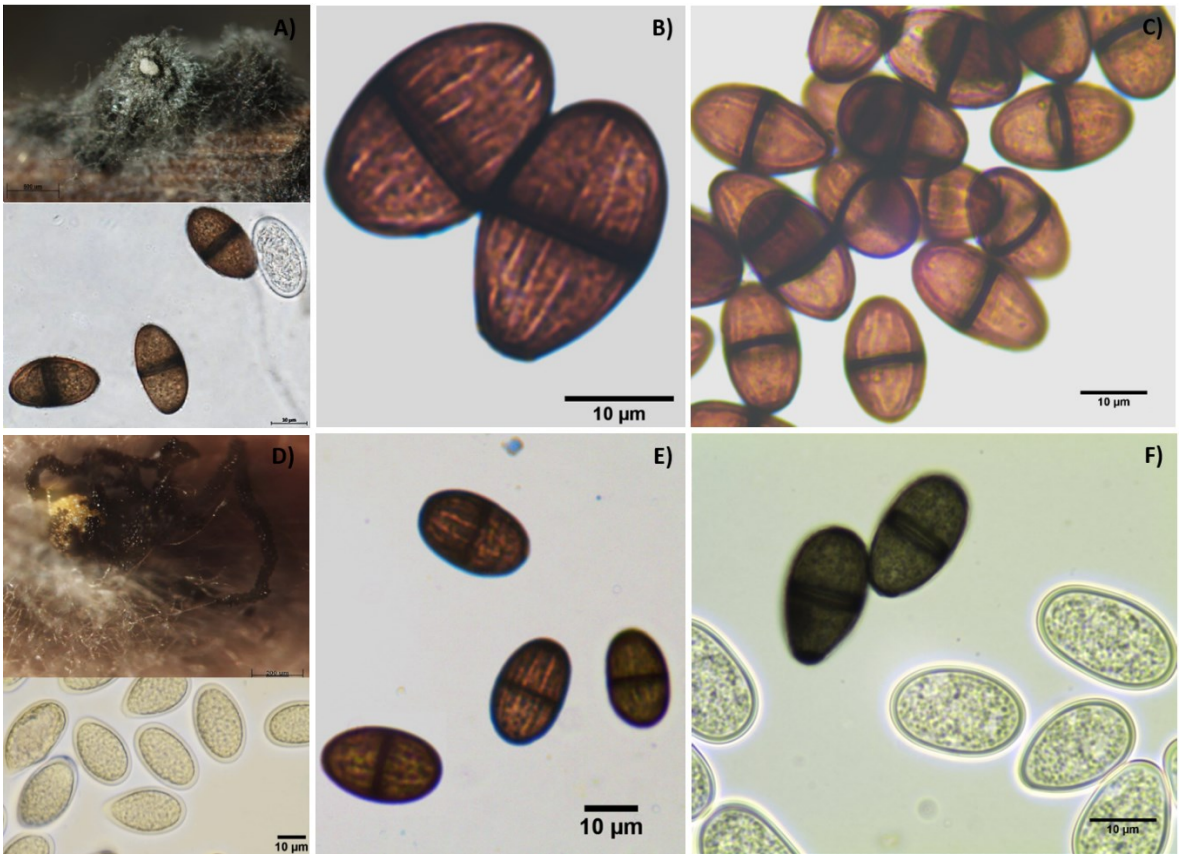

Figure 3. Conidia morphology of *Lasiodiplodia* spp. isolates. A) *L. brasiliensis* MXBCL28, B) *L. brasiliensis* MXVS18, C) *L. exigua* MXVS21b, D) *L. gilanensis* MXBCCS01 E) *L. crassispora* MXVS1b, F) *L. exigua* MXVS5a.

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

Isolate	Origin	Conidia Size*	Mean±SD**
Lasiodiplodia brasiliensis[b]			
MXBCL28	Valle de Guadalupe, B.C.	(21.9-)24-28.4x(12.8-)13.6-14.7	24.3±1.4x13.7±0.7
MXVSCC1	Hermosillo, Sonora	(20.4-)24.6-27.1x(11.3-)12.5-14.8	23.7±1.7x12.8±0.8
MXVS15a	Hermosillo, Sonora	(20.3-)22.3-24.6x(11.5-)12.5-14.4	22.8±1x12.5±0.7
MXVS16a	Hermosillo, Sonora	(22.1-)26.8-27.6x(10.6-)11.7-13.1	24.7±1.6x11.9±0.5
MXVS18	Hermosillo, Sonora	(21.3-)24.8-29.4x(11.3-)13.5-15.2	24.7±2x13.3±0.8
MXVS19a	Hermosillo, Sonora	(20.1-)23.3-26.4x(11.4)13.4-16.8	23.2±1.7x13.3±1.3
Lasiodiplodia crassispora[c]			
MXBCV5	Valle de Guadalupe, B.C.	(23-)24.4-29.9x(13.3-)16.7-20.2	26.1±2.2x17.5±1.7
MXVS1b	Hermosillo, Sonora	(23.7-)24.6-27.1x(13-)14.7-16.7	25.0±0.9x14.7±1.1
Lasiodiplodia exigua[a]			

MXBCV4	Valle de Guadalupe, B.C.	(18.6-)21.1-24.8x(11-)12-13.9	21.5±1.6x12.2±0.8
MXBCV6	Valle de Guadalupe, B.C.	(18.4-)19.2-22.5x(10.5-)11.4-12.7	20.2±1.1x11.2±0.7
MXBCV7	Valle de Guadalupe, B.C.	(19.1-)20.1x21.7(12.0-)12.9-14.2	20.3±0.7x12.9±0.5
MXVS5a	Hermosillo, Sonora	(21.1-)22.5-25.6x(11.7-)13.2-16	22.7±1.1x13.9±1
MXVS6a	Hermosillo, Sonora	(21-)23.4-24.6x(11.9-)12.9-13.9	22.8±1x13±0.5
MXVS2Ta	Hermosillo, Sonora	(19.7-)21.3-22.8x(11.3-)12.3-12.9	21.3±0.9x12.2±0.5
MXVS16b	Hermosillo, Sonora	(19.6-)23-26.9x(11.1)13-14.9	22.5±2x12.9±0.9
MXVS20	Hermosillo, Sonora	(20.2)21.9-23.7x(11.2-)12.7-13.9	22.2±0.9x12.8±0.7
MXVS21a	Hermosillo, Sonora	(18.4-)19.6-23.8x(10.1-)12.5-13.9	20.6±1.5x12.5±0.9
MXVS21b	Hermosillo, Sonora	(19.3-)20.3-23.2x(10.7-)11.9-13.4	21±1x12±0.7
MXVSV1	Hermosillo, Sonora	(19.1)20.8-23.4x(10.2)12-12.8	20.6±1x11.6±0.7
MXVSSC1	Hermosillo, Sonora	(18.2-)19.8-24.1x(10.5-)11.5-13.5	20.8±1.9x11.7±0.6
MXVSS2	Hermosillo, Sonora	(18.3-)20-23x(11.4-)11.9-14.2	20.5±1.2x12.5±0.7
Lasiodiplodia gilanensis[d]			
MXBC50	Valle de Guadalupe, B.C.	(25.6-)28-33.8x(15-)17.1-18.1	28.5±1.7x16.6±0.6
MXNCCS01	Valle de Guadalupe, B.C.	(25.4-)28.9-33x(13.8-)15.4-18.7	30.2±1.8x15.6±1.2

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

2.3.2. Molecular identification of *Lasiodiplodia* isolates

The ITS region and *tef*1 loci, sequences obtained were approximately 500 and 263 bp, respectively. The combined dataset comprised 832 characters including gaps after alignment (541 corresponded to ITS gene and 291 corresponded to *tef*1 gen) and 72 taxa. *Diplodia mutila* (CBS 136015) and *Diplodia seriata* (CBS 112555) were used as the outgroup taxon. Maximum parsimony analysis yielded one most parsimonious tree (length=151, CI=0.711864 (0.677885), RI=0.922197, and RC=0.714550 (0.656479) for all sites and parsimony-informative sites. Maximum likelihood analysis using 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, T=0.23783; and a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5665)) The phylogenetic analysis of the ITS region and *tef*1-α revealed that the isolates belong to four different *Lasiodiplodia* spp. (Figure 4). Most of the isolates belonged to *L. exigua* (syn. *Lasiodiplodia mahajangana*) (MXBCV4, MXBCV7, MXBCV6, MXVSV1, MXVS5a, MXVSSC1, MXVSS2, MXVS2Ta, MXVS6a, MXVS16b, MXVS20, MXVS21a, and MXVS21b).

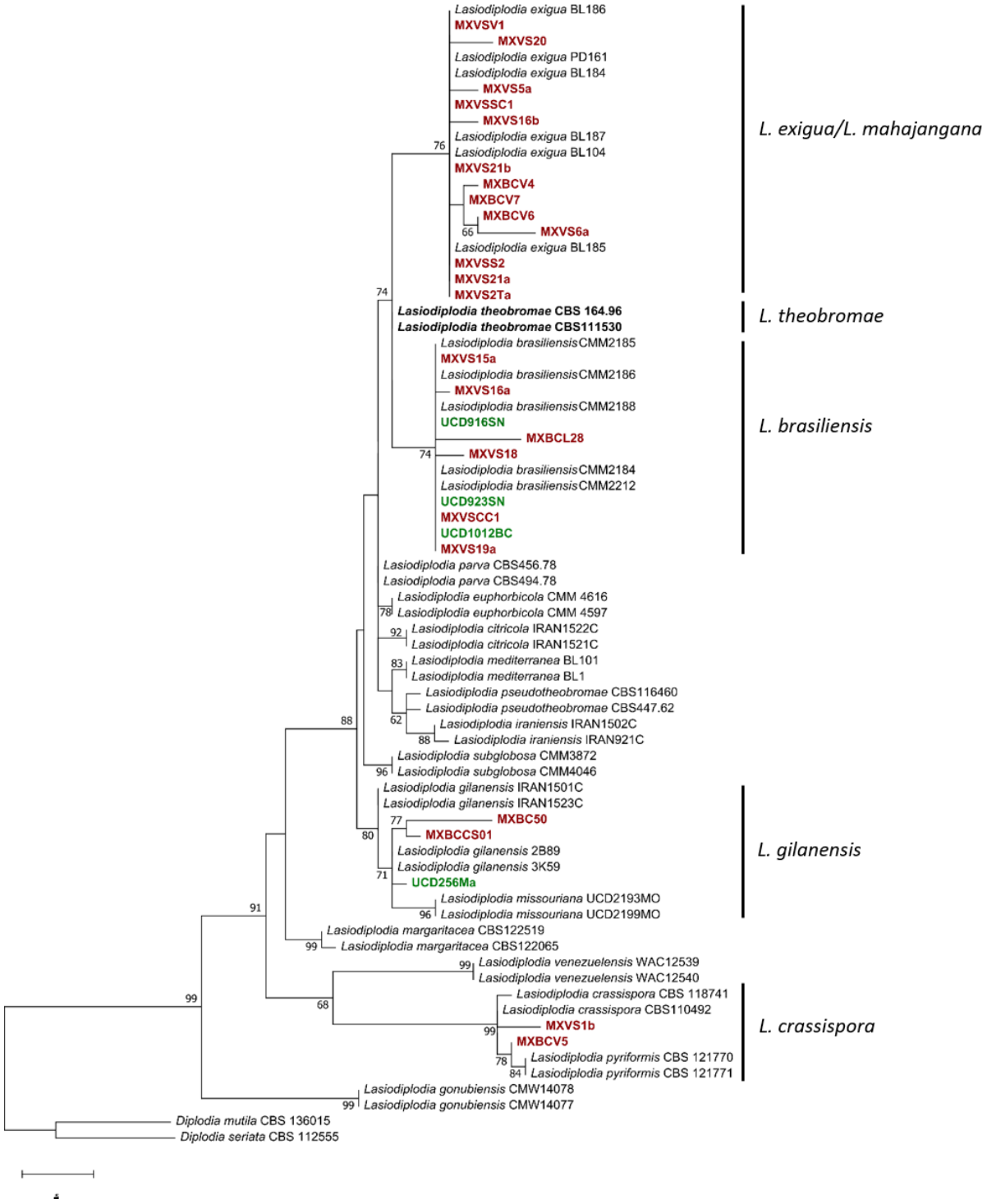

Figure 4. Phylogenetic analyses of *Lasiodiplodia* spp**.** Most-parsimonious tree (length = 151) obtained from analysis of ITS, and *tef1* concatenated dataset. Bootstrap values from 1000 replicates greater than 50 are indicated at the nodes. The tree is rooted with *Diplodia mutila* (CBS 136015) and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

Six isolates were identified as *L. brasiliensis* (MXBCL28, MXVSCC1, MXVS15a, MXVS16a, MXVS18, and MXVS19a); two isolates as *L. gilanensis* (syn. *Lasiodiplodia missouriana*) (MXBCCS01 and MXBC50); and two isolates as *L. crassispora* (syn. *Lasiodiplodia pyriformis*) (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 reference 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, Supplementary 1).

2.3.3. Optimal growth temperature and aerial mycelium production of *Lasiodiplodia* spp.

The *Lasiodiplodia* selected isolates displayed an optimal temperature for growth at 28 °C. However, most of them had a growth rate above 20 mm/day at 30 °C (Table 3). *Lasiodiplodia exigua* showed a mycelial growth rate of up to 24.6 mm/day at 37°C and it was the only species that showed growth at 40 °C. *Lasiodiplodia gilanensis* had the lowest mycelial growth rate, with a maximum growth rate of 19.8 mm/day at 28 °C.

Table 3. Optimal mycelial growth temperatures of Mexican *Lasiodiplodia* isolates.

	Temperature						
Isolate	**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

All *Lasiodiplodia* isolates produced aerial mycelium, but in *L. gilanensis* it was shorter (0.8±0.4 mm). The most abundant and longer aerial mycelium was observed in *L. exigua* MXVS5a (16±4.8 mm), followed by *L. brasiliensis* (9.0±2.56 mm). The species *L. crassispora* produced less abundant aerial mycelium (5.4±2.3 mm) than the other species and it became melanized in a shorter time (Figure 5).

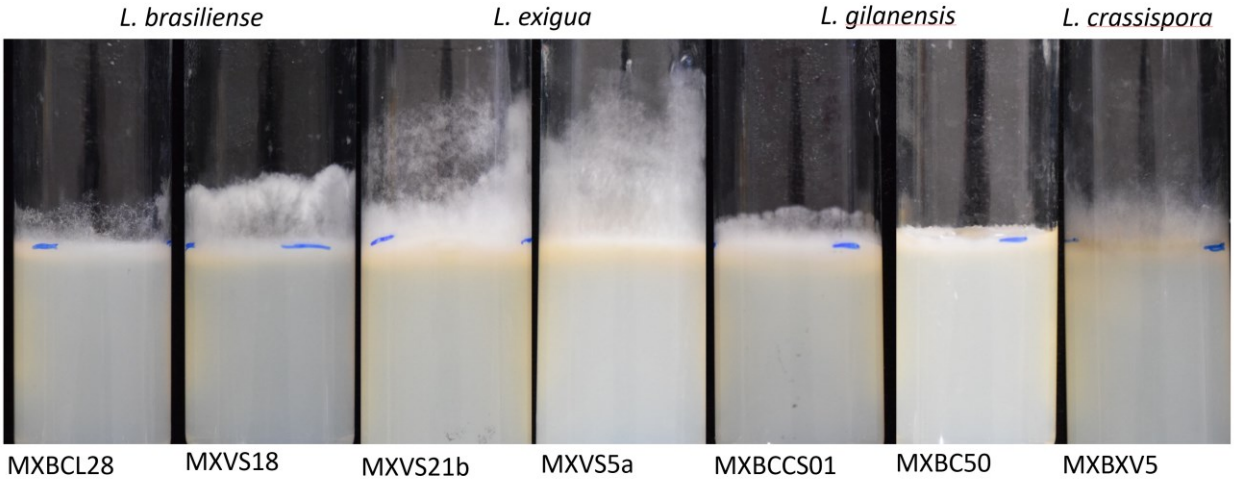

Figure 5. Aerial mycelium growth of *Lasiodiplodia* spp. isolated from grapevines in Mexico. Fungal isolates were grown on glass tubes containing PDA media during 5 days at 28 °C.

2.3.4. 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 6C, 6D, 6F). They induced necrotic lesions in the woody shoots up to 6 cm in 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 (Figures 6 and 7). *L. crassispora* MXBCV5 and MXVS1b caused lesions less than 1 cm in length (Figures 6 and 7) and showed a non-significant difference in comparison to control plants. All isolates were recovered from the inoculation site 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 green tissue was found, which indicated tissue regeneration of the caused wound.

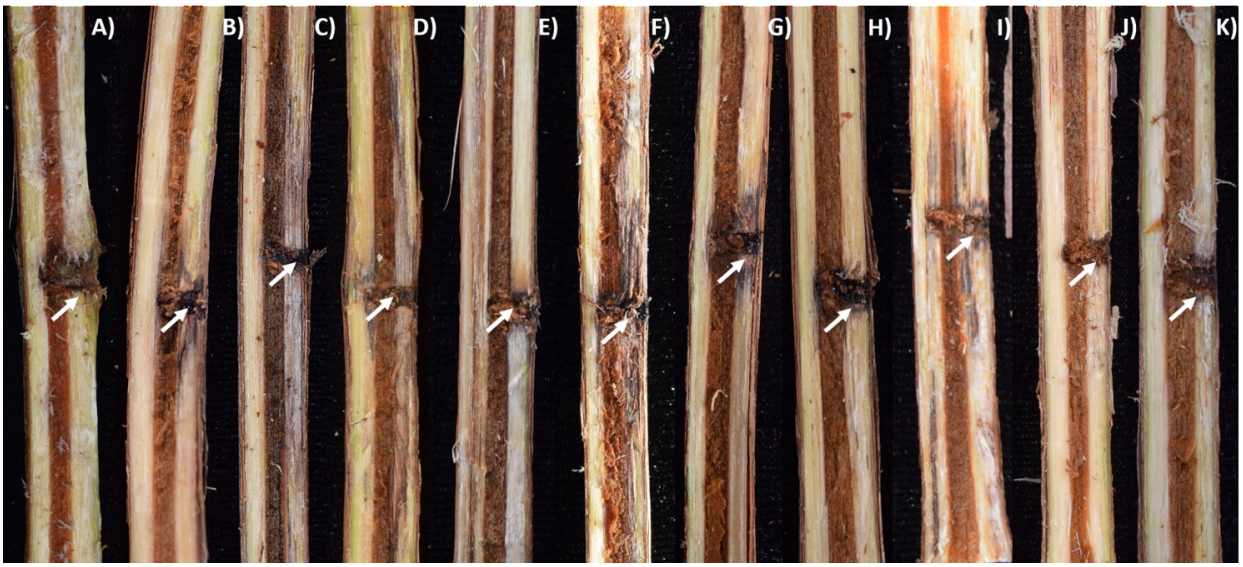

Figure 6. Grapevine woody shoots showing dark-brown lesions at 2-months post inoculation with *Lasiodiplodia* isolates**.** A) Control plant (PDA), B) *L. gilanensis* UCD256Ma, C) *L. brasiliensis* MXBCL28, D) *L. brasiliensis* MXVS18, E) *L. brasiliensis* MXVS16a F) *L. gilanensis* MXBCCS01, G) *L. 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.

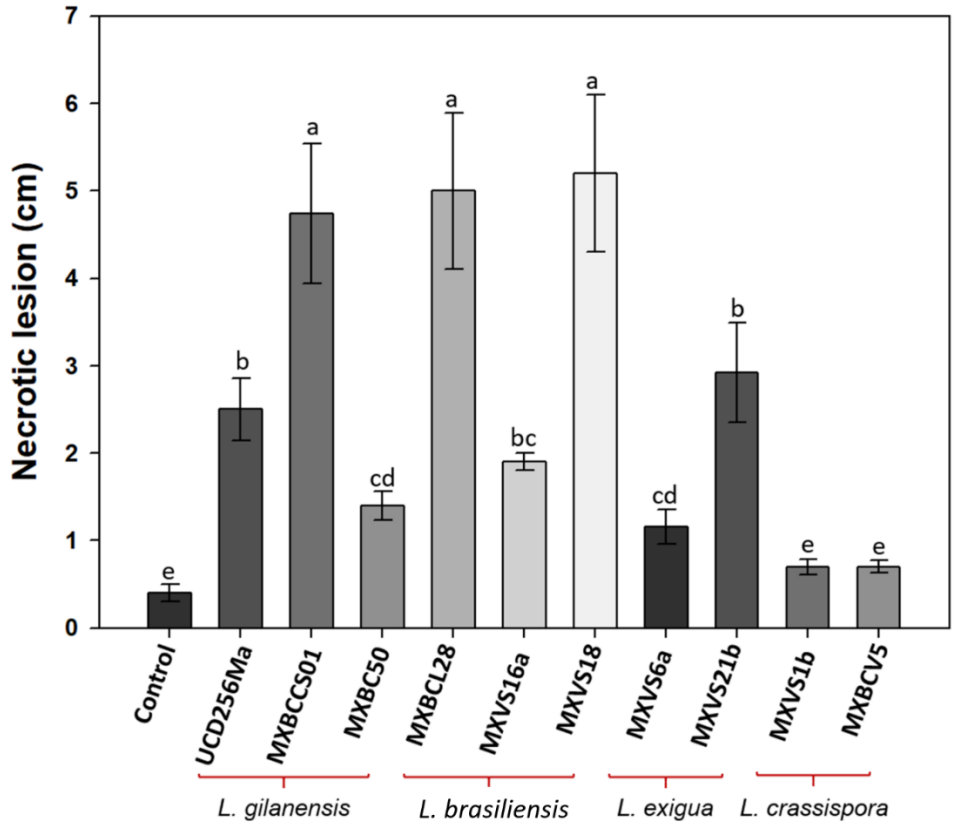

Figure 7. Lesion length caused by *Lasiodiplodia* isolates in grapevine plants 2-months post inoculation under greenhouse conditions. Bars indicate the standard deviation of each treatment. Significance letters were grouped based on Fisher's analysis ($P<0.05$); Bars indicate standard deviations. Means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

2.4. Discussion

In this study, four *Lasiodiplodia* species causing Botryosphaeria dieback symptoms were identified in Mexican vineyards. *Lasiodiplodia theobromae,* the type species of the genus *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, it was discovered that *L. theobromae* is a complex of cryptic species (Alves *et al.,* 2008), which led to taxonomic revision of the genus *Lasiodiplodia*. As a result, fungal isolates previously reported as *L. theobromae* have been reclassified as new species (Dissanayake *et al.,* 2016; Cruywagen *et al.,* 2017; Mehl *et al.,* 2017; Tibpromma *et al.,* 2018), and more recently, some species were 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., it shows a low interspecific variation. The translation elongation factor 1-α (*tef*1-α) is a more variable locus and has been recommended as a secondary barcode region to estimate the species identity for the Botryosphaeriaceae family (Lawrence *et al.,* 2017) and allowed us to segregate the *L. brasiliensis* from *L. theobromae.*

Pathogens associated with wood dieback diseases are generally found in vineyards that are at least ten-year-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 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 analysis indicated that those isolates clustered with *L. brasiliensis*, suggesting that the later species has been in Mexico for a long time.

Morphologically, the production of a reddish-pink pigment by the isolates of *L. brasiliensis* and *L. gilanensis* was observed. This characteristic has been reported in other species as *L. pseudotheobromae*, *L. parva*, and *L. theobromae* (Alves *et al.,* 2008; Abdollahzadeh *et al.,* 2010). Although *L. missouriana* was reduced to synonymy with *L. gilanensis* (Zhang *et al*., 2021), conidial dimensions of the Mexican isolates of *L. gilanensis* (MX50, MXSC01) and California, USA (UCD256Ma) were larger (av. = 29.6 x 15.6 µm) than in *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) presents conidia dimensions similar to *L. brasiliensis* (av. ± SD = 26.01±1.36 x 14.64±1.16 µm) (Netto *et al.,* 2014), difficult to distinguish based solely on morphological traits. In this study, aerial mycelium height was another morphological

characteristic evaluated, and the observed differences suggest that this trait could help with the differentiation of *Lasiodiplodia* species.

According to the pathogenicity test, the *L. brasiliensis* isolates MXBCL28 and MXVS18, and *L. gilanensis* MXCS01 were the most virulent to grapevine plants cv. Cabernet Sauvignon, causing necrotic lesions to the host vascular system at two-months post-inoculation. *L. brasiliensis* was also reported for the first time on grapevine in Brazil, and it was found to be the most virulent species on green shoots, followed by *L. theobromae* (Correia *et al.,* 2016). On the other hand, *L. gilanensis* was described for the first time in Iran from an unknown tree showing branch dieback, cankers, and fruit rot (Abdollahzadeh *et al.,* 2010). Considering that the isolate UCD256Ma, formerly identified as *L. theobromae* (Úrbez-Torres *et al.,* 2006), belongs to this species, this data supports taxonomic reassignment. Recently, *L. missouriana* was reduced to synonymy with *L. gilanensis* (Zhang *et al*., 2021). *Lasiodiplodia missouriana* was isolated from grapevines in 2011 and proved to be one of the most aggressive species to grapevine (Úrbez-Torres *et al*., 2012), which is in line with our results.

Isolates from *L. exigua*, MXVS6a and MXVS21b showed a significant difference in virulence. This species was first isolated from broom bush (*Retama raetam*) in Tunisia (Linaldeddu *et al.,* 2015) and was recently reported causing brown discoloration and streaks in grapevine wood (Akgül *et al.,* 2019). The *L. crassispora* isolates MXBCV5 and MXVS1b from this study were the least virulent, agreeing with previous reports (Correia *et al.,* 2016).

Grapevine plants are susceptible to many wood pathogens during the pruning period, and thus 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). The spread of fungal pathogens involved in Botryosphaeria dieback within the vineyard is linked with rainfall and associated wind dispersal (Mehl *et al.,* 2017); *Lasiodiplodia* has been reported to be more prevalent in regions with high temperatures and low precipitation (Úrbez-Torres, 2011; Gispert et al., 2020). The isolates from this study have an optimal temperature of 28 °C, but all were able to grow up to 37 °C, and the isolates of *L. exigua* even grew at 40°C. This might be an adaptation of *L. exigua* to extreme hot weather conditions. This species is the most commonly found in Baja California and Sonora's grape-growing regions. Curiously, even when the rest of the isolates did not grow at 40 °C, they recovered their average growth once they were transferred to room temperature, except for *L. gilanensis* MXBC50. Therefore, these fungi seem to enter a dormant state that recovers when the temperature drops. This information could explain why *L. gilanensis* is the most prevailing species in Baja California and Sonora, where prevalent climate conditions

are annual precipitation of 280 mm and a temperature above 40 °C during the summer, seem to favor their growth. More studies need to be done of these fungal species under extreme growing conditions; however, this study contributes to recognizing GTDs species present in Mexico's most economically important viticulture region, representing the first step of epidemiological studies to control the spread of these pathogens.

Chapter 3. Characterization of Botryosphaeriaceae species associated with grapevine in Mexico

3.1. Introduction

Botryosphaeria dieback is a degenerative disease caused by fungi from the Botryosphaeriaceae family, part of the complex of Grapevine trunk diseases (GTDs). This disease is one of the most destructive GTDs affecting productivity and causing significant economic losses (Fontaine et al., 2016; Gramaje et al., 2018; Hrycan et al., 2020).

Around the world, several genera of Botryosphaeriaceae have been associated with this disease in grapevine, *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia, Neofusicoccum*, *Neoscytalidium*, *Sphaeropsis,* and *Spencermartinsia* (Rolshausen *et al.,* 2013; Stempien *et al.,* 2017; Gramaje et al., 2018). These fungi cause necrotic lesions in the vascular system, stunted growth, wedge-shaped cankers in the woody trunk, dieback, and eventually the death of the plant in grapevine (Gramaje and Armengol, 2011; Úrbez-Torres, 2011; Bertsch et al., 2013; Hrycan et al., 2020). The primary source of inoculum of these pathogens is conidia, released and dispersed into the environment under humid conditions, rainfall, and wind currents; conidia penetrate the plants mainly through pruning wounds (Agustí-Brisach and Armengol, 2013; Gramaje et al., 2018; Waite et al., 2018).

Botryosphaeriaceae spp. can live as endophytes in asymptomatic plant tissues for extended periods (Slippers and Wingfield 2007) as latent pathogens, representing a serious problem since infected plants can remain undetected in the field or nurseries (Hrycan et al., 2020). Botryosphaeria dieback generally occurs in older vineyards (Gubler et al., 2005), but recently, it has been found in young grapevines (Gramaje and Armengol, 2011; Bertsch et al., 2013). Under climate change, plants are under more constant heat and water stress, being more susceptible to these pathogens (Fontaine et al., 2016; Mehl et al., 2017). Among the factors affecting the spread of these diseases are the poor cultural practices such as the inadequate protection of pruning wounds and low sanitary care of the propagation material (Graniti et al., 2000; Fontaine et al., 2016).

In Mexico, the grapevine growing area is of great economic importance; more than 480,000 tonss of grape were produced in 2019, being Sonora, Zacatecas, Baja California, Aguascalientes, and Coahuila the leading producers (SIAP, 2019). Nonetheless, there is a flawness of legislation regarding vine importations and therefore a constant risk to also import pathogens from abroad. In addition, the status of GTDs in the

region are understudied. In 2008, Úrbez-Torres et al. reported the presence of *L. theobromae* and *D. seriata* associated to Botryosphaeria dieback in Baja California and Sonora; Paolinelli-Alfonso et al. in 2015, reported *Eutypella microtheca* associated to Eutypa dieback in Baja California; and Rangel-Montoya et al. in 2021, reported *L. crassispora*, *L. brasiliensis*, *L. exigua*, and *L. gilanensis* associated to Botryosphaeria dieback in Baja California and Sonora. This study aims to identify the Botryosphaeriaceae species, different from *Lasiodiplodia*, present in vineyards of Mexico and compare their pathogenicity in grapevine.

3.2. Materials and methods

3.2.1. Fungal isolation and morphological characterization

Samples of grapevine exhibiting Botryosphaeria dieback symptoms were taken from seven vineyards in the main growing areas of the States of Baja California, one vineyard from Chihuahua, and three vineyards from Coahuila. Small pieces of symptomatic plant tissue were obtained from the diseased plant, immersed in 95% ethanol, quickly flamed, and then placed onto potato dextrose agar (PDA Difco) supplemented with 25 $mg \cdot mL^{-1}$ chloramphenicol to avoid bacteria growth. Plates were incubated at 30 °C until fungal growth was observed. Fungal colonies that showed smoke-gray growth with abundant aerial mycelium were sub-cultured on PDA plates to obtain pure cultures and then preserved at -4 °C in 20% glycerol.

Pure cultures were grown on PDA and incubated at 30°C for seven days to determine morphological characteristics of fungal isolates as pigmentation and aerial mycelium. Pycnidia production was induced using liquid Minimal Medium 9 (MM9) ($g \cdot L^{-1}$: 10.0 glucose, 1.0 NH_4Cl, 0.5 NaCl 2.5 K_2HPO_4, 2.5 KH_2PO_4) supplemented with sterile pine needles (5% w/v). Flasks were incubated at room temperature under a nearby ultraviolet electromagnetic radiation lamp, using 12 h in light irradiation and 12h darkness for 15 days. Formed pycnidia were taken and suspended in 0.5% Tween 20 to obtain conidiospores, then observed under a light microscope (AxioVert200 Zeiss). Conidia dimensions (length and width) of 30 conidia per isolate were measured. Images of the conidia were taken with a camera AxioCam HRc from Zeiss and analyzed using AxioVision 4.8.2. software. Statistical analysis was performed using STATISTICA 8.0 to compare spore size across species.

3.2.2. DNA extraction and PCR amplification

All Botryosphaeriaceae isolates were grown in PDB at 30°C for three days, and mycelium was recovered by filtration. Total genomic DNA was extracted using the CTAB protocol (Wagner et al. 1987). To characterize Botryosphaeriaceae spp. as recommended in TrunkDiseaseID.org (http://www.grapeipm.org/d.live/) (Lawrence et al. 2017), the primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´TCCTCCGCTTATTGATATGC3´) were used to amplify the ITS region of the nuclear ribosomal DNA, including the 5.8S gene (White et al. 1990); and EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'- TACTTGAAGGAACCCTTA CC -3') were used to amplify part of the translation elongation factor-1α (*tef*-1α) gene (Carbone and Kohn 1999). Each PCR reaction contained 2.5 µL of 10X PCR buffer with 15 mM $MgCl_2$, 0.5µL of 20 mM dNTPs, 0.625 µL of 10 µM of each primer, 0.125 µL of Taq DNA polymerase (GoTaq® DNA polymerase, Promega) at 5 units·μL^{-1}, 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 1:30 min. Both programs had a final cycle of 72°C for 10 min. Once observed by gel electrophoresis, amplicons were purified using the GeneJet PCR purification kit (Thermo Scientific). The purified products were sequenced by Eton Bioscience Inc.

3.2.3. Phylogenetic analysis

The sequences were analyzed using BioEdit v.7.0.5.3 (Hall 1999), and a BLASTn analysis was carried out. Sequences with the highest similarity were downloaded from the GenBank (Table 4) 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), and 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) analysis and Maximum Parsimony (MP) analysis 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 K2+G+I model was used

for the ML analysis (Kimura 1980). 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 4. List of GenBank and culture accession numbers of Botryosphaeriaceae spp. used in this study for phylogenetic analysis.

Species	Isolate	Host	Origin	GeneBank accession number	
				ITS	*tef*1-α
B. agaves	MFLUCC11-0125	*Agave* sp.	Thailand	JX646791	JX646856
B. agaves	MFLUCC10-0051	*Agave* sp.	Thailand	JX646790	JX646855
B. dothidea	CMW8000	*Prunus* sp.	Switzerland	AY236949	AY236898
B. dothidea	CBS 110302	*V. vinifera*	Portugal	AY259092	AY573218
B. dothidea	**RJM2MX**	***V. vinifera***	**Mexico**	**MZ312534**	**MZ397922**
B. dothidea	**RJM9MX**	***V. vinifera***	**Mexico**	**MZ312535**	**MZ397923**
B. dothidea	**RJM19MX**	***V. vinifera***	**Mexico**	**MZ312536**	**MZ397924**
B. dothidea	**RJM22MX**	***V. vinifera***	**Mexico**	**MZ312537**	**MZ397925**
B. dothidea	**RJM23MX**	***V. vinifera***	**Mexico**	**MZ312538**	**MZ397926**
B. dothidea	**RJM25MX**	***V. vinifera***	**Mexico**	**MZ312539**	**MZ397927**
D. corticola	CBS 112549	*Q. suber*	Potugal	AY259100	AY573227
D. corticola	CBS 112547	*Q. ilex*	Spain	AY259110	DQ458872
D. mutila	CBS 112553	*V. vinifera*	Portugal	AY259093	AY573219
D. mutila	CBS230.30	*P. dactylifera*	USA	DQ458886	DQ458869
D. sapinea	CBS393.8	*P. nigra*	Netherlands	DQ458895	DQ458880
D. sapinea	CBS109725	*P. patula*	South Africa	DQ458896	DQ458881
D. seriata	CBS 112555	*V. vinifera*	Portugal	AY259094	AY573220
D. seriata	CBS119049	*Vitis* sp.	Italy	DQ458889	DQ458874
D. seriata	**RF05MX**	***V. vinifera***	**Mexico**	**MZ312540**	**MZ397928**
D. seriata	**RF07MX**	***V. vinifera***	**Mexico**	**MZ312541**	**MZ397929**
D. seriata	**BY06MX**	***V. vinifera***	**Mexico**	**MZ312542**	**MZ397930**
D. seriata	**SASI19MX**	***V. vinifera***	**Mexico**	**MZ312543**	**MZ397931**
D. seriata	**ER1MX**	***V. vinifera***	**Mexico**	**MZ312544**	**MZ397932**
D. seriata	**16P2MX**	***V. vinifera***	**Mexico**	**MZ312545**	**MZ397933**
D. scrobiculata	CMW 189	*P. resinosa*	USA	AY253292	AY624253
D. scrobiculata	CBS109944	*P. greggii*	Mexico	DQ458899	DQ458884

L. theobromae	CBS 164.96	Fruit along coral reef	PNG	AY640255	AY640258
L. theobromae	CBS111530	Unknown	Unknown	EF622074	EF622054
N. australe	CMW6837	*Acacia* sp.	Australia	AY339262	AY339270
N. australe	CMW6853	*S. giganteum*	Australia	AY339263	AY339271
N. australe	**BT10MX**	***V. vinifera***	**Mexico**	**MZ312546**	**MZ397934**
N. australe	**BT12MX**	***V. vinifera***	**Mexico**	**MZ312547**	**MZ397935**
N. australe	**5P5MX**	***V. vinifera***	**Mexico**	**MZ312548**	**MZ397936**
N. eucalypticola	CMW6539	*E. grandis*	Australia	AY615141	AY615133
N. eucalypticola	CMW6217	*E. rossi*	Australia	AY615143	AY615135
N. luteum	CBS110299	*V. vinifera*	Portugal	AY259091	AY573217
N. luteum	CBS 110497	*V. vinifera*	Portugal	EU673311	EU673277
N. mediterraneum	PD312	*Eucalyptus* sp.	Greece	GU251176	GU251308
N. mediterraneum	CBS121558	*V. vinifera*	USA	GU799463	GU799462
N. parvum	CMW9081	*P. nigra*	New Zealand	AY236943	AY236888
N. parvum	CBS 110301	*V. vinifera*	Portugal	AY259098	AY573221
N. parvum	**14P4MX**	***V. vinifera***	**Mexico**	**MZ312549**	**MZ397937**
N. parvum	**24P2MX**	***V. vinifera***	**Mexico**	**MZ312550**	**MZ397938**
N. parvum	**RJM6MX**	***V. vinifera***	**Mexico**	**MZ312551**	**MZ397939**
N. parvum	**RJM15MX**	***V. vinifera***	**Mexico**	**MZ312552**	**MZ397940**
N. parvum	**RJM16MX**	***V. vinifera***	**Mexico**	**MZ312553**	**MZ397941**
N. parvum	**CHP08EMX**	***V. vinifera***	**Mexico**	**MZ312554**	**MZ397942**
N. viticlavatum	STE-U 5044	*V. vinifera*	South Africa	AY343381	AY343342
N. viticlavatum	STE-U 5041	*V. vinifera*	South Africa	AY343380	AY343341
N. vitifusiforme	STE-U 5252	*V. vinifera*	South Africa	AY343383	AY343343
N. vitifusiforme	STE-U 5050	*V. vinifera*	South Africa	AY343382	AY343344
N. vitifusiforme	**SACH23MX**	***V. vinifera***	**Mexico**	**MZ312555**	**MZ397943**
N. vitifusiforme	**SACH24MX**	***V. vinifera***	**Mexico**	**MZ312556**	**MZ397944**
N. vitifusiforme	**CNA1MX**	***V. vinifera***	**Mexico**	**MZ312557**	**MZ397945**
Mycosphaerella pini	CMW14822	*P. ponderosa*	USA	AY808300	AY808265

Isolates from this study are highlighted using text in bold.

3.2.4. Determination of the optimal growth temperature of Botryosphaeriaceae spp.

The optimal growth temperature of different Botryosphaeriaceae spp. was assesed by selecting at least two isolates of different species. Isolates were grown on PDA plates by inoculating a 3-mm diameter plug of a 2-day-old colony on the edge of the plate. Then, plates were incubated at 20, 25, 28, 30, 35, 37 and 40°C. The colony radio was measured every 24h for four days. The optimal growth temperature, calculated according to equation (1), was the temperature where the maximum mycelial growth rate was observed (mm/day):

$$GR = \frac{R_f - R_i}{T_f - T_i} \quad (1)$$

Where: *GR*= Growth rate, R_f= final radial growth in mm, R_i= initial radial growth in mm, T_f= final time in which fungal growth is measured, and T_i= Initial time (day 1). Three replicates of each isolate for each temperature were included. Statistical analyses were carried out using STATISTICA 8.0 to compare the rate growth of each isolate.

3.2.5. Pathogenicity test

Grapevine plants of cv. Merlot were used to evaluate the pathogenesis of different Botryosphaeriaceae isolates. For inoculation, a wound was made in the woody tissue with a drill bit of 2 mm in diameter. Then a mycelial plug was placed inside the hole. Each selected isolate was inoculated in five plants. Plugs of sterile PDA were used in control plants. All wounds were covered with Parafilm® to protect them from dryness. The grapevine plants were left in greenhouse conditions for two months. Then, samples were taken to measure the length of the necrotic lesion caused by Botryosphaeriaceae isolates. To fulfill Koch's postulates, tissue from all infected plants was recovered, flamed, inoculated into PDA and incubated at 30 °C. The experiments in plants were conducted twice.

3.3. Results

3.3.1. Morphological characterization of Botryosphaeriaceae isolates

The collected plants in Baja California, Chihuahua, and Coahuila showed wedge-shaped cankers and necrotic lesions in the vascular bundles. From them, 24 fungal isolates with a similar phenotype to Botryosphaeriaceae spp. were obtained. Nine isolates from Baja California, ten isolates from Chihuahua, and four from Coahuila. According to their morphological characteristics, these isolates were identified within the genera *Botryosphaeria*, *Diplodia*, and *Neofusicoccum*. Colonies were whitish to gray or olivaceous with moderate aerial mycelium and produced globose and dark pycnidia in PDA with age (Figure 8A-E). Some isolates produced a yellow pigmentation in the center of the colonies within the first 24h of incubation, which is a characteristic of certain *Neofusicoccum* species.

Significant differences in spore size were observed among the analyzed Botryosphaeriaceae spp. (Table 5). Isolates identified as *B. dothidea* showed hyaline, narrow conidia with fusiform base, and granular content. Some of them had a septum (Figure 8J), and presented larger conidia than the other Botryosphaeriaceae species observed (av. = 26.6x5.0 µm). Isolates of *D. seriata* showed dark brown conidia aseptate and ovoid (Figure 8K) and wider conidia (av. = 23.3x10.05 µm). *Neofusicoccum australe* isolates showed hyaline conidia with fusiform base and granular content without septum (Figure 8F) (av. = 19.4x5.7 µm). Isolates identified as *N. parvum* showed conidia ellipsoidal with apex and base flat, most of them were hyaline (Figure 8G) (av. = 21.3x5.2 µm), and some oldest conidia become light brown with 1-2 septa with the middle cell darker (Figure 8H). *Neofusicoccum vitifusiforme* isolates showed hyaline conidia ellipsoid widest in the upper and apex and subtruncate bases (av. = 20.7x5.5 µm) (Figure 8I).

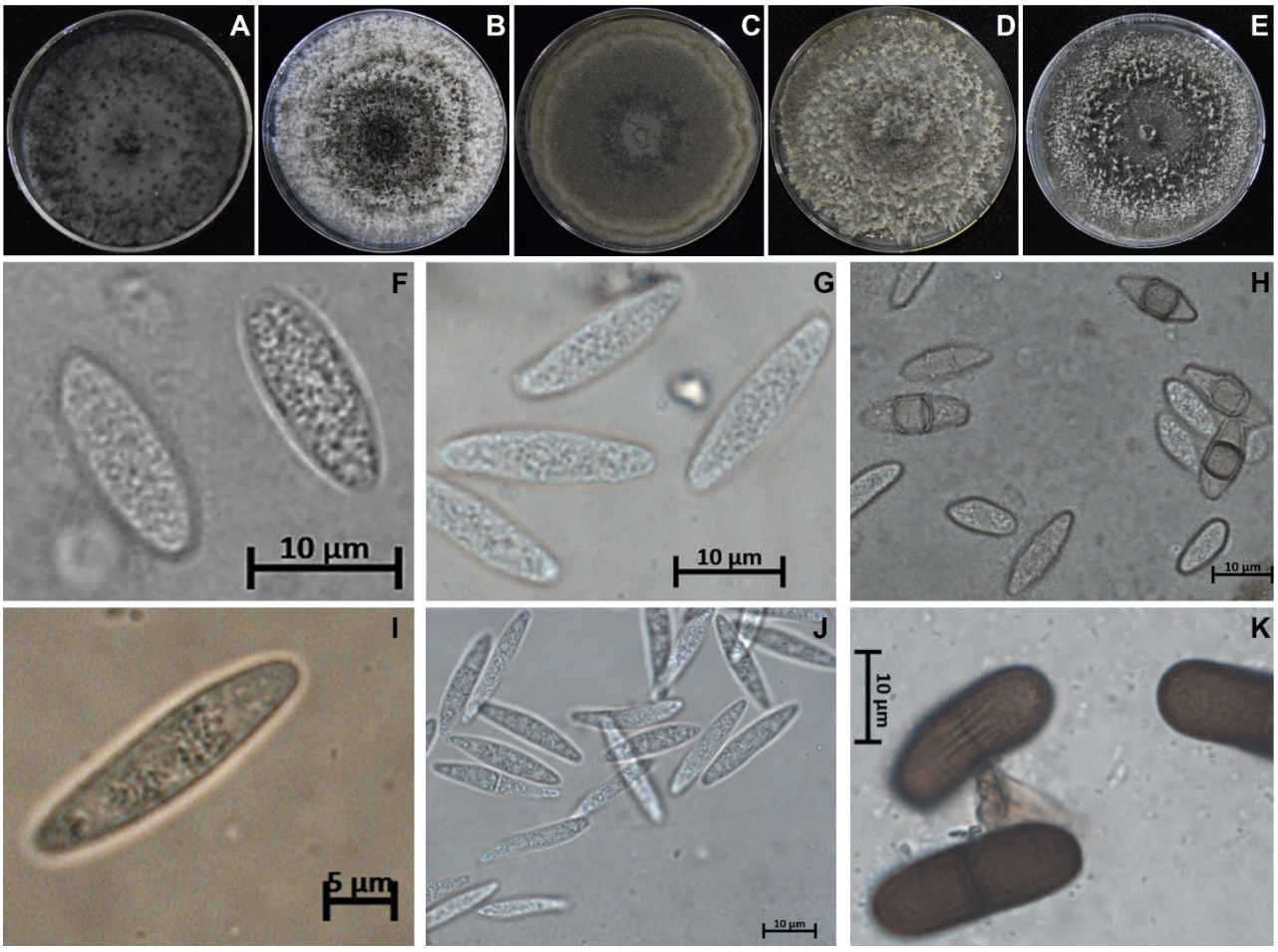

Figure 8. Macroscopic and microscopic characteristics of Botryosphaeriaceae spp. isolates grown on PDA at 30°C for 7 days. A) *Neofusicoccum australe* 5P5MX, B) *Neofusicoccum parvum* 14P4MX, C) *Neofusicoccum vitifusiforme* SACH24MX, D) *Botryosphaeria dothidea* RJM22MX F) *Diplodia seriata* BY06MX, G) *Neofusicoccum australe* conidia 5P5MX, H) *Neofusicoccum parvum* conidia 14P4MX I) *Neofusicoccum parvum* pigmented conidia with septa RJM16MX J) *Neofusicoccum vitifusiforme* conidia SACH24MX, K) *Diplodia seriata* conidia BY06MX.

Table 5. Conidial dimensions of the Botryosphaeriaceae spp. isolates from this study.

Isolate	Origin	Conidia Size µm*	Mean±SD**
***Neofusicoccum australe*[a]**			
BT10MX	Baja California	(17.8-)18.7-20.1x(5.7-)6.3-7.2	18.9±1.3x5.6±1.6
BT12MX	Baja California	(17.9-)18.5-20.3x(6.8-)6.4-7.5	18.6±1.2x6.7±0.6
5P5MX	Coahuila	(17.1-)21.1-24.0x(4.3-)5.0-6.4	20.9±1.9x5.0±0.5
***Neofusicoccum parvum*[b]**			
14P4MX	Coahuila	(19.5-)20.3-24.0x(4.5-)5.1-5.5	21.9±1.3x5.0±0.2
24P2MX	Coahuila	(19.0-)21.9-23.9x(4.9-)5.2-6.3	21.4±1.4x5.5±0.4
RJM6MX	Chihuahua	(17.9-)21.7-23.8x(4.4-)5.3-5.8	21.8±1.3x5.0±0.4

RJM15MX	Chihuahua	(18.5-)20.3-22.9x(4.8-)5.3-5.8	21.0±1.3x5.3±0.2
RJM16MX	Chihuahua	(16.9-)19.6-23.6x(4.4-)5.8-7.1	20.3±1.9x5.6±0.8
CHP08EMX	Chihuahua	(18.1-)21.1-24.4x(4.4-)5.1-6.2	21.7±1.6x5.2±0.5
Neofusicoccum vitifusiforme[a]			
SACH23MX	Baja California	(19.4-)21.2-23.9x(5.2-)5.9-6.8	21.5±1.2x5.8±0.5
SACH24MX	Baja California	(16.7-)20.5-23.0x(5.1-)5.3-6.5	21.0±1.5x5.6±0.4
CNA1MX	Coahuila	(16.8-)19.2-23.6x(4.7-)5.1-5.8	19.7±1.4x5.3±0.3
Diplodia seriata[c]			
RF05MX	Baja California	(21.0-)23.9-27.5x(8.4-)9.6-10.0	23.6±1.4x9.5±0.6
RF07MX	Baja California	(21.3-)23.4-25.7x(8.6-)10.3-10.9	23.4±1.1x10.0±0.5
BY06MX	Baja California	(21.9-)24.9-27.2x(9.3-)11.7-13.4	24.7±1.2x11.8±1.0
SASI19MX	Baja California	(19.0-)20.0-22.8x(8.0-)9.3-11.2	20.3±1.1x9.7±0.9
ER1MX	Baja California	(20.1-)22.9-28.9x(8.0-)8.9-11.2	24.1±2.0x9.2±0.8
16P2MX	Coahuila	(20.5-)23.2-28.6x(8.2-)9.8-12.1	23.7±2.2x10.1±0.9
Botryosphaeria dothidea[d]			
RJM2MX	Chihuahua	(24.0-)26.3-31.2x(4.2-)5.6-6.4	26.3±1.9x5.5±0.5
RJM9MX	Chihuahua	(24.7-)26.1-31.1x(4.1-)5.3-6.0	26.8±1.6x5.0±0.5
RJM19MX	Chihuahua	(23.5-)26.1-32.4x(4.1-)4.8-5.5	27.0±2.1x4.7±0.3
RJM22MX	Chihuahua	(24.3-)28.1-30.0x(4.1-)5.1-6.1	26.9±1.6x5.1±0.5
RJM23MX	Chihuahua	(24.1-)24.6-27.4x(4.1-)4.8-5.4	25.7±1.1x4.6±0.4
RJM25MX	Chihuahua	(24.2-)27.8-31.9x(4.3-)5.5-6.1	27.1±1.5x5.3±0.4

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

3.3.2. Molecular identification of Botryosphaeriaceae isolates

Sequences obtained from the ITS region and *tef*1 locus were approximately 550 and 255 bp, respectively. The concatenated dataset comprised 859 characters, including gaps after alignment (553 corresponded to ITS gene and 306 corresponded to *tef*1 gen) and 55 taxa. *Mycosphaerella pini* (CMW 14822) was used as the outgroup taxon. Maximum likelihood analysis using Kimura 2-parameter model resulted in a tree with the log likelihood value of -2509.24, and estimated base frequencies were: A=0.21506, T=0.23181, C=0.29218, G=0.26094. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6685)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 31.23% sites). Maximum parsimony analysis yielded one most parsimonious tree (length=354, CI=0.643382, RI=0.914387, and RC=0.663834 (0.588300) for all sites and parsimony-informative sites.

According to the phylogenetic analysis of the ITS region and *tef*1-α, Mexican isolates belong to five different Botryosphaeriaceae spp. (Figure 9). Six isolates belonged to *B. dothidea* (RJM2MX, RJM9MX, RJM19MX, RJM22MX, RJM23MX, RJM25MX); six isolates to *D. seriata* (RF05MX, RF07MX, BY06MX, SASI19MX, ER1MX, 16P2MX); six isolates to *N. parvum* (14P4MX, 24P4MX, RJM6MX, RJM15MX, RJM16MX); three isolates to *N. australe* (BT10MX, BT12MX, 5P5MX); and three isolates to *N. vitifusiforme* (SACH23MX, SACH24MX, CNA1MX). *Botryosphaeria dothidea* was found only in samples from Chihuahua, while *N. australe* and *N. vitifusiforme,* and *D. seriata* were isolated from samples from Baja California and Coahuila, and *N. parvum* was found in samples from Chihuahua and Coahuila.

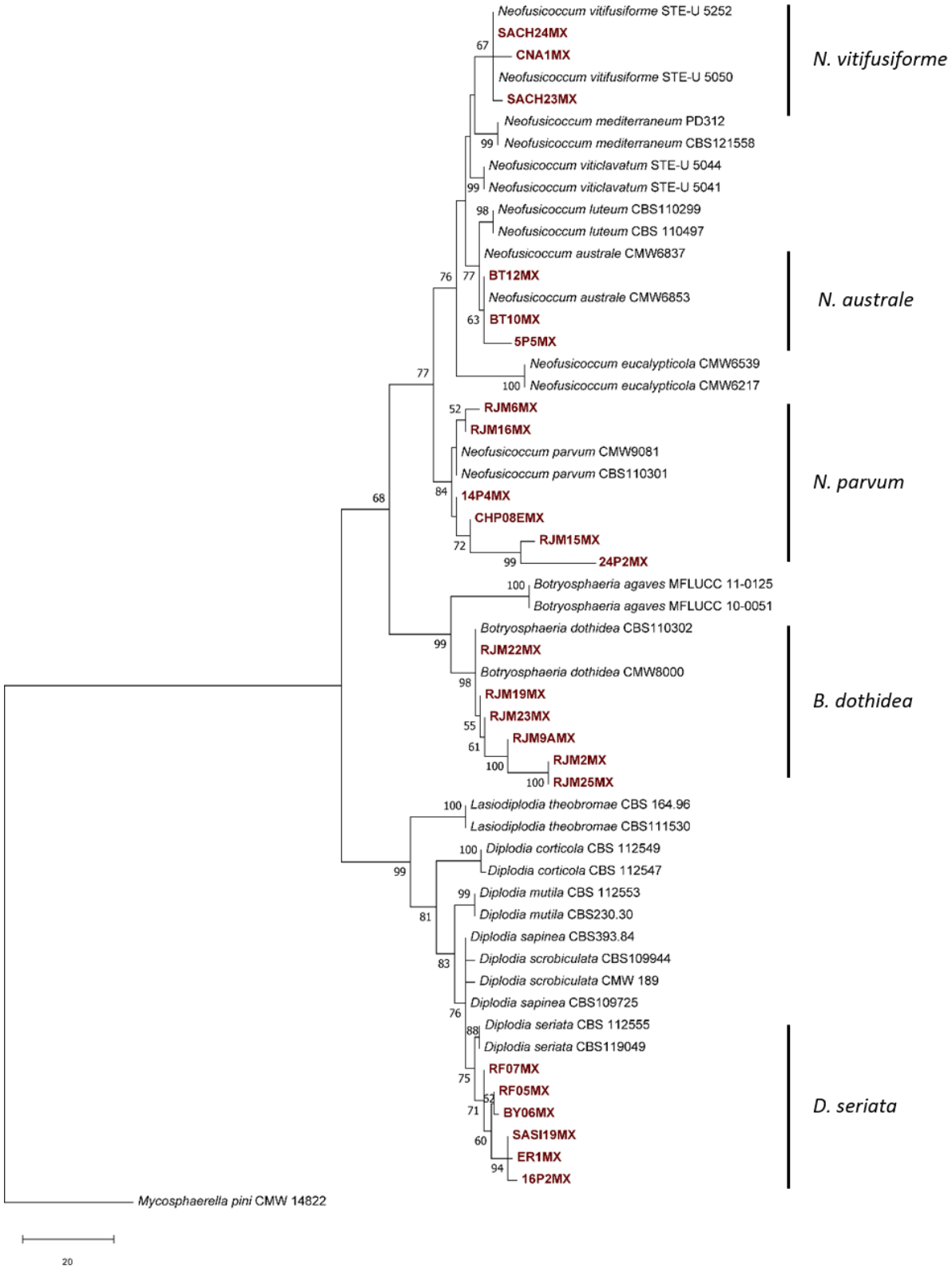

Figure 9. Phylogenetic analyses of Botryosphaeriaceae spp. Most-parsimonious tree (length = 354) obtained from analysis of ITS, and *tef1* concatenated dataset. Bootstrap values from 1000 replicates greater than 50 are indicated at the nodes. The tree is rooted with *Mycosphaerella pini* (CMW 14822). The isolates from this study are indicated in red bold.

3.3.3. Optimal growth temperature of Botryosphaeriaceae spp.

The selected isolates RJM6MX and RJM16MX of *Neofusicoccum parvum*, and RJM22MX of *B. dothidea* had an optimal temperature for growth in a range of 25-30 °C; but *B. dothidea* RJM25MX grew in a range of 28-35°C. *Diplodia seriata* and *N. australe* isolates showed the highest growth rate at 25 °C. The *N. vitifusiforme* isolate SACH23MX had an optimal growth temperature at 30 °C, and SACH24MX at 25 °C. *Botryosphaeria dothidea* RJ22MX had the highest growth rate of 16.4±1.7 mm/day at 28 °C (Table 6). The isolates from the five different Botryosphaeriaceae spp. did not grow at 37 °C and 40 °C. Only *D. seriata* isolates recovered when 4-days at 37 °C, or 40 °C plates were switched at room temperature.

Table 6. Mycelial rate growth at different temperature of Mexican Botryosphaeriaceae isolates (mm/day).

	Temperature						
Isolate	**20 °C**	**25 °C**	**28 °C**	**30 °C**	**35 °C**	**37 °C**	**40 °C**
N. parvum							
RJM6MX	12.6±2.6	15.8±2.1	15.3±2.3	15.1±2.2	3.1±0.1	*NG*	*NG*
RJM16MX	11.0±0.5	15.1±1.5	15.9±0.8	15.4±1.0	3.3±0.01	*NG*	*NG*
N. vitifusiforme							
SACH24MX	10.4±0.5	15.0±2.5	13.2±3.7	13.3±4.4	2.2±1.1	*NG*	*NG*
SACH23MX	9.9±0.1	9.3±0.5	9.7±0.5	10.0±0.01	2.8±0.8	*NG*	*NG*
N. australe							
BT12MX	6.8±1.6	9.6±1.8	7.4±1.2	4.6±.1.0	2.8±0.5	*NG*	*NG*
BT10MX	11.3±0.8	14.4±0.3	9.3±0.2	4.3±0.3	0.9±0.2	*NG*	*NG*
D. seriata							
BY06MX	12.4±0.8	14.1±0.6	12.8±0.9	11.4±1.0	8.9±0.7	*NG*	*NG*
16P2MX	12.8±0.5	13.6±0.1	13.0±0.4	12.4±0.8	9.0±0.9	*NG*	*NG*
B. dothidea							
RJ22MX	6.9±0.3	15.3±2.2	16.4±1.7	15.6±0.9	9.3±0.5	*NG*	*NG*
RJM25MX	6.1±0.9	7.3±0.3	7.9±0.7	8.2±1.5	8.6±0.2	*NG*	*NG*

NG: non mycelial growth

3.3.4. Pathogenicity assay

Plants inoculated in green tissue with *N. parvum* 14P4MX, 24P2, RJM6MX, RJM16MX, and *B. dothidea* RJM22MX showed the larger necrotic lesions 15-days post inoculation (dpi) (Figure 10A). Two-months post inoculation *N. parvum* isolates 14P4MX, 24P2, RJM6MX, and RJM16MX were also the most virulent in the woody tissue (Figures 10B and 11). Plants developed lesions larger than 4 cm in length, whereas isolates of *B. dothidea, D. seriata, N. australe*, and *N. vitifusiforme* were less virulent, causing lesions less than 2 cm in length. The Koch's postulates were confirmed by recovering the inoculated fungi from diseased plants. The control plants did not show necrotic lesions; instead, green tissue was observed closing in the wound.

Figure 10. Grapevine shoots inoculated with Botryosphaeriaceae spp. showing dark-brown lesions at A) 15-days post inoculation in green tissue, and B) 2-months post inoculation in woody tissue with Botryosphaeriaceae isolates**.**

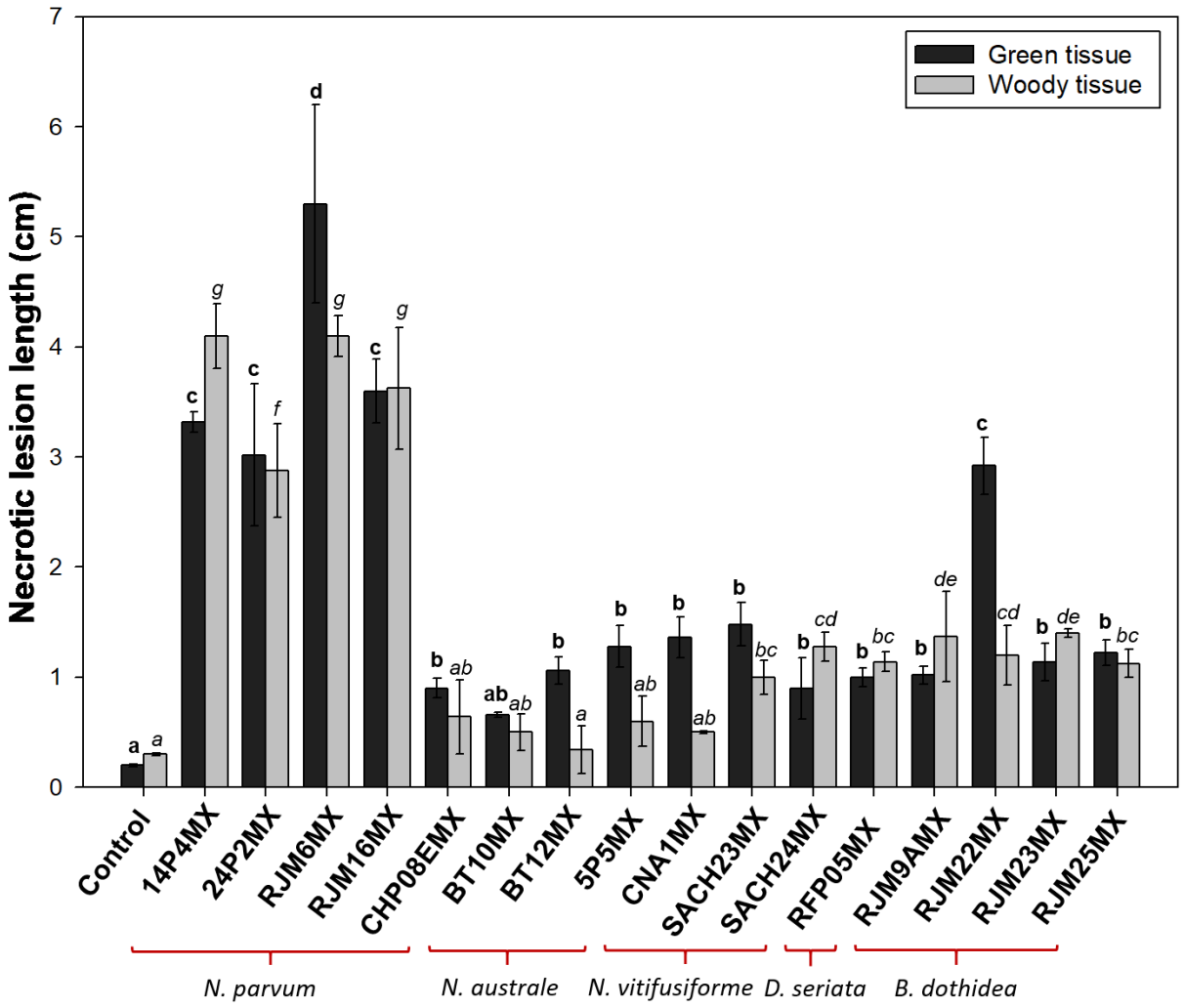

Figure 11. Lesion caused by Botryosphaeriaceae isolates in grapevine plants 15-days post inoculation in green tissue and 2-months post inoculation in woody tissue under greenhouse conditions. Significance letters were grouped based on Fisher's analysis (P<0.05) (bold letters for green tissue, and italic letters for woody tissue); bars indicate standard deviations. Means accompanied by the same letters are not significantly different ($\alpha < 0.05$).

3.4. Discussion

Fungi from the Botryosphaeriaceae family are recognized among the most aggressive vascular plant pathogens worldwide in a wide variety of hosts, most commonly of woody plants (Batista et al., 2021). In the current study, five Botryosphaeriaceae species were isolated and identified from vineyards of Baja California, Chihuahua, and Coahuila, Mexico, associated with Botryosphaeria dieback in grapevine. The

genera *Neofusicoccum* was the most prevalent. Six isolates of *N. parvum*, *B. dothidea*, and *D. seriata* were obtained and three of *N. australe*, and *N. vitifusiforme* (Figure 9). Previously, we identified four species of *Lasiodiplodia* in the regions of Baja California and Sonora (Rangel-Montoya et al., 2021). This study contributes to broadening the epidemiological knowledge of Botryosphaeriaceae species in the vine-growing regions of Mexico.

The taxonomy of Botryosphaeriaceae has changed since Theissen & Sydow (1918) introduced it as a subfamily in the Pseudosphaeriaceae. Currently, for the molecular identification of Botryosphaeriaceae fungi, the fungal rDNA internal transcribed spacer region (ITS) as primary barcode, and *tef1* as a secondary 'group-specific' barcode (Lawrence et al., 2017) are being used. Previously, the genus *Neofusicoccum* was accommodated in *Botryosphaeria* (Crous et al. 2006), and recently several cryptic species were reduced to synonymy, especially the *N. parvum* species complex. *Botryosphaeria dothidea*, was another species in which six species were reduced to synonymy (Zhang et al., 2021).

Conidia morphology and size could help distinguish between some genera and species of the Botryosphaeriaceae family (Phillips et al., 2013). In some cases, significant differences between conidia dimensions were observed (Table 5), e.g. conidia of *N. parvum* isolates (av. = 21.3x5.2 µm) and *B. dothidea* (av. = 26.6x5.0 µm), present similar characteristics, but the conidia of *B. dothidea* are longer and narrower. *Neofusicoccum vitifusiforme* (av. = 20.7x5.5 µm) and *N. australe* (av. = 19.4x5.7 µm) have similar conidia dimensions, but their morphology can distinguish them. *N. vitifusiforme* has fusoid to ellipsoid conidia, widest in the upper third with an obtuse apex and flattened, and subtruncate base; and in contrast, in *N. australe,* conidia are fusiform, base subtruncate to bluntly rounded, and non-septate (Phillips et al., 2013). *Diplodia seriata* isolates present different conidia morphology and dimensions (av. = 23.3x10.05 µm), are initially hyaline and become dark brown, and are aseptate and ovoid with wall externally smooth and roughened on the inner surface (Phillips et al., 2013).

The isolates of *N. parvum* 14P4MX, 24P4MX, RJM6MX, and RJMX16 were the most virulent in both green and woody tissues (Figures 10 and 11); while the isolate CHP08EMX, from the same species, was weakly virulent, which shows that isolates of the same species may present differences in their virulence. *Neofusicoccum parvum* was reported associated to grapevine in 2002 as *Botryosphaeria parva* (Phillips, 2002); since then, it is one of the most commonly isolated species from grapevine and one of the most virulent to this crop along with several species of *Lasiodiplodia* (Úrbez-Torres et al., 2006). The isolates of *N. australe* (BT10MX and BT12MX), *N. vitifusiforme* (SACH23MX and SACH24MX), *D. seriata* (BY06MX) and *B. dothidea* (RJM9AMX, RJM22MX, RJM23MX, and RJM25MX) were less virulent (Figure 11). Some plants

inoculated in woody tissue with *N. australe* and *B. dothidea* did not show damage; instead, tissue regeneration was observed at the site of the mechanical wound (Figure 10B). *Diplodia seriata* was reported in grapevine for first time in 1998 in Portugal with low virulence (Phillips, 1998). *Neofusicoccum vitfusiforme* was reported for first time in grapevine in 2004 as a weak pathogen (van Niekerk et al., 2004), and it was thought be restricted to *Vitis* spp. (Phillips et al., 2013), until it was isolated in Italy from *Olea europaea*, where was considered one of the most aggressive species (Lazzizera et al. 2008).

Fungi from the Botryosphaeriaceae family might behave as endophytes in their host for long periods. When the host plant is under hydric or heat stresses transition to a pathogenic stage occurs (Burgess and Wingfield 2002; Slippers et al. 2007; Czemmel et al. 2015). *Dothiorella* spp. and *L. theobromae* were isolated from the stem of healthy mango plants (Johnson et al. 1992), while *Botryosphaeria dothidea* was found as endophyte in the bark of white cedar (Xiao et al. 2014). Recently, *B. dothidea* has been considered as latent pathogen of global importance in woody plants, because it can present a prolonged endophytic phase before causing decay symptoms in the plant (Marsberg et al., 2017). According to their pathogenicity, some isolates from this study might behave as endophytes in grapevine plants and represent a future problem for vineyards in Mexico; therefore, it would be advisable to evaluate their pathogenicity in vine plants at a time greater than 2-mpi.

Most of the isolates from this study showed an optimal growth at 25 °C and could not grow above 37 °C. The optimal growth temperature for species of the genera *Neofusicoccum*, *Diplodia*, and *Botryosphaeria* spp. is in a range of 25-30 °C (Phillips et al., 2013). Interestingly, only the isolates evaluated of *D. seriata* (16P2MX from Coahuila and BY06MX from Baja California) incubated at 37 °C and 40 °C for 4-days, recovered their growth after being transferred to room temperature. In a previous study, we found that isolates of *Lasiodiplodia gilanensis*, *L. crassispora*, and *L. brasiliense* grew at 37 °C while isolates of *L. exigua* grew up to 40 °C. Like the isolates of *D. seriata* from this study, species of *Lasiodiplodia* did not grow at 40 ° C but recovered once left at room temperature (Rangel-Montoya, et al., 2021). Differences in high-temperature tolerance could be related to the virulence observed among different Botryosphaeriaceae spp., since several species of *Lasiodiplodia* have been considered highly virulent.

The diversity, distribution, the wide range of hosts, and the several factors that favored the distribution of conidia (wind, rain, and insects), make Botryosphaeriaceae spp. highly problematic plant pathogens (Slippers and Wingfield 2007; Mehl et al., 2017). Some species such as *B. dothidea*, *D. seriata*, *N. parvum*, and *L. theobromae* are globally distributed, as part of the international trade of plants without appropriate quarantine measures. Reports of the distribution and pathogenicity of Botryosphaeriaceae species in

different countries provide helpful information on the frequency and diversity of hosts of these species (Batista et al., 2021). The current study broadens the knowledge on the incidence of Botryosphaeriaceae spp. in the expanding viticulture regions and provides an important benchmark for future work on GTD epidemiology and disease management in Mexico.

Chapter 4. Colonization process of *Lasiodiplodia brasiliensis* in grapevine plant

4.1. Introduction

Grapevine plants are threatened by many pathogenic microorganisms that negatively impact their health and consequently reduce the yield and the quality of their fruits (Armijo et al., 2016). Grapevine Trunk Diseases (GTDs) are considered the most destructive grapevine diseases worldwide, responsible for significant economic losses for the grape industry. These diseases are caused by one or more xylem inhabiting fungi, including Botryosphaeriaceae spp., *Eutypa lata*, *Eutypella* spp. *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* (Bertsch et al., 2013; Fontaine et al., 2016; Claverie et al., 2020). In general, the symptoms of GTDs include the death of arms and spurs, spots on the leaves, and vascular discoloration; which eventually leads to the death of the plant due to the formation of cankers and necrosis in the vascular tissue (Gubler et al., 2005; Hrycan et al., 2020). Physiologically, these diseases mainly affect the metabolism of carbohydrates in the plant and the defense mechanisms in different organs (Fontaine et al., 2016; Batista et al., 2021).

In response to pathogens invasion, grapevine plants have several defense mechanisms, which the phytopathogens must overcome (Adrian et al., 2012; Armijo et al., 2016). Some are constitutive defenses, including structural barriers such as the cell wall, the cuticle, and the cortex, or induced defense such as the production of chemical toxins, hydrolytic enzymes, and the release of suicide cells (Freeman and Beattie 2008; Mithöfer and Boland 2012). Physical defenses, such as the formation of tyloses in the vascular bundles or suberin deposits in the cell wall, block the advance of pathogenic fungi by compartmentalizing woody tissue (Pouzoulet et al., 2013; Gómez et al., 2016). The production of phenolic compounds such as phenylpropanoids, flavonoids, and benzoic acid derivatives was found to play an important role in grapevine defense mechanisms (Adrian et al., 2012). The accumulation of phenolic compounds such as resveratrol and viniferin has been reported in plants infected with *Phaomoniella chlamydospora* and *Phaeoacremonium* spp. (Magnin-Robert et al., 2016; Rusjan et al., 2017). Similarly, Spagnolo et al. (2014) reported an accumulation of phenolic compounds and stilbenes in grapevine cv. Chardonnay, Gewurztraminer, and Mourvèdre with symptoms of Botryosphaeria dieback.

Botryosphaeria dieback is a degenerative GTDs caused by fungi from the Botryosphaeriaceae family. Several species within the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia, Neofusicoccum*,

Neoscytalidium, *Sphaeropsis,* and *Spencermartinsia* haves been associated to this disease in grapevine (Rolshausen *et al.,* 2013; Stempien *et al.,* 2017; Gramaje et al., 2018). Botryosphaeriaceae spp. are opportunistic pathogens that affect mainly water or heat-stressed plants (Slippers and Wingfield, 2007). During winter, these fungi form fruiting bodies called pycnidia on infected woody tissues. Conidia produced within these structures are released and spread in the vineyards by the action of the wind, rainwater, or irrigation (Úrbez-Torres, 2011; Bertsch et al., 2013). Subsequently, conidia can penetrate plants through pruning wounds, the main route of infection (Gubler et al., 2005; Spagnolo et al., 2014).

The genus *Lasiodiplodia* is one of the most aggressive pathogens to grapevine (Úrbez-Torres et al., 2006), reported so far in 749 hosts (Batista et al., 2021). Analyses of the transcriptome and genome of the genus *Lasiodiplodia*, revealed that the expression of gene families of cell wall degrading enzymes (CWDE) and carbohydrate metabolism, are mainly induced under heat stress conditions (Paolinelli et al., 2016; Yan et al., 2018; Félix et al., 2019; Gonçalves et al., 2019; Garcia et al., 2021; Nagel et al., 2021). Nonetheless, the mechanisms by which Botryosphaeriaceae fungi infect grapevine have been understudied. Recently, in Mexico *Lasiodiplodia brasiliensis* was reported with high virulence and frequently isolated from the states of Baja California and Sonora (Rangel-Montoya et al., 2021). This study aimed to evaluate the colonization process of *L. brasiliense* in grapevine.

4.2. Materials and methods

4.2.1. Fungi isolate and culture conditions

The fungus *L. brasiliensis* MXBCL28 was isolated from a diseased grapevine in Baja California (Rangel-Montoya et al., 2021), and it was preserved in 20% glycerol at 4°C and cultivated in PDA Difco medium at 30°C.

4.2.2. Plant material

Grapevine plants of cv. Cabernet Sauvignon were obtained from a local nursery. Plants were inoculated with *L. brasiliensis* MXBCL28 through a mechanical wound in the woody tissue made with a drill bit of 2 in

mm diameter. A mycelial plug of the fungus was placed inside the just-made hole and covered with Parafilm®. Plugs of sterile PDA medium were used in control plants. Plants were kept in greenhouse conditions for 2-months. Then, samples were taken two centimeters above and below the inoculation site and fixed in FAA solution (Formaldehyde, Acetic acid, Ethyl Alcohol; 5/5/9) for 24 h at 4 °C, then rinsed and preserved in 80% ethanol at 4 °C until use.

4.2.3. Histology of grapevine plants infected with *Lasiodiplodia brasiliensis* MXBCL28

To evaluate the colonization and the interaction of *L. brasiliensis* MXBCL28 with grapevine, longitudinal and transverse sections were made of the fixed tissues using a manual microtome. Approximately 70 µm thick slices were obtained. Sections were stained (Supplementary 2) with 0.1% Toluidin B (TBO) to observe the production of phenolic compounds, and with 5 % iodine and 10 % potassium iodide to investigate the starch in the plant; suberin deposits were observed using 0.00 1% Sudan black IV (Ruzin, 1999; Yeung, 2015). The presence of lignin in the woody tissue was observed by using 0.1 % phloroglucinol-HCl (Phl-HCl) and Mäule stain (Nakano and Meshitsuka, 1992). 0.02 % Calcofluor M2R White and 0.5% Congo Red were used to detect polysaccharides of cellulose and hemicelluose (Ruzin, 1999; Mitra and Loqué, 2014). Hyphae of *L. brasiliensis* colonizing the plant tissue was observed using the Fontana-Masson stain (Lillie, 1965). Stained sections were observed using a microscope Nikon Eclipse E200 with a camera AxioCam HRc (Zeiss) and with an AxioVert200 with a camera RisingCam® U3CMOS. Epifluorescence microscopy was conducted on an AxioVert200 microscope supplied with a HBO100 100W Mercury Lamp with ebq100 power. Cellulose and suberin in the plant tissue were observed using a DAPI filter (excitation at 330–380 nm, emission at 420 nm), and a TEXAS RED filter (excitation at 542–595 nm, emission at 644 nm) was used to analyze hemicellulose and also suberin. Images were analyzed using AxioVision 4.8.2, RisingView, and ImageJ 1.49v.

4.2.4. Evaluation of carbon sources for growth of *Lasiodiplodia brasiliensis* MXBCL28

The ability of *L. brasiliensis* to use different plant constituents as carbon sources was evaluated as follows. The fungus was grown in Minimal Medium 9 (MM9) ($g \cdot L^{-1}$ 3.0 K_2HPO4, 3.0 KH_2PO4, 0.5 NaCl, 1.0 NH_4Cl, 15.0 agar) supplemented either with glucose 1%, xylose 1%, lignin 0.1%, starch 1%, cellulose 1%, xylene

1%, glycerol 1%, pectin 1%, or tannic acid 0.1%, as carbon sources. After inoculation with the fungus, plates were incubated at 30 °C for 7 days, and the fungal growth was marked every 24 h. The experiment was done in triplicate

Fungal biomass was measured by inoculating with a mycelium disk of *L. brasiliense* test tubes containing MM9-liquid supplemented with the different carbon sources listed above. All cultures were incubated at 30 ° C for 7 days. Subsequently, the mycelium of each growth condition was recovered by filtration. The biomass data were calculated by weighing the mycelium obtained from each growth condition and subtracting the weight of the mycelium disk used for inoculation; in the case of treatments with grapevine wood of ground wood contained in the sample was carefully removed. The experiment was done in triplicate.

4.3. Results

4.3.1. Colonization of *Lasiodiplodia brasiliensis* MXBCL28 in grapevine plants

After 2-month post-inoculation, grapevine plants infected with *L. brasiliensis* showed necrotic lesions along the xylem up to 7 cm in length (Figure 12B). In comparison, the control plants presented short lesions in the xylem tissue surrounding the wound area and tissue regeneration of the wound (Figure 12A).

Cross-sections of control plants stained with iodine-potassium iodide showed a black coloration indicating the presence of starch in the ray parenchyma and even in the wound area (Figure 12C). In contrast, plants infected with *L. brasiliensis* MXBCL28 showed the lack of the black staining, indicating the complete starch depletion in the ray parenchyma and the pith (Figure 12D and E).

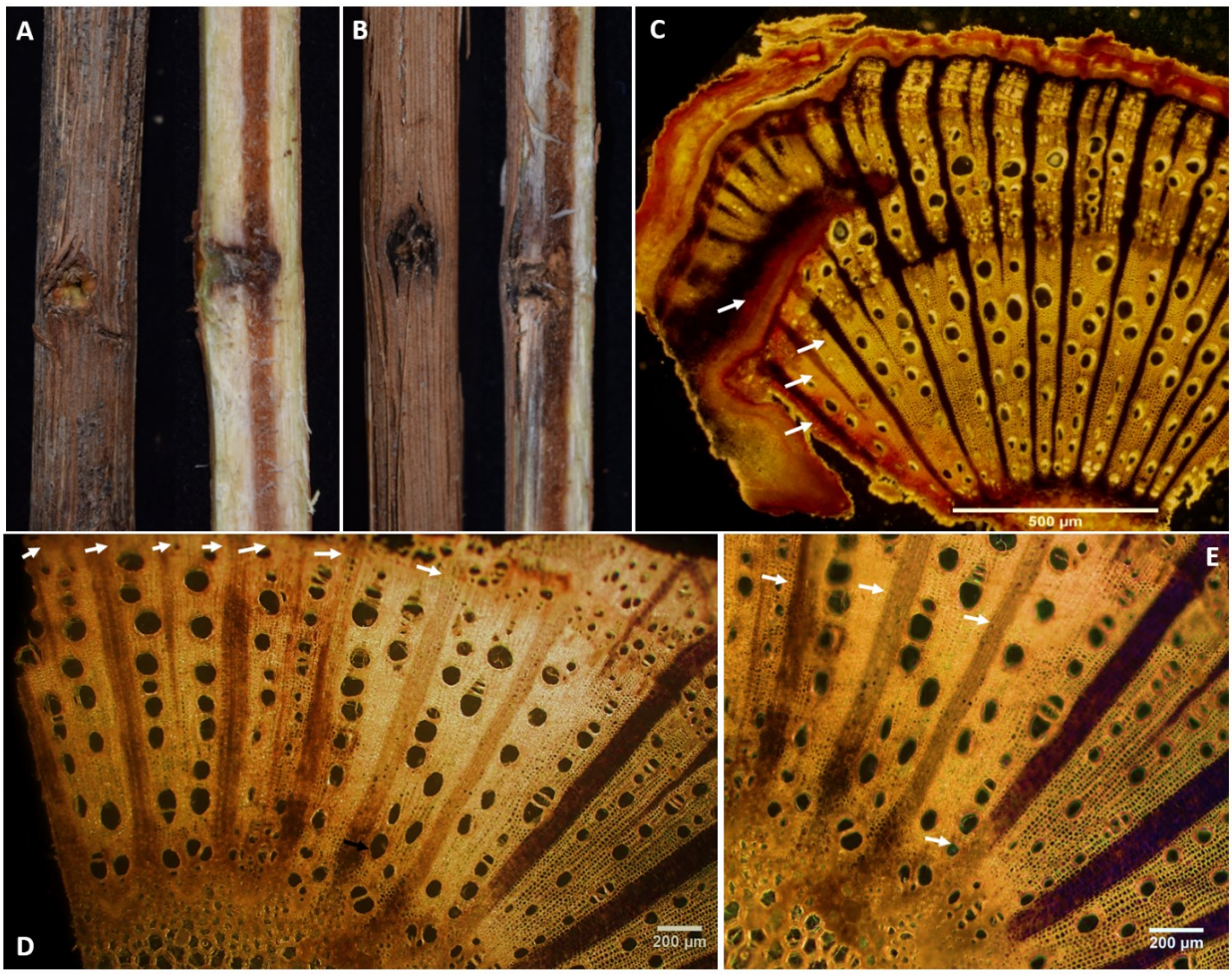

Figure 12. Grapevine plants cv. Cabernet Sauvignon two months after inoculation with *Lasiodiplodia brasiliensis* stained for starch. A) Wound recovery in control plants (left: outer view; right: inside longitudinal view). B) Necrotic lesion caused by *L. brasiliensis* MXLBC28 (left: outer view; right: longitudinal section). C-E) Iodine-potassium iodide stain for analyzing the presence of starch on grapevine plants cv. C) Cross section of control plants showing starch as black zones in the ray parenchyma. D-E) Cross-section of infected plants showing starch depletion in the ray parenchyma of the necrotic tissue. Arrows indicate the ray parenchyma in the wound area and necrotic tissue.

Cell wall components of grapevine plants were observed using Calcofluor White M2R for cellulose and Congo Red for cellulose and hemicellulose. Under epifluorescence microscopy, the control plants stained with Calcofluor White M2R showed brownish areas in the wound area (Figure 13A and D). In contrast, plants infected with *L. brasiliense* showed dark zones indicating the depletion of cellulose in the necrotic tissue (Figure 13B and E). Also, under epifluorescence microscopy, tyloses were observed next to and inside of the necrotic vascular bundles (Figure 13C). In sections stained with Congo Red, dark zones in the infected tissue (Figure 13H), vascular bundles (Figure 13F), and in the ray parenchyma (Figure 13I) were observed in comparison with the control plants (Figure 13G).

Regarding the presence of lignin in the woody tissue, the cinnamaldehyde end groups of lignin react with Phloroglucinol-HCl giving lignin a pink-purple color (Adler, 1977; Liljegren et al., 2010). No changes in lignin content were observed in control plants in the wound site since the pink color intensity was similar than in the rest of the tissue, and lignin was also observed in the regenerated tissue zone (Figure 14A). In contrast, infected plants showed a lighter pink color in the lesion area and the vascular cambium (Figure 14B), indicating that the fungus uses lignin, although lignin deposits were observed in the fibers surrounding the vascular bundles (Figure 14C). The Mäule stain gives to lignin a red color, due to the reaction with the syringyl lignin units (Meshitsuka and Nakano, 1977; Yamashita, et al., 2016). In the control plants, lignin was observed in the regenerated tissue and the ray parenchyma; slight discoloration was found only in the tissue surrounding occluded vascular bundles near the wound site (Figure 14D). In plants infected with *L. brasiliensis*, discoloration was observed in most of the xylem, the ray parenchyma (Figure 14E-F), and the fibers surrounding occluded vascular bundles (Figure 14G), suggesting changes in the lignified tissue.

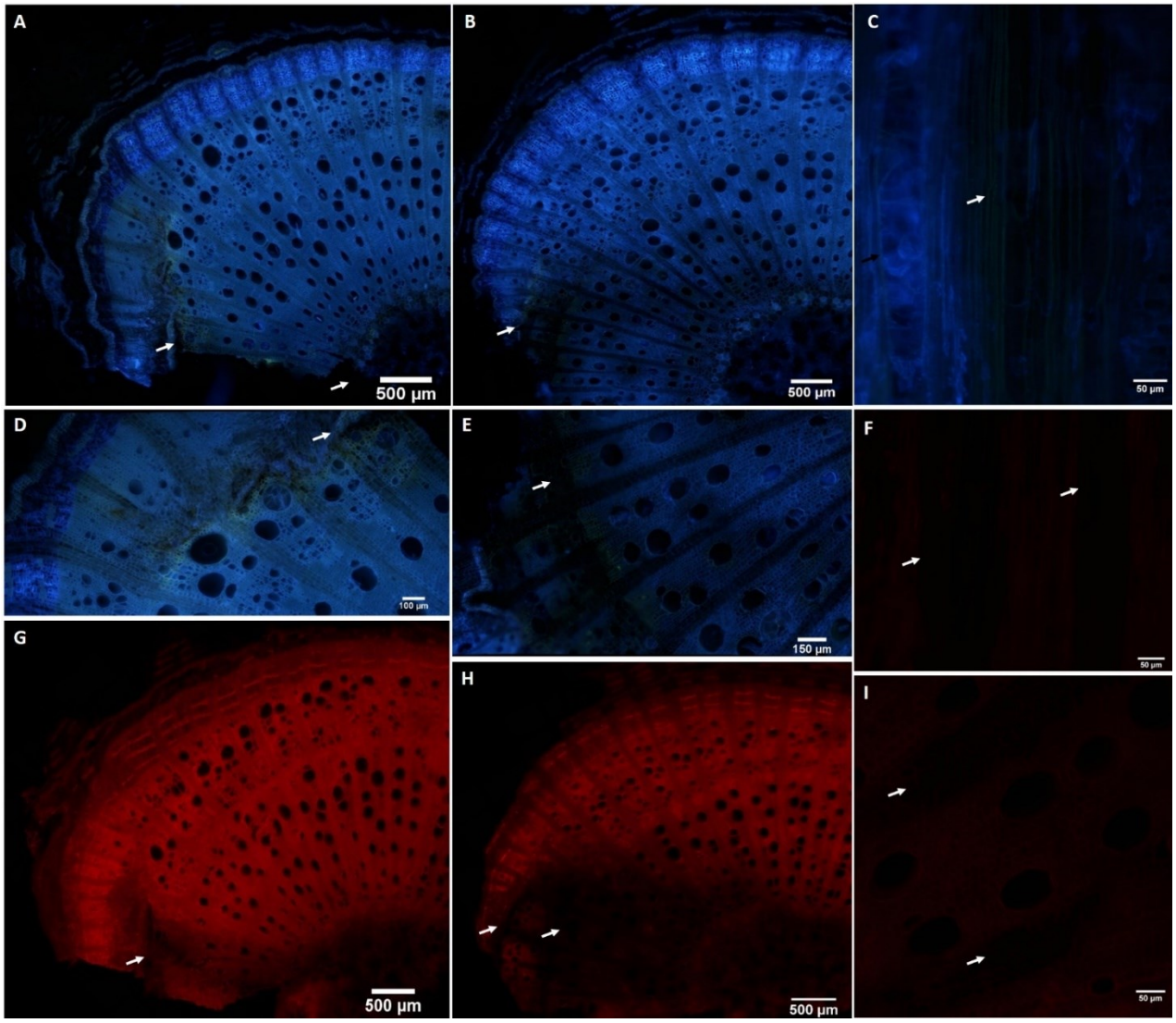

Figure 13. Epifluorescence microscopy of grapevine plants cv. Cabernet Sauvignon stained to observe cellulose and hemicellulose in the woody two months after inoculation with *Lasiodiplodia brasiliensis*. A-E) Sections stained with Calcofluor White M2R. In control plants (A, D, and G) brownish areas were observed in the wound area. Plants infected with *L. brasiliensis* (B, C, E, F, H, I) showed dark zones without cellulose in the necrotic tissue. Sections stained with Congo Red (F-I) show dark zones in the infected tissue (H), vascular bundles (F), and in the ray parenchyma (H) in comparison to control plants (G). White arrow indicates the zones without fluorescence. Black arrow indicates fluorescent occlusions.

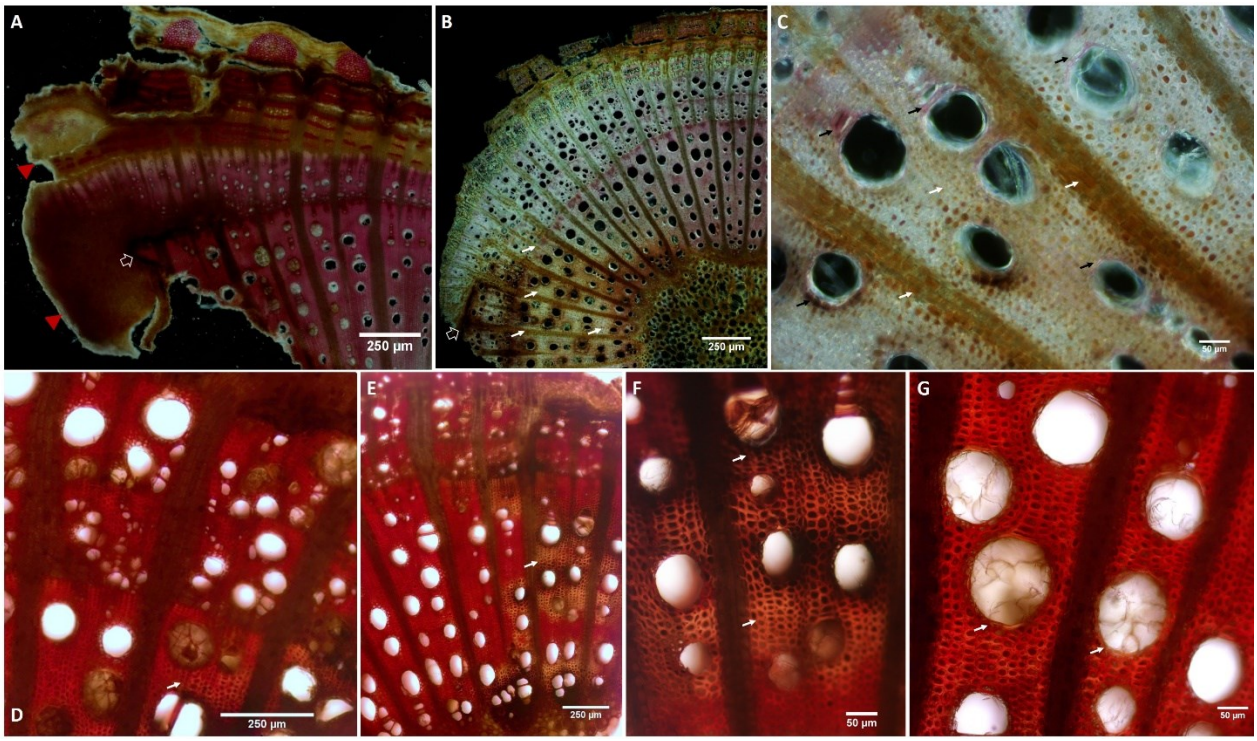

Figure 14. Cross sections of grapevine plants cv. Cabernet Sauvignon stained the presence of lignin with Phloroglucinol-HCl (A-C) and Mäule method (D-G). A) Control plants showing no change in the pink intensity in all tissue. B) Infected plants with *L. brasiliensis* 2-mpi showing lighter pink color in the lesion area and vascular cambium. C) Infected plants with *L. brasiliensis* presenting lignin deposits were observed in the fibers surrounding the vascular bundle. D) Control plants with slight discoloration in the tissue surrounding occluded vascular bundles near the wound site. E-G) Infected plants with *L. brasiliensis* 2-mpi showing discoloration in the xylem, ray parenchyma and the fibers surrounding occluded vascular bundles. Hollow arrow indicates inoculation area; red arrow indicates tissue regenerated; with arrow indicates zones without lignin; black arrow indicates lignin surrounding vessels.

Suberin deposits were observed in the cambium cork in the wound area (Figure 15A) and the ray parenchyma in control plants (Figure 15B). In contrast, suberin was observed in the vascular cambium, ray parenchyma, cambium cork, and pith in response to the injury caused by the fungus in the infected plants. Suberin was not observed in necrotic rays (Figure 15C-F).

Toluidine blue O allowed to observe phenolic compounds as plant response to the wound in the vascular cambium (Figure 16A-C) and mainly to the infection in the phloem, vascular cambium, vessel occlusions, and in the pith of plants inoculated with *L. brasiliensis* (Figure 16D-H).

Fontana-Masson stain allowed the observation of melanized mycelium in the inoculation zone, in the vascular cambium of the plant, as well as in the vascular bundles, even in those with occlusions (Figure 17).

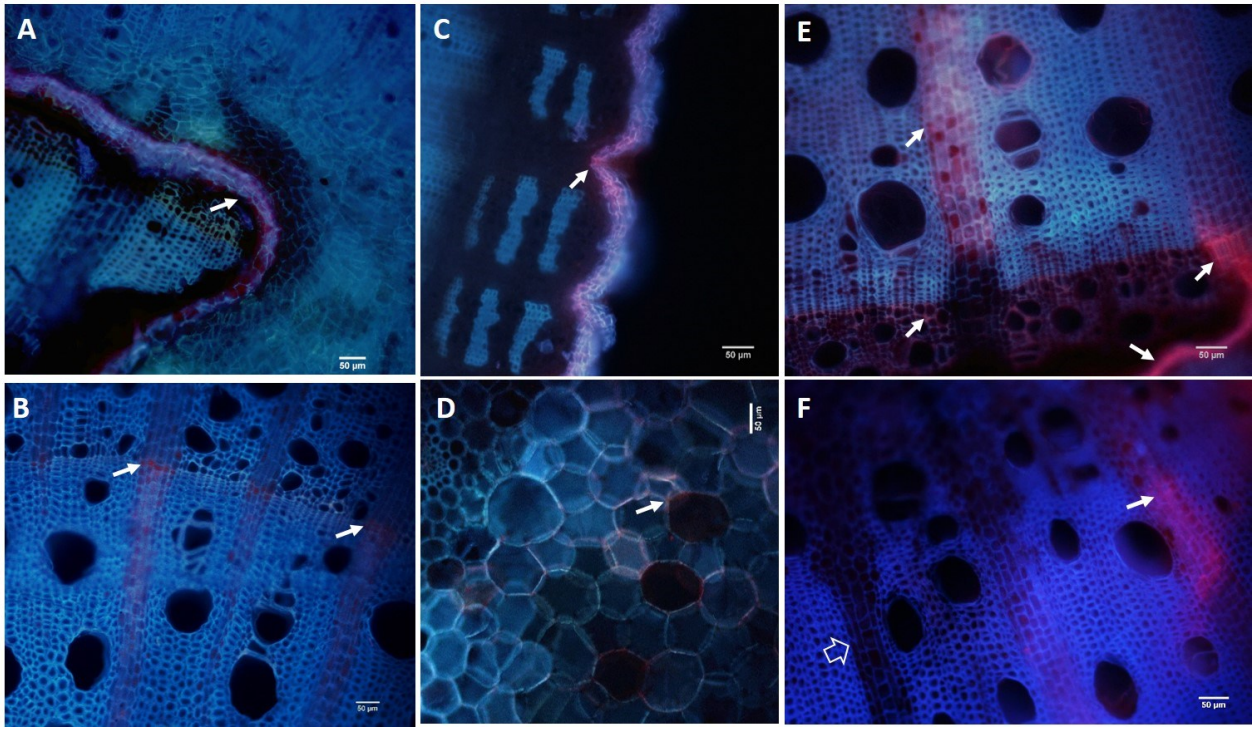

Figure 15. Epifluorescence of Grapevine plants cv. Cabernet Sauvignon showing suberin localization. A-F) Sudan IV stained sections observed under TEXAS RED and DAPI filters. A-B) Control plants presenting suberin in the cambium cork and ray parenchyma near the wound area. C-F) Infected plants with *Lasiodiplodia brasiliensis* 2-mpi showing suberin deposits in the cork (C), pith (D), vascular cambium, occlude vessels (E), and ray parenchyma (E-F). With arrow indicates the presence of suberin. Hollow arrow indicates ray parenchyma in the necrotic area.

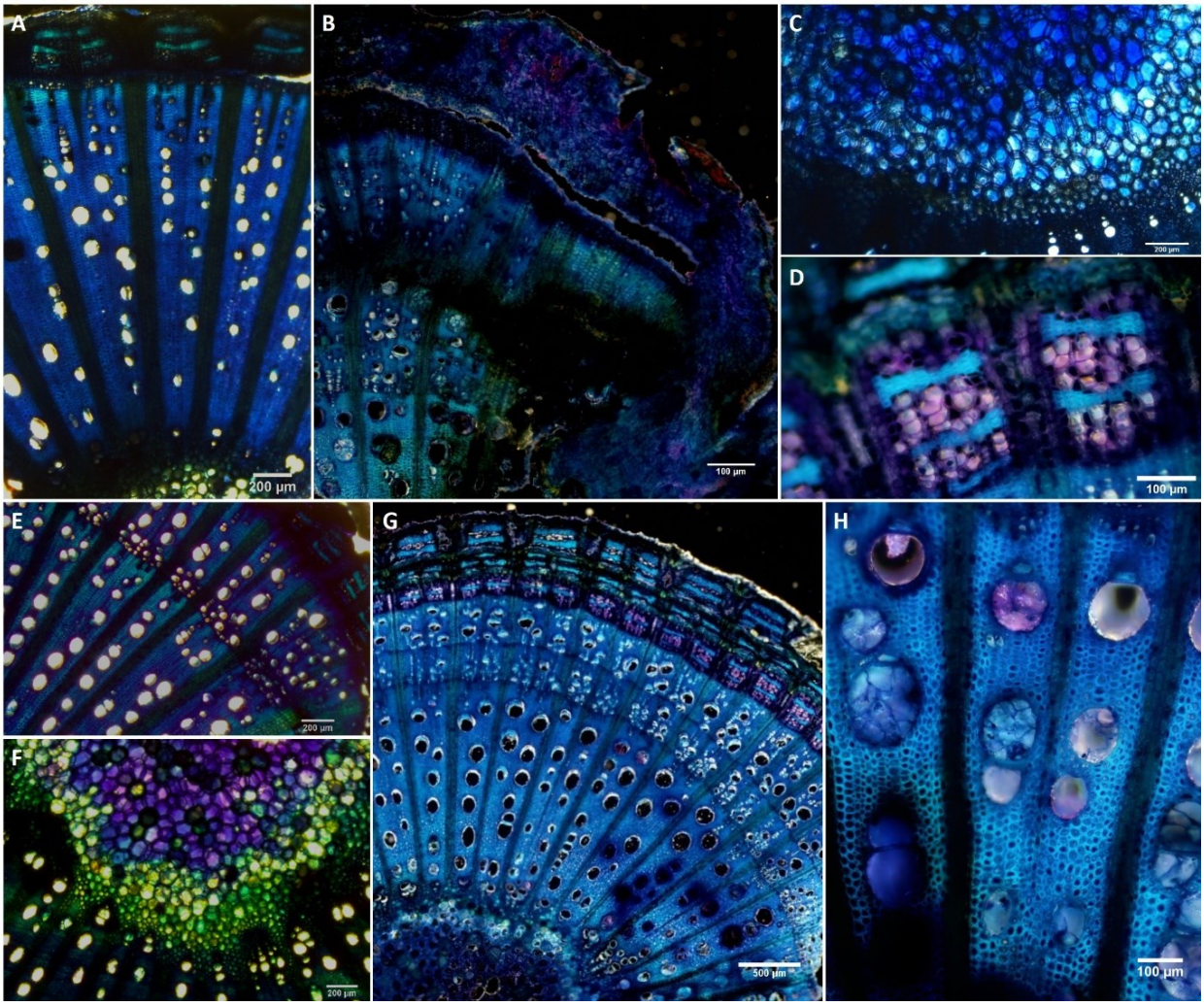

Figure 16. Toluidine blue O (TBO) stains of cross-sections of grapevine plants cv. Cabernet Sauvignon. A-C) Control plants showing phenolic compounds as response to the wound in vascular cambium and tissue regenerated. D-H) Infected plants with *Lasiodiplodia brasiliensis* showing phenolic compounds in the vascular cambium, phloem (D, E, G), pith (F), and vessel occlusions (H).

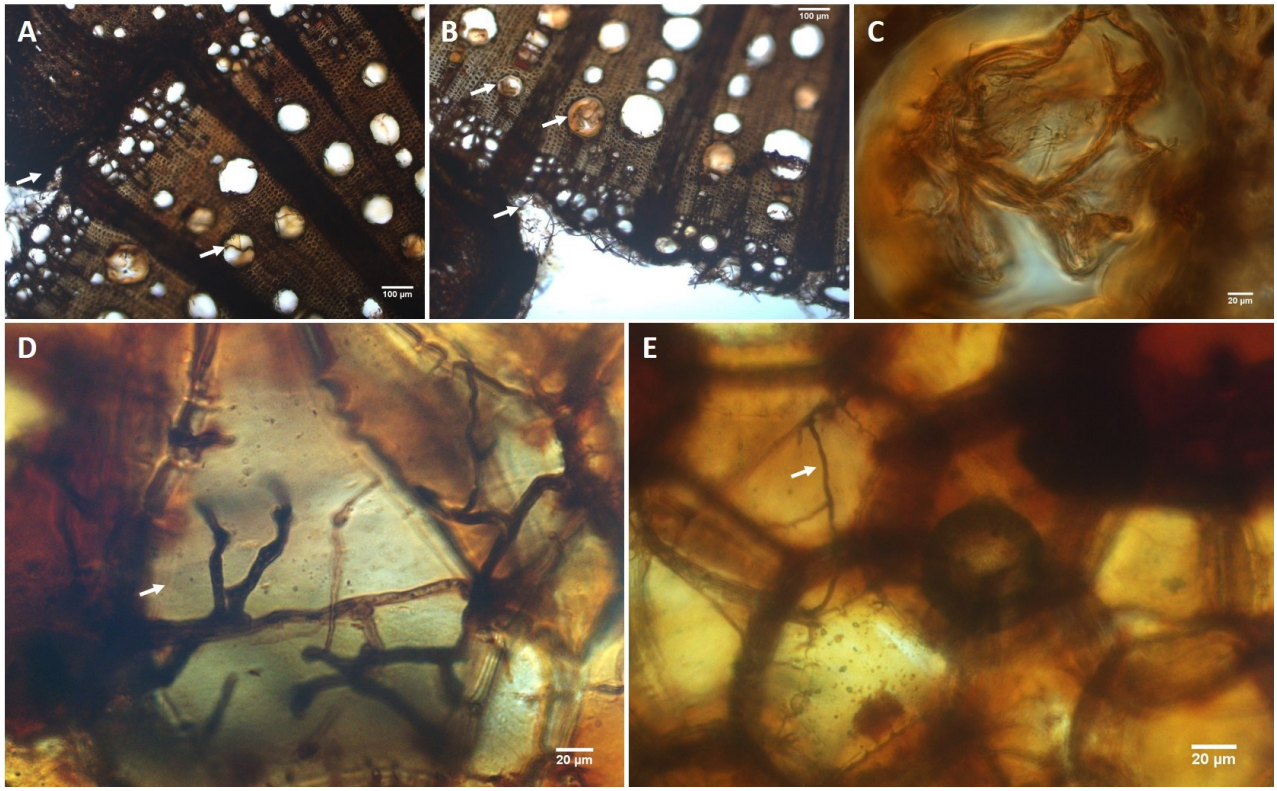

Figure 17. Melanin staining of *Lasiodiplodia brasiliensis* in grapevine plants cv. Cabernet Sauvignon 2-mpi using the Masson-Fontana method. A-B) Melanized fungus colonizing the vascular cambium and vascular bundles. C) Vessel occlusion. D-E) Melanized fungus colonizing the plant piths. Withe arrow indicates melanized hyphae.

4.3.2. Evaluation of carbon sources for growth of *Lasiodiplodia brasiliensis* MXBCL28

The growth of *L. brasiliensis* MXBCL28 without a carbon source was feeble, the mycelium stunted (Figure 18A), and biomass production was deeply affected (Table 7). *Lasiodiplodia brasiliensis* had a significantly higher growth rate and biomass production using pectin as a carbon source (Figure 18C) or ground grapevine wood (Figure 18K). The fungus used xylan, xylose, and starch as carbon sources, similarly to glucose (Figure 18B, D-F). In all these carbon sources, the fungus produces dense aerial mycelium and dark gray coloration. The fungus produced less aerial mycelium with glycerol and tannic acid as a carbon source (Figure 18G-H). The lower growth rate and biomass was observed using cellulose and lignin (Figure 18I-J) as a carbon sources.

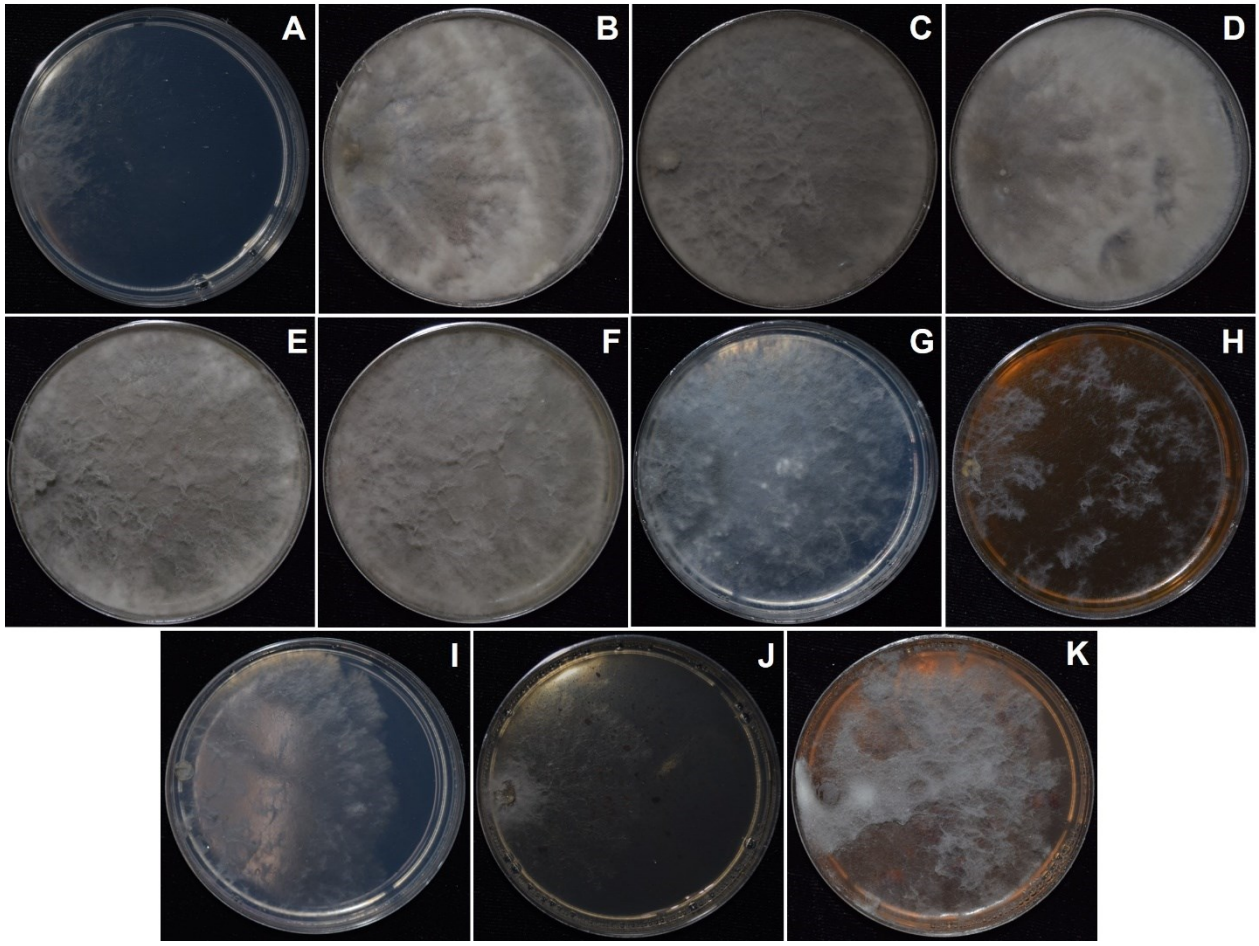

Figure 18. Growth of *Lasiodiplodia brasiliensis* MXBCL28 in different carbon sources. A) MM9 without carbon source (MM9-C), B) MM9+Glucose, C) MM9+Pectin, D) MM9+Xylose E) MM9+Xylan, F) MM9+Starch, G) MM9+Glycerol, H) MM9+Tannic Acid, I) MM9-Cellulose J) MM9+Lignin K) MM9+Wood.

Table 7. Biomass production and rate growth of *Lasiodiplodia brasiliensis* MXBCL28 using different carbon sources.

Carbon source	Biomass (mg)	Rate growth (mm/day)
Glucose	42.3±11.2 cd	14.7±0.5 b
Pectin	57.6±3.9 b	17.7±0.8 a
Starch	33.7±6.9 de	9.7±0.2 d
Xylan	47.6±7.7 bc	11.7±0.6 c
Xylose	40.4±2.4 cd	9.3±0.8 d
Glycerol	27.9±9.0 ef	8.4±0.7 de
Lignin	21.9±5.4 ef	4.8±0.2 f
Tannic acid	19.9±5.7 f	12.6±0.5 c
Cellulose	28.4±1.6 ef	7.0±0.9 e
Grapevine wood	70.3±12.6 a	18.7±1.764 a
No carbon source	7.2±1.0 g	N/A

4.4. Discussion

Fungi from the *Lasiodiplodia* spp. are among causal agents of Botryosphaeria dieback and one of the most aggressive pathogens of grapevine (Úrbez-Torres, 2011). The disease process and its establishment in the plants' vascular system are not fully documented (Claverie et al., 2020). In this study, the colonization process of *L. brasiliensis* was analyzed by microscopy, comparing grapevine plants wounded mechanically with those infected with *L. brasiliensis* MXBCL28. Regarding the presence of starch, whereas control plants recovered from the mechanical wound regenerating tissue to close the wound (Figure 1A) and showed stored starch granules (Figure 1C), plants infected with *L. brasiliensis* had a complete starch depletion in the ray parenchyma (Figure 1D-E). This observation and the dense aerial mycelium produced in media supplemented with starch indicate that *L. brasiliensis* uses starch as a carbon source. In woody plants, carbohydrate reserves are storage mainly as starch in the ray parenchyma; this is of great importance in the survival of the plant during the winter and for its vegetative growth the following spring (Noronha et al., 2018; Călugăr et al., 2019), and might be related to the activation of the host defense system (Shigo and Tippett, 1981). The ability to degrade grapevine starch has been reported in *Eutypa lata*, other fungi

associated with GTDs as the causal agent of Eutypa dieback (Rolshausen et al., 2008). Also, in grapevine plants infected with *Phaeomoniella chlamydospora*, a causal agent of Esca, partial starch depletion in parenchyma cells was observed next to occluded vessels and a severe depletion next to moderately occluded vessels (Pouzoulet et al., 2017). In the genome and transcriptome analyses of different *Lasiodiplodia* species, genes involved in the degradation of starch were found; in *L. gilanensis,* the expression of a putative amylase induced in the presence of grapevine wood was reported (Paolinelli-Alfonso et al., 2016), and in *L. theobromae* genes involved in starch metabolism were induced during infection (Yan et al., 2018). Thus starch seems to play an essential role in the colonization process of the fungus within the plant, which can use it as an energy source, degrading parenchyma cells and reducing the energy needed for the grapevine to active its defense system.

The plant cell wall is a complex structure network of structural support, plays an essential role in plant development and stress responses, and serves as a barrier for pathogens (Houston et al., 2016). Cell walls are composed of layers of different thicknesses of cellulose (50%), hemicelluloses (xylan, glucuronoxylan, xyloglucan, arabinoxylan, glucomannan) (20-25%), a matrix of lignin (25%) and pectin (1-4 %). In woody plants, lignin represents the most significant barrier to decay (Schwarze, 2007). Here, epifluorescent microscopy of plants infected with *L. brasiliensis*, stained with Calcofluor White M2R and Congo Red, showed dark zones without cellulose or hemicellulose closer to necrotic tissue, indicating that when the fungus colonizes the plant, it degrades the components of the cell wall (Figure 12). Hyphae of *L. gilanensis* have been observed invading grapevine parenchyma cells after 48 h of inoculation (Obrador-Sáncez and Hernandez-Martinez, 2020). The degradation of hemicellulose by *E. lata* when colonizes grapevine has also been reported (Rolshausen et al., 2008), and several genes involved in the plant cell wall degradation have been found in the genome and transcriptome of *L. gilanensis* and *L. theobromae* (Paolinelli-Alfonso et al., 2016; Yan et al., 2018; Félix et al., 2019; Gonçalves et al., 2019; Garcia et al., 2021; Nagel et al., 2021). This information supports the importance of plant cell wall-degrading enzymes (PCWDE) in these fungi to overcome the plant barrier defense and use cell wall components for growth during their establishment in the plant tissues.

Another important component of the plant cell wall is pectin, linked to hemicellulose forming a gel phase. In woody plants, a significant part of the pectic substances occurs as polygalacturonic acid in the middle lamella. This polysaccharide has several biological functions in plants, such as development, defense, cell-cell adhesion, wall structure, signaling, wall porosity, and binding of ions (Schwarze et al., 2007; Ochoa-Villarreal et al., 2012). Our results indicate that *L. brasiliensis* uses pectin as a carbon source since the fungus had a higher growth rate of 17.7 mm/day, exceeded only with ground grapevine wood in the

medium (18.7 mm/day), and the biomass production was higher than in glucose (Table 7). The expression of a pectate lyase was found up-regulated during the first stages of *L. gilanensis* infection in grapevine (Paolinelli-Alfonso, et al., 2016). According to this, the metabolism of pectin is of great importance for the vegetative growth of the fungus and during the colonization of the plant. Furthermore, in this assay, *L. brasiliensis* also had high growth in xylose (9.3 mm/day) and xylan (11.7 mm/day), producing dense pigmented aerial mycelium, similar as in glucose, pectin, or starch. A lower growth rate and biomass production of the fungus was observed in cellulose (7.0 mm/day) and lignin (4.8 mm/day) (Table 7, Figure 17); therefore, even though the fungus has the ability to degrade cellulose, it may not be necessary in the first steps of colonization.

In woody plants, compartmentalization of tissue is a general defense response to wounds and stops or restricts the spread of pathogens. The model of compartmentalization of decay in trees (CODIT) describe how woody tissues are sectioned resulting in four walls of defense against decay: 1) the occlusion of xylem vessels (callus, tyloses, gels), 2) the vascular cambium, 3) the ray parenchyma, and 4) the barrier zone (depositions of lignin, suberin, and phenolic compounds) (Shigo and Marx, 1977; Tippett and Shigo 1981; Pearce, 1996; Pearce, 2000). In the barrier zone, the cell wall suffers modifications by the deposition of lignin or suberin; lignified cell walls inhibit the radial movement of pathogens (Morris et al., 2016). The stain of lignin using phloroglucinol-HCl and Mäule method showed changes in lignified tissue in the necrotic area of infected plants with *L. brasiliensis*, mainly surrounding occluded vascular bundles and parenchyma cells (Figure 13). A lower growth rate and biomass production of the fungus was observed in the presence of lignin in the medium (4.8 mm/day) (Table 7, Figure 17). Genes involved in lignin degradation were found down-regulated in grapevine plants infected with *L. gilanensis* until 15-dpi, suggesting that lignin break-down is not required in the first steps of infection (Paolinelli-Alfonso et al., 2016). However, once the fungus establishes in the secondary xylem tissues, it could degrade lignin, weakening the secondary wall of the vascular tissue.

Vessel occlusion was observed in both the control and infected plants, but mainly in those inoculated with *L. brasiliensis,* where the accumulation of phenolic compounds was observed (Figure 15H). These occlusions are formed into vascular bundles by depositions of resins, pectin, polysaccharides, crystals, and phenolic compounds (De Micco et al., 2016). Furthermore, accumulation of phenolic compounds was observed along the vascular cambium, phloem, and pith tissues (Figure 15D-G). Several genes involved in phenolic metabolism were found in *L. gilanensis* during *in vitro* response to heat shock in the presence of grapevine wood (Paolinelli-Alfonso et al., 2016); and in *L. theobromae* infecting grapevine plants (Gonçalves et al., 2019).

Suberin is a lipid-phenolic biopolymer glycerol-based consisting of a polyaliphatic polyester linked with phenolic components, especially ferulic acid, and embedded waxes (Vishwanath, et al., 2014). Suberin is deposited in the cell walls of certain plant tissues serving as a protective barrier against pathogens. The amount of suberin and lignin can be related to phenols accumulation in parenchyma (Morris et al., 2020). Grapevine plants infected with *L. brasiliensis* showed suberin deposits mainly in vascular cambium, vascular cork, and ray parenchyma next to occluded vessels, where suberin was also observed in tyloses and the xylem fiber of vascular bundles (Figure 14). In *P. chlamydospora* infecting grapevine plants, suberin accumulation was observed in ray parenchyma and vessels walls; and cell wall modifications in xylem fibers and ray parenchyma have been associated with suberin rather than lignin deposits, this could efficiently restrict the fungus spread since *P. chlamydospora* is not able to alter secondary cell wall (Pouzoulet et al., 2013, Pouzoulet et al., 2014; Pouzoulet et al., 2017).

Nonetheless, suberin-degrading enzymes might be necessary for wood decay fungi to penetrate the highly resistant periderm surface (Pearce, 1996). Suberin degradation has been reported in *Fusarium solani* f. sp *pisi* that uses suberin from potato tuber periderm as a carbon source and has a cutinase-like esterase (Fernando et al., 1984). *Rosellinia desmazieresii,* which causes ring-dying in *Salix repens,* also degrades suberin (Ofong and Pearce, 1994). *Lasiodiplodia brasiliensis* grew using glycerol as a carbon source with a rate growth of 8.4 mm/day (Table 7). Some esterase genes have been reported in *L. gilanensis* and *L. theobromae* (Paolinelli et al., 2016; Yan et al., 2018), suggesting that *Lasiodiplodia* spp. degrades suberin of plant tissues during the colonization process.

Melanized hyphae of *L. brasiliensis* were observed in the vascular cambium near the inoculation point, vascular bundles, occlusions, and mainly in the pith (Figure 16). Similar observations were made in *L. gilanensis* colonizing grapevine plants one-month post-inoculation (Rangel-Montoya et al., 2020). Melanin is considered a virulence factor in some phytopathogenic fungi (Eisenamn et al., 2020). In *L. gilanensis* several genes involved in the synthesis of different melanin pathways were found differentially expressed in the presence of grapevine wood and heat shock (genes involved in DHN-melanin and pyomelanin pathways) and without heat shock (genes involved in DOPA-melanin pathway) (Paolinelli-Alfonso et al., 2016). In plants, phenylalanine is a precursor of the synthesis of lignin and suberin (Lewis et al., 1987) and could be a precursor of DOPA-melanin and pyomelanin pathways being converted to tyrosine (Eisenman and Casadevall, 2012; Schmaler-Ripcke et al., 2009). In *L. gilanensis,* melanin protects against UV radiation, enzymatic lysis, and reactive oxygen species; and the fungus can grow using tyrosine as carbon and nitrogen sources and uses this amino acid as a DOPA-melanin precursor (Rangel-Montoya et al., 2020).

Therefore, melanin might play an important protective role for the fungus during colonization, and it could use phenylalanine or tyrosine of the plant in its metabolism.

Lasiodiplodia seems to overcome plant defenses and modify the cell walls of grapevine. Therefore, we propose that once *Lasiodiplodia* conidia penetrate the grapevine plant through a wound and germinate, the fungus initiates the production of PCWDE. Hemicellulose seems to be preferred by the fungus over cellulose. Melanin synthesis inside the plant might protect the fungus from the plant defense mechanisms while it continues the colonization process using mainly pectin and starch as carbon sources and producing different secondary metabolites that induce phenolic compounds of the plant. Eventually, the fungus also degrades lignin and suberin, moving from ray to ray, which induces the formation of the typical shape of Botryosphaeria canker.

Chapter 5. Biological control of *Lasiodiplodia* species

5.1. Introduction

Botryosphaeria dieback is one of the most destructive grapevine trunk diseases (GTDs) caused by fungi from the Botryosphaeriaceae family (Bertsch et al., 2013; Fontaine et al., 2016). These fungi affect a wide range of economically important woody plants globally. In grapevine, main symptoms include vascular discoloration, cankers in shoots and cordons, dieback, and eventually the death of the plant (Úrbez-Torres, 2011). The primary source of inoculum is conidia that, when released into the environment dispersed under humid conditions, rain and wind currents; penetrate the plants mainly through pruning wounds (Spagnolo et al., 2014). Once inside, the conidia germinate and colonize the vascular system (van Niekerk et al., 2006; Úrbez-Torres et al., 2010). These GTDs pathogens reduce yields and grapevine longevity (Gramaje et al., 2018; Billones-Baaijens and Savocchia, 2019), causing significant economic losses. For instance, in South Australia up to AUD$2,800 per ha (Wicks and Davies 1999) and in California up to $USD260 million per year (Siebert 2001).

Several fungal species from the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neoscytalidium, Neofussicoccum*, *Phaeobotryosphaeria*, and *Spencermartinsia* have been identified associated with Botryosphaeria dieback (Úrbez-Torres 2011; Rolshausen *et al.,* 2013; Stempien *et al.,* 2017; Gramaje *et al.,* 2018). *Lasiodiplodia theobromae* has been reported as the most virulent pathogens within the family (Úrbez-Torres and Gubler, 2009).

Currently, there are no efficient treatments for trunk diseases, mainly due to the complexity of fungal species associated (Mondello et al., 2018). The management of GTDs is based on preventive strategies through cultural practices such as the use of disease-free propagation material, performing pruning in dry seasons, avoiding deep wounds, application of protective paint on pruning wounds, retraining of new shoots, cutting and removing infected plants to avoid the risk of reinfection and spread of pathogens (Buckley and Gould, 2000; Epstein et al., 2008).

Sodium arsenite was used to control these diseases, but due to its toxicity, it was banned in 2001 (van Niekerk et al., 2006). Among several evaluated fungicides, benomyl, tebuconazole, prochloraz, magnesium chloride, fusilazole, and fenarimol have demostrated an inhibitory effect on the mycelial growth of *Neofusicoccum australis*, *Diplodia obtusa*, *Neofusicoccum parvum* and *Botryosphaeria rhodina* (Bester and

Fourie, 2005; van Niekerk et al., 2006). Methyl thiophanate has 80% protection against fungal species of the Botryosphaeriaceae family when applied to prune wounds (Rolshausen et al., 2010). The fungicides carbendazim, fluazinam, and tebuconazole have also been tested against *D. seriata* and *D. mutila* to protect fresh pruning wounds reducing infection by 65% (Pitt et al., 2012). Nonetheless, chemical products can be toxic and represent a danger to human health.

The use of biological control agents (BCAs) is an alternative to chemical treatments. BCAs have different mechanisms against fungal pathogens, which can be physical and chemical such as antibiosis through the production of toxic compounds, competition for the substrate or nutrients, and parasitism (Köhl et al., 2019). Among the BCAs tested as wound protectants for the control of grapevine trunk diseases are different species of the genus *Trichoderma* (Mortuza and Ilag, 1999; Sultana and Ghaffar, 2010; Kotze et al., 2011; Bhadra et al., 2014) and *Bacillus subtilis* (Zhang et al., 2014; Sajitha et al., 2016). *Bacillus* species are ubiquitous, present in the soil and the rhizosphere of plants, produce a wide array of compounds to inhibit the growth of plant pathogens, induce plant systemic resistance and promote plant growth (Fira et al., 2018; Khan et al., 2021).

The management of Botryosphaeria dieback disease is limited and poorly efficient; thus, it is necessary to search for new strategies to mitigate these diseases, being the use of BCAs a potential alternative. Several species of *Bacillus* have been reported to have potential as BCAs against GTDs fungi, however, most of the evaluation has been carried out only *in vitro* conditions (Mondello et al., 2018), and the search for microorganisms adapted to the environmental conditions where they will be applied is necessary. This study aimed to evaluate the use of *Bacillus* strains as biological control agents against *Lasiodiplodia* spp.

5.2. Materials and Methods

5.2.1. Microorganisms and culture conditions

5.2.1.1. Fungal isolates.

The fungal isolates used were *L. brasiliensis* MXBCL28, MXVS16a, and MX VM18, *L. gilanensis* MXBCCS01, and *L. exigua* MXVS21b isolated from Baja California and Sonora (Rangel-Montoya et al., 2021); and *L.*

gilanensis UCD256Ma isolated from Madera Country, California (Úrbez-Torres et al., 2006). All fungal isolates were preserved in 20% glycerol at 4°C and cultivated in PDA Difco medium at 30°C.

5.2.1.2. Bacterial isolates.

Bacterial isolates were recovered from the collection of the plant pathology laboratory of CICESE. BsA3MX and BsC11MX obtained from San Quintin, a semi-arid region in Baja California, showed inhibition against *Macrophomina phaseolina* in a previous study. Those isolates were identified using primers 27F and 1492R (Lane, 1991) to amplify the 16Sr RNA gene region. Genomic DNA was extracted using CTAB protocol (Lee et al., 1988). PCR reaction was carried out in a total volume of 25 µL, containing 1 µL of genomic DNA (30 $ng \cdot \mu L^{-1}$), 2.5 µL of Taq Buffer10X, 0.5 µL of dNTP mix (20 mM), 0.625 µL of each primer (10µM), 0.125 µL of Taq DNA polymerase (GoTaq® DNA polymerase, Promega, 5 $units \cdot \mu L^{-1}$) and adjusted to final volume with distilled water. Amplification reactions were carried out in a Bio-Rad T-100 thermal cycler set to the following conditions: an initial step of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1:30 min, and a final cycle of 72°C for 10 min. PCR reactions were purified using GeneJet PCR purification kit and sequenced in the Clemson University Genomics Institute. The sequences were analyzed using BioEdit Sequence Alignment Editor and deposited in the GenBank (http://www.ncbi.nlm.nih.gov). A BLASTn was performed against the GenBank 16S Ribosomal RNA sequences database, and those with the highest similarity for each phylogenetic marker were used (Supplementary 3, Table 10) to construct the alignment using ClustalW. Maximum Likelihood (ML) and Maximum Parsimony (MP) analysis were performed using MEGA-X (Kumar et al., 2018) with Bootstrap values based on 1,000 replicates. Gaps were treated as missing data. The tree was visualized in MX: Tree Explorer.

5.2.2. Growth characterization of bacterial isolates

For bacteria strains, cultural, physiological, and biochemical characteristics were determined as follows. Bacterial colonies were grown on TSA ($g \cdot L^{-1}$: 15.0 Tryptone, 5.0 soytone, 5.0 NaCl, 15.0 agar, pH was adjusted to 7.0) at 30 °C for 2 days and then examined for Gram reaction and endospore formation. Activities for hemolysis, oxidase, catalase, protease, and urease were evaluated; as well as the use of citrate, and motility (Cowan, 2004). Acid production from sugars (glucose, dextrose, arabinose, mannitol,

xylose, lactose, trehalose, amylose, and methyl-α-D-glucoside) was evaluated following standard procedures (Cowan, 2004). Then, bacteria isolates were grown in trypticase soy broth (TSB) ($g \cdot L^{-1}$: 15.0 Tryptone, 5.0 soytone, 5.0 NaCl, pH was adjusted to 7.0) at different salt concentrations to test salt tolerance (0–15 % NaCl), different temperatures (4–55°C), and different pH values (4-10).

5.2.3. Plant growth-promoting activities

5.2.3.1. Biofilm formation

Bacteria isolates were grown in 96-well polystyrene microplates in TSB medium. The plates were incubated at 30 °C and 50 rpm for 48 h. Biofilm formation was evaluated by adding 75 µL of 10% crystal violet to each microplate dish well containing bacterial culture- at room temperature for 15 min, and the excess colorant was removed by rinsing with distilled water. The presence of a violet ring on the surface of the culture indicated biofilm formation (O'Toole et al., 1999).

5.2.3.2. Siderophores production

Siderophore production was evaluated using the Chrome azurol S (CAS) agar method (Schwyn and Neilands, 1987). Strains were streaked on the CAS agar and incubated at 30 °C for 48 h. The presence of a yellow halo around the bacterial colonies was a positive result for siderophore production.

5.2.3.3. Indole-3-acetic-acid (IAA) production

The production of Indole-3-acetic-acid (IAA) was evaluated by growing the bacterial isolates in TSB medium supplemented with 500 $\mu g \cdot mL^{-1}$ tryptophan at 30 °C and 110 rpm for 48 h (Ahmad et al., 2008). Bacterial cells were harvested at 10,000 rpm for 3 min. An aliquot of 200 µL of the free-cell supernatant was placed in 96 wells microplates, following by the addition of 100 µL of Salkovsky's reagent (Gordon and Weber, 1951). The appearance of pink-orange color after 25 min indicated IAA synthesis.

5.2.3.4. HCN production

Hydrogen cyanide (HCN) production by the bacterial isolates was analyzed growing bacterial isolates in 96 wells microplates with TSA medium. After growth, a filter paper soaked in a 0.5% sodium carbonate solution in 0.5% of Picric acid was placed on the top. The microplates were sealed with parafilm and incubated for 48 h at 30 °C. The development of orange-red color in the filter paper indicated HCN production (Ahmad et al. 2008).

5.2.3.5. 1-Aminocyclopropane-1-carboxylate (ACC)-deaminase production

The minimum medium DF ($g \cdot L^{-1}$: 4.0 KH_2PO_4, 6.0 Na_2HPO_4, 0.2 $MgSO_4 \cdot 7H_20$, 2.0 glucose, 2.0 gluconic acid, 2.0 citric acid, 1.0 mL trace element solution ($mg \cdot 100\ mL^{-1}$: 1.0 $FeSO_4 \cdot 7H_20$, 10.0 H_3BO_3, 11.19 $MnSO_4 \cdot H_20$, 124.6 $ZnSO_4 \cdot 7H_2O$, 78.22 $CuSO_4 \cdot 5H_2O$, 10.0 MoO_3) 15.0 agar, pH was adjusted to 7.2) supplemented with a 0.5 M ACC solution was used to evaluate ACC deaminase enzyme activity (Dworkin and Foster, 1958). DF medium supplemented with $(NH_4)_2SO_4$ ($2\ g \cdot L^{-1}$) was used as positive control and DF medium as negative control (Penrose and Glick, 2003). Bacteria were plated on each medium and incubated at 30 °C for 4-days. The uniform growth of the isolates on the DF medium supplemented with ACC was considered a positive result (Govindasamy et al., 2009).

5.2.3.6. Nitrogen fixation

Nitrogen fixation by bacteria was evaluated according to Baldani et al. (2014). Solution A (950 mL: 5.0 g malic acid, 0.5 g K_2HPO_4, 0.5 g $FeSO_4$, 0.010 g $MnSO_4$, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.002 g Na_2MoO_4, 0.02 g $CaCl_2$, 0.002 g bromothymol blue, 1.75 g agar) and Solution B (4 g KOH in 50.0 mL) were sterilized separately and mixed when they reached a temperature between 45 °C and 50 °C. The medium supplemented with NH_4Cl ($2.5\ g \cdot L^{-1}$) as a nitrogen source was used as a positive control. Plates containing each medium were inoculated with 10 µL of a 24 h bacterial culture and incubated at 30 °C for 8-days. A change in color of the medium from yellow to green was considered a positive result.

5.2.3.7. Phosphorous solubilization.

Phosphorus solubilization activity by bacterial isolates was determined using Pikovskaya medium ($g \cdot L^{-1}$: 5.0 yeast extract, 10.0 glucose, 5.0 $Ca_3(PO_4)$, 0.5 $(NH_4)_2SO_4$, 0.2 KCl, 0.1 $MgSO_4$, 0.0001 $MnSO_4$, 0.0001 $FeSO_4$, 0.01 bromocresol purple, 15.0 agar; pH was adjusted to 7.2) (Pikovskaya, 1948; Corrales-Ramírez et al., 2014). Plates containing the medium were inoculated with 10 µL of a 24 h bacterial culture and then incubated at 30 °C for 72 h. A change of color of the medium from purple to yellow was considered a positive result.

5.2.3.8. Potassium solubilization

Potassium solubilization activity by bacterial isolates was determined using modified Pikovskaya medium ($g \cdot L^{-1}$: 5.0 yeast extract, 10.0 glucose, 5.0 KNO_3, 0.5 $(NH_4)_2SO_4$, 0.2 KCl, 0.1 $MgSO_4$, 0.0001 $MnSO_4$, 0.0001 $FeSO_4$, 0.002 bromocresol green, 15 g agar; pH was adjusted to 7.2). Plates containing the medium were inoculated with 10 µL of a bacterial culture of 24 h, incubated at 30 °C for 72 h. A color change of the medium from blue to yellow t was considered a positive result.

5.2.3.9. Zinc solubilization.

Zinc solubilization activity by bacterial isolates was determined using modified Pikovskaya medium ($g \cdot L^{-1}$: 5.0 yeast extract, 10.0 glucose, 5.0 KNO_3, 0.5 $(NH_4)_2SO_4$, 0.02 KCl, 0.1 $MgSO_4$, 0.0001 $MnSO_4$, 0.0001 $FeSO_4$, 12.0 ZnO, 0.25 bromothymol blue, 15.0 agar; pH was adjusted to 7.0) (Komal and Kalavati, 2018; Tagele et al., 2019). Plates containing the medium were inoculated with 10 µL of a 24 h bacterial culture, incubated at 30 °C for 48 h. A color change from blue to yellow of the medium was considered a positive result.

5.2.4. Production of cell wall degrading enzymes

5.2.4.1. Chitinases

For chitinase activity detection, bacterial isolates were grown on a basal medium supplemented with colloidal chitin ($g \cdot L^{-1}$: 0.3 $MgSO_4.7H_2O$, 3.0 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 1.0 citric acid monohydrate, 4.5 colloidal chitin, 0.15 bromocresol purple, 200 µL Tween-80 and 15.0 agar; pH was adjusted to 4.7) (Agrawal and Koyasthane, 2012). Plates were incubated at 30 °C for 48 h. A positive result was considered when the color of the medium changed from yellow to purple.

5.2.4.2. Cellulases

For cellulase activity detection, bacterial isolates were grown on MM medium supplemented with carboxymethyl-cellulose (CMC) ($g \cdot L^{-1}$: 1.0 glucose, 2.5 yeast extract, 1% CMC, 15.0 agar) (Mazzucotelli et al., 2013). Plates were incubated at 30 °C for 48 h and stained with 0.2% Congo red dye. A positive result was considered when a yellow halo formation (8 mm or more) against red background was observed

5.2.4.3. Pectinases

For pectinase activity detection, bacterial isolates were grown on pectin agar ($g \cdot L^{-1}$: 1.0 $NaNO_3$, 1.0 KCl, 1.0 K_2HPO_4, 0.5 $MgSO_4$, 0.5 yeast extract, 10.0 pectin, 15.0 agar; pH was adjusted to 7.0) (Kumar et al. 2012). Plates were incubated at 30 °C for 48 h and flooded with Lugol's iodine solution. The presence of clear zones around the colonies after 5 minutes A was considered a positive result.

5.2.4.4. Amylases

For amylase activity detection, bacterial isolates were grown on starch agar ($g \cdot L^{-1}$: 10.0 peptone, 5.0 yeast extract, 5.0 NaCl, and 2.0 starch, 15.0 agar; pH was adjusted to 7.0) (Hankin and Anagnostakis, 1975).

Plates were incubated at 30 °C for 48 h and flooded with Lugol's iodine solution. The presence of clear zones around the colonies after 5 minutes A was considered a positive result.

5.2.5. Bacterial antagonistic activities against *Lasiodiplodia* spp.

5.2.5.1. Inhibition dual tests *in vitro*

The biocontrol potential of bacterial isolates was evaluated by performing dual inhibition assays. A loop of each bacteria was inoculated approximately 2 cm from each edge of the Petri dish in a straight line of around 4 cm, followed by the inoculation of a 5 mm diameter mycelial disc in the center of the different fungal pathogens (UCD256Ma, MXCS01, MXLBC28, MXVS16a, and MXVS18). Control plates were inoculated with the bacterial isolates and the pathogen separately. The plates were incubated at 30 °C for around 5-days until control plates were entire covered by the fungal pathogens. Mycelial growth was monitored every 24 h by marking the edge of the colony. Phenotypic characteristics of the colony were recorded, such as pigmentation and aerial mycelium formation. Three measures in different directions were made to obtain the radial growth of each fungal pathogen, and the inhibition percentage was calculated using the formula: inhibition percentage (%) = ((R1 – R2)/R1) x 100 (Fokkema, 1976). R1 is the radial growth of different *Lasiodiplodia* isolates in control plates, and R2 is the radial growth of different *Lasiodiplodia* isolates in the presence of bacteria.

5.2.5.2. Inhibition by non-volatile compounds

The production of non-volatile compounds as antagonism mechanism was evaluated growing the bacteria isolates in TSB media at 30 °C and 120 rpm for 72 h. Bacterial cultures were centrifuged, and the recovered supernatant filtered. Cell-free supernatant (0.1, 0.5, and 1 mL) was spread on PDA plates and left to dry. Then, a mycelial plug of 5 mm diameter of each *Lasiodiplodia* isolate (UCD256Ma, MXCS01, MXLBC28, MXVS16a, and MXVS18) was inoculated at the center of the plate. Fungal pathogens were inoculated on PDA plates as controls. All plates were incubated at 30 °C for 5-days, and the mycelial growth was monitored every 24 h. Experiments were carried out in triplicate.

5.2.5.3. Inhibition by volatile compounds

Lasiodiplodia isolates (UCD256Ma, MXCS01, MXBCL28, MXVS16a, and MXVS18) were inoculated at the center of PDA plates using a mycelial plug of 5 mm of diameter. A PDA plate spread of a bacterial isolate replaced the lid. The two plates were sealed with insulating tape to avoid the escape of any volatile compound. The plates were incubated at 30 °C for 5-days, and the growth of the fungus recorded every 24 h. In the control plates, both the base and the lid contained the fungal pathogen or the bacterial strain. Mycelial growth and colony morphology were recorded every 24 h. Experiments were carried out in triplicate.

5.2.6. Effect of *Bacillus amyloliquefaciens* in the conidia germination of *Lasiodiplodia*

The production of pycnidia in *L. gilanensis* MXBCCS01 was induced using minimal medium 9 (MM9) agar supplemented with wood sticks under a white light fluorescence lamp period of 1.5 h in light irradiation and 30 min in darkness for 15 days. Conidia were collected under a stereoscopic microscope (Zeigen) using a dissection needle. The inhibition of the germination of conidia was evaluated by spreading 0.1 mL of 24 h culture of each bacterium on PDA plates. Cell-free supernatant of the bacterial isolates was obtained by filtering a bacterial culture of 24 h through a 0.22 µm filter. The inhibition of conidia germination by diffusible compounds produced by the bacteria was evaluated by adding 30% of cell-free supernatant to PDA media. Then, around 30 conidia were inoculated in each plate. Plates were incubated at 30 °C, and the percentage of conidia germination was evaluated after 24 h using a light microscope (Nikon Eclipse E200). Images of conidia were taken with a camera AxioCam HRc from Zeiss and analyzed using AxioVision 4.8.2. Experiments were done in triplicate.

5.2.7. Biocontrol assays *in planta*

Two assays were performed, one in 2019 and the other in 2020, to evaluate the biocontrol potential of bacterial isolates *in planta.*

In 2019, grapevine plants cv. Merlot were used to evaluate the biocontrol potential of bacterial isolates *in planta*. Bacteria isolates were used separately or mixed. Before inoculation, stems were surface-sterilized with 70% ethanol, and then a mechanical wound was made by drilling a hole of 2 mm in diameter. Two inoculation methods were made for preventive treatments. In the first method, 50 µL of a bacterium culture of $1x10^6$ CFU was inoculated in the fresh wound and left to dry. In the second method, cuttings were inoculated by pouring 50 mL of a bacterial suspension of $1x10^6$ CFU at the crown of each plant. In all treatments, stem wounds were made on the same day and covered with Parafilm® for protection. A second bacterial inoculation was made one week after, either into the wound or the soil, depending on the preventive method. Two weeks after the first bacteria inoculation, a mycelial disc of 2 mm diameter of MXBCL28 was inoculated over the wound and covered with Parafilm®. As controls, plants inoculated with discs of PDA medium and plants inoculated only with bacteria in the wound site or the soil were used. After a month, a third bacterial application was made into the wound or to the soil. Grapevine plants were kept in a greenhouse for two months. After, samples were taken by cutting the stem at the base of the plant to make a cross-section and assess the total size of the lesion. The number of leaves, the height of the plant, the weight of the fresh and dry foliage was also evaluated.

In 2020, grapevine plants cv. Cabernet Sauvignon were used. Bacteria isolates were evaluated independently or mixed. In this bioassay, the commercial product BACILLIOSS®, a biofungicide based on *Bacillus subtilis* spores, was also used to compare our bacterial isolates. All stems were surface-sterilized with 70% ethanol; then, a mechanical wound was made by drilling a hole of 2 mm diameter. Two preventive inoculations at soil or to the wound were made. For soil inoculation, 50 mL of bacterial suspension of $1x10^6$ CFU was poured at the crown of each plant. Stem wounds in the plants were made on each plant and were covered with Parafilm® for protection. One week later, a second bacterial inoculation of BsA3MX, BsC11MX, and BACILLIOSS® was made in the soil, and the fungal pathogens, *L. brasiliensis* MXL28 and *L. gilanensis* UCD256Ma, were inoculated into the stem wound using a mycelial disc of 2 mm of diameter. The wound was covered with Parafilm®. As controls, discs of PDA medium and plants inoculated only with bacteria in soil were used. A third bacterial inoculation was made after a month of the first inoculation. Grapevine plants were left in the greenhouse for two months. Samples were taken by cutting the stem at the base of the plant to make a cross-section and assess the total size of the lesion. Besides, the number of leaves, the height of the plant, the weight of the fresh and dry foliage was evaluated.

5.3. Results

5.3.1. Identification of *Bacillus* isolates

Bacterial isolates were identified based on the 16S ribosomal RNA gene; the sequences obtained were approximately 1,168 bp, the dataset comprised 1,276 characters, including gaps, after alignment, and 29 taxa. *Alicyclobacillus acidocaldarius* (DSM 446) was used as the outgroup taxon. Maximum likelihood analysis using Tamura 3-parameter model resulted in a tree with a log-likelihood value of -3201.92. The phylogenetic analysis of the 16S ribosomal RNA gene clustered the two fungal isolates, BsA3MX (MW651769) and BsC11MX (MW651770), within the *Bacillus amyloliquefaciens* clade (Figure 19).

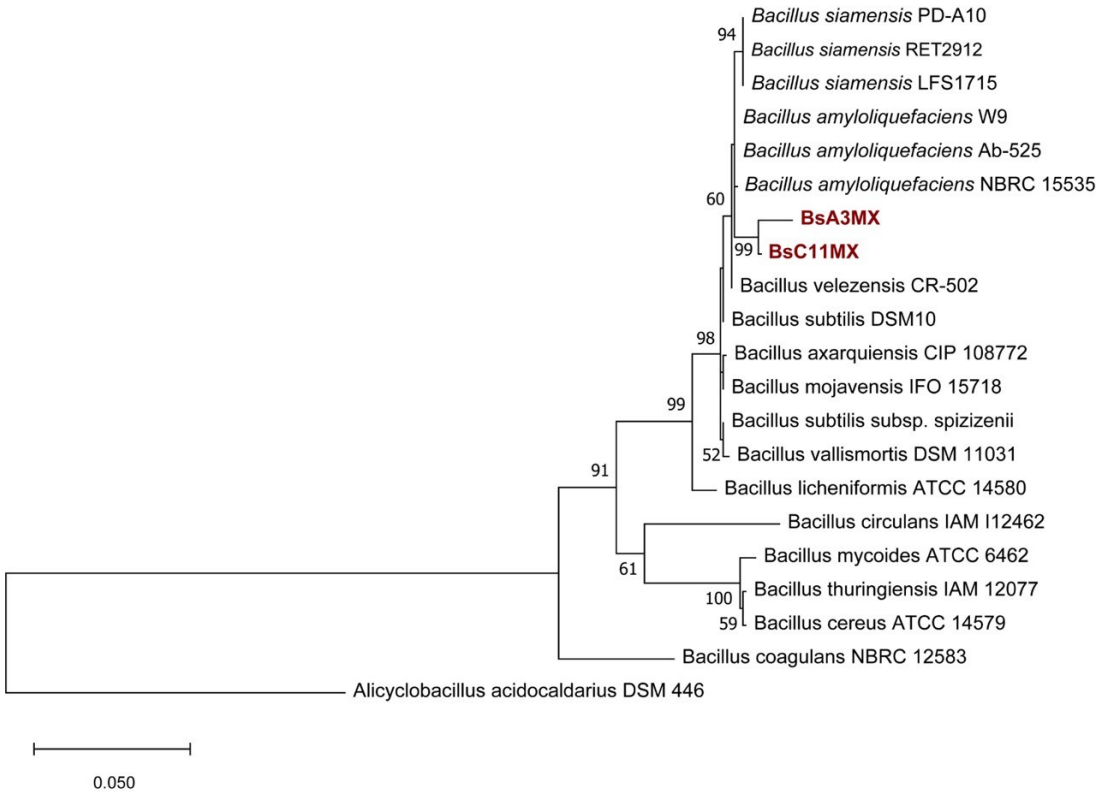

Figure 19. Phylogenetic analysis of *Bacillus amyloliquefaciens* isolates. Maximum likelihood tree with the highest log likelihood (-3201.92) obtained from the 16S ribosomal RNA gene dataset. The tree was rooted with *Alicyclobacillus acidocaldarius* (DSM 446). Bootstrap values greater than 50 are indicated at the nodes, and the isolates from this study are indicated in bold.

5.3.2. Characterization of *Bacillus amyloliquefaciens* isolates

Bacterial isolates BsA3MX and BsC11 were Gram-positive, motile, and endospore-forming rods. They formed irregular, flat, dry, and dull colonies creamy white on TSA medium with undulate margins and a thin biofilm at the surface on TSB after 24h incubation at 30 °C. Both isolates were halotolerant, able to grow in the presence of NaCl up to 10% (w/v) and in a minimum pH 5 and maximum pH 9. In addition, they grew at temperatures up to 50 °C (Table 7). Tests for hemolytic activity, urease, and citrate utilization were negative for both isolates, while tests for oxidase, catalase, and indole were positive. The preferential utilization of carbohydrates was different for each isolate (Table 7). BsA3MX preferred glucose, trehalose, dextrose glycogen, followed by xylose, mannitol, lactose, arabinose, and amylose, while BsC11MX preferred glucose, arabinose, dextrose, mannitol, and glycogen, followed by lactose, xylose, amylose, and trehalose. The use of methyl-α-D-glucoside was negative for both bacteria isolates.

Table 8. Characteristics of *Bacillus amyloliquefaciens* isolates.

Characteristic	**BsA3MX**	**BsC11MX**
Gram stain	+	+
	Irregular, flat, dry and dull colonies creamy white on TSA	
Colony morphology		
Form	Single rod-shaped	Single rod-shaped
Motility	+	+
Endospore formation	2-terminals	2-terminals
NaCl		
Maximum	10%	10%
Temperature		
Maximum	50 °C	50 °C
pH		
Minimum	5	5
Maximum	9	9
Hemolytic activity	-	-
Urease	-	-
Oxidase	+	+
Catalase	+	+
Protease	+	+
Citrate utilization	-	-
Indole test	+	+
Acid from:		

Glucose	+++	+++
Lactose	+	++
Arabinose	+	+++
Trehalose	+++	+
Dextrose	+++	+++
Amylose	+	+
Xylose	++	++
Mannitol	++	+++
Glycogen	+++	+++
Methyl-α-D-glucoside	-	-

5.3.3. Characterization of *Bacillus amyloliquefaciens* as plant growth promoter and production of hydrolytic enzymes

Both *Bacillus amyloliquefaciens* isolates produced siderophores and indole-3-acetic acid (IAA), presented ACC-deaminase activity and solubilized phosphate and zinc, and produced biofilm. They were negative for the production of cyanhydric acid, nitrogen fixation, and potassium solubilization. Strains BsA3MX and BsC11MX produced proteases (Table 8) and amylases and were negative for chitinase, cellulase and pectinase production (Table 9).

Table 9. Plant growth promotion characteristics and hydrolytic activity of *Bacillus amyloliquefaciens* isolates.

Characteristic	**BsA3MX**	**BsC11MX**
Siderophore production	+++	+++
IAA production	++	+
ACC-deaminase production	+	+
Nitrogen fixation	-	-
Cyanhydric acid production	-	-
P solubilization	+	+
K solubilization	-	-
Zn solubilization	+	+
Biofilm formation	+	+
Chitinase	-	-
Cellulase	-	-
Pectinase	-	-
Amylase	+	+

5.3.4. Antagonisms bioassays *in vitro*

The growth of the *Lasiodiplodia* isolates was inhibited up to 65 % by the bacterial isolates. UCD256Ma caused 65.82% growth inhibition, followed by MXVS16a with 62 %, MXBCL28 with 59.49 %, MXCS01 with 57.26 %, MXVS21b with 56.2 %, and MXVS18 with 56.3 3%. The presence of both bacteria induced the production of a pink pigment in all fungal isolates, which has been observed when these fungi grow under abiotic stress conditions as high temperature, and lack of nutrients (Alves *et al.,* 2008; Abdollahzadeh *et al.,* 2010; Rangel-Montoya et al., 2020). Besides, the fungal isolates formed a melanin barrier in the interaction zone with the bacteria as protection (Figure 20).

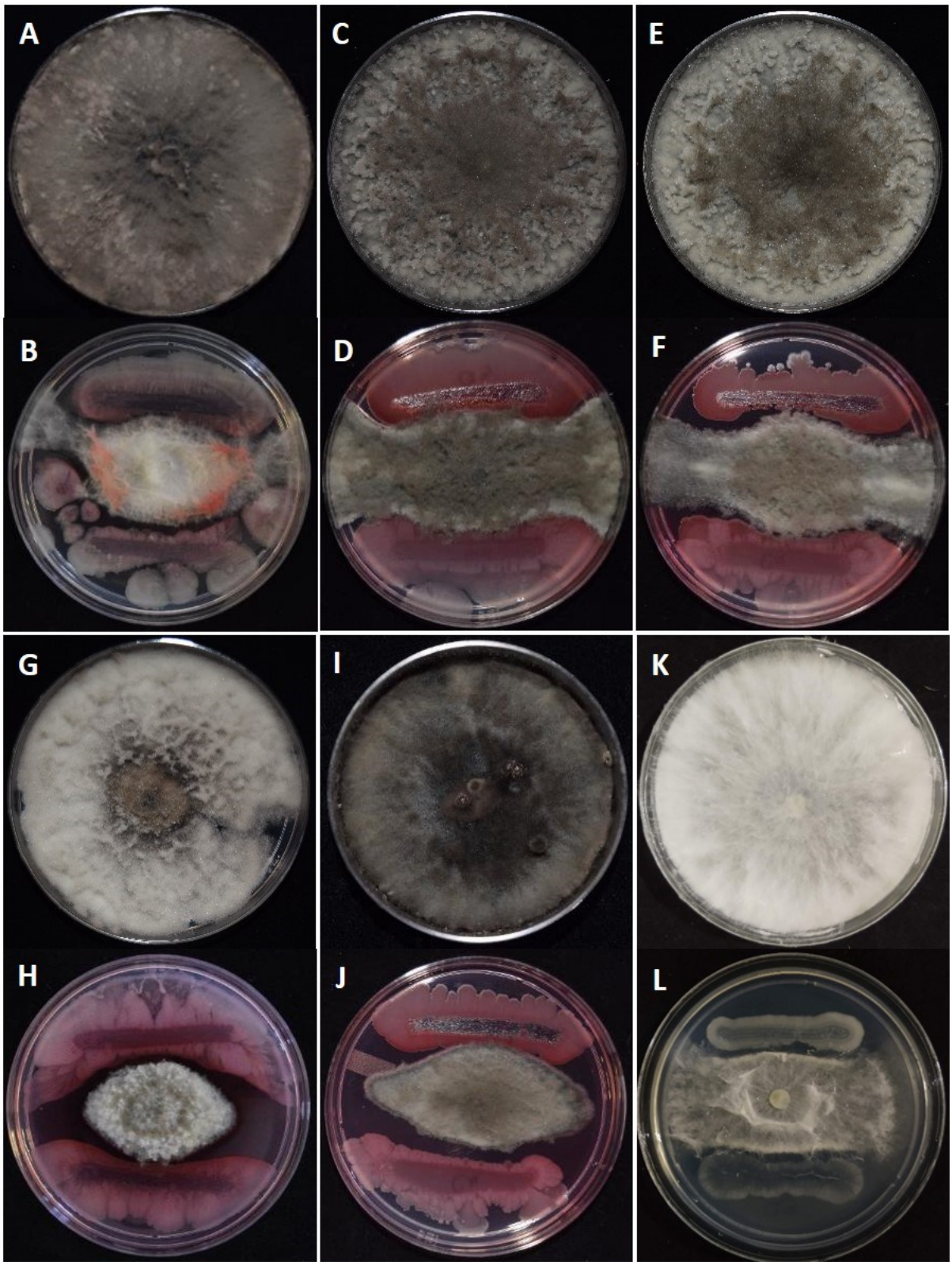

Figure 20. Biocontrol assays *in vitro Bacillus amyloliquefaciens* against *Lasiodiplodia* spp. A, B) *L. brasiliensis* MXBCL28; C, D) *L. brasiliensis* MXVS16a; E, F) *L. brasiliensis* MXVS18; G, H) *L. gilanensis* UCD256Ma; I, J) *L. gilanensis* MXCS01; K, L) *L. exigua* MXVS21b. A, C, E, G, I, K: Control; B, D, F, H, J, L: *B. amyloliquefaciens* against fungi (BsA3MX above and BsC11MX down).

5.3.5. Mechanisms of antagonisms of *Bacillus amyloliquefaciens*

5.3.5.1. Production of non-volatile compounds.

Besides the production of volatile compounds, *B. amyloliquefaciens* BsA3MX and BsC11MX produced non-volatile compounds that inhibited the growth of *Lasiodiplodia* isolates and affected the morphology of the colonies, as well as induced the production of a pink pigment in the fungi. In the control plates, *Lasiodiplodia* spp. covered all plate agar surface (Figure 21).

5.3.5.2. Production of volatile compounds.

B. amyloliquefaciens BsA3MX and BsC11MX produced volatile compounds that reduce the growth and pigmentation mycelia of *Lasiodiplodia* isolates. Mainly, volatile compounds inhibited the production of aerial mycelium, contrasting with the control treatments in which *Lasiodiplodia* isolates also grew on the cover plate (Figure 22).

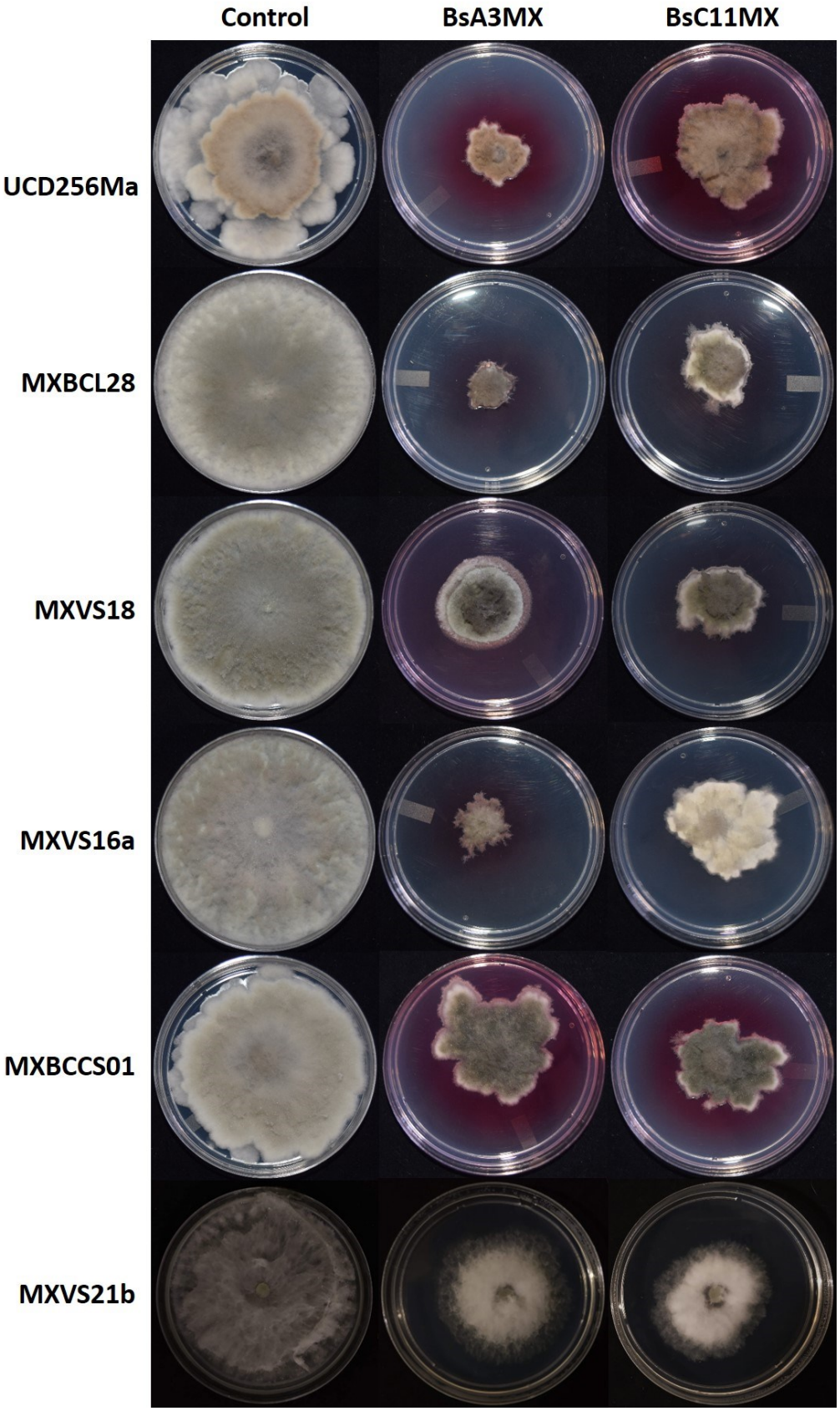

Figure 21. Growth inhibition of *Lasiodiplodia* spp. using 500 µL of cell-free supernatant on PDA of *Bacillus amyloliquefaciens* BsA3MX and BsC11MX.

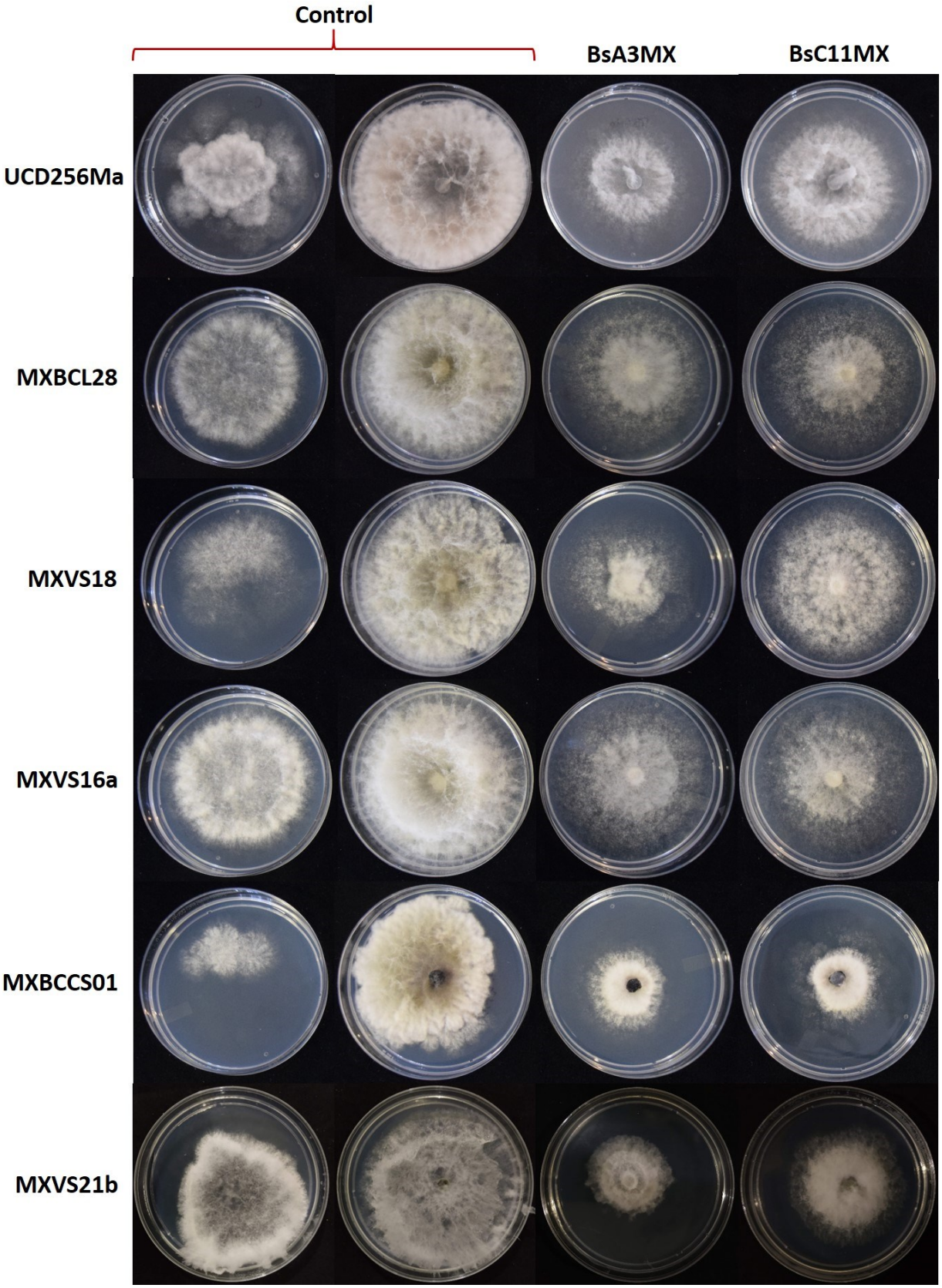

Figure 22. Growth inhibition of *Lasiodiplodia* spp. by volatile compounds produced by *Bacillus amyloliquefaciens* BsA3MX and BsC11MX.

5.3.6. Effect of *Bacillus amyloliquefaciens* in *Lasiodiplodia* conidia germination

Conidia inoculated in PDA medium germinated at 24 h of incubation (Figure 23A). The presence of both bacterial isolates plated in the culture medium completely inhibited the germination of *L. gilanensis* conidia (Figure 23F and G). In the presence of 30% cell-free bacterial supernatant in the medium, the germination conidia suffered swelling and cellular lysis (Figure 23B-E).

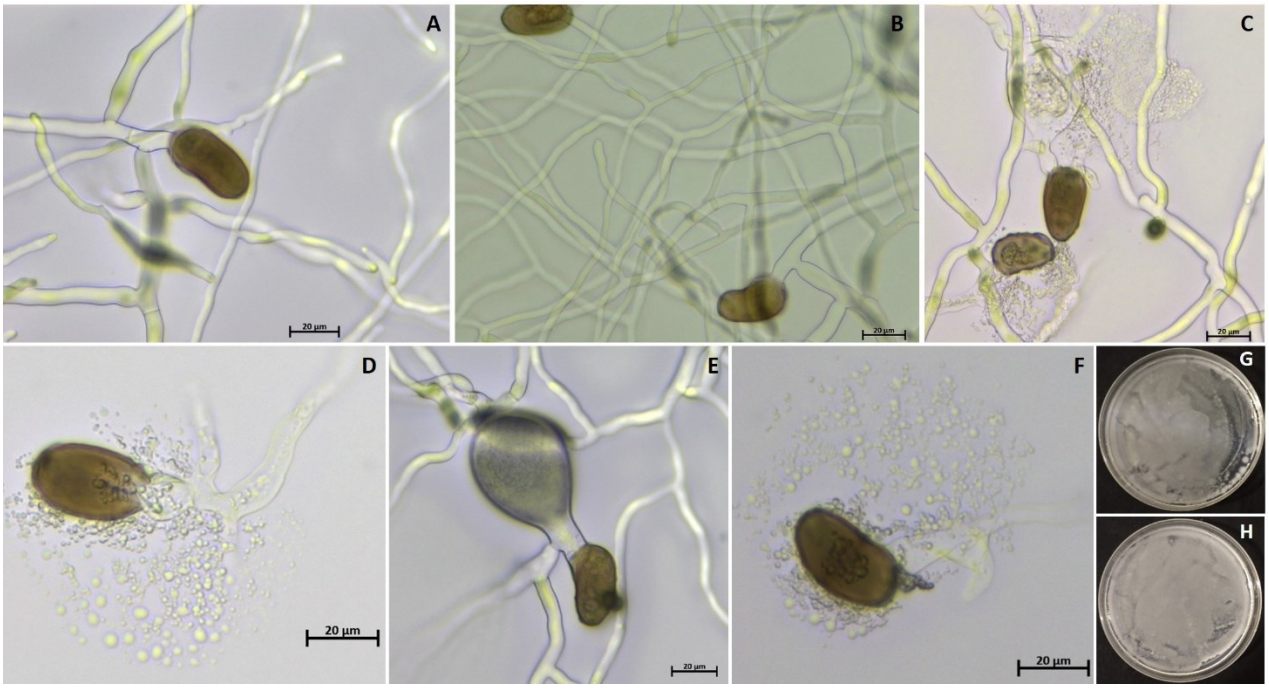

Figure 23. Effect of *Bacillus amyloliquefaciens* on the germination of *Lasiodiplodia gilanensis* conidia at 24 h. A-B) Conidia germinated on PDA medium (control), C-D) Conidia germinated using BsA3MX 30% CFS, E-F) Conidia germinated using BsC11MX 30% CFS, G) Conidia non germinated on BsA3MX, H) Conidia non germinated on BsC11MX. CFS: cell-free supernatant.

5.3.7. Biocontrol assay *in planta*

5.3.7.1. 2019 biocontrol assay

Plants inoculated only with *L. brasiliensis* MXBCL28 showed lesions up 6 cm of length (Figure 24, 25A) above and below the inoculation point. Plants inoculated only with bacteria either into the fresh wound or in the soil showed no evident damage and tissue regeneration, similar to control plants. Plants inoculated with bacteria showed reduced necrotic lesions in both preventive treatments (Figure 24). There was no difference in the number of leaves among treatments. Regarding height, plants inoculated with *L.*

brasiliensis MXBCL28 were similar to the control; those inoculated with the bacterial isolates were higher, especially the plants inoculated in soil using BsA3MX, but without significant differences. Similarly, plants inoculated only with the fungus had similar foliage to the control, but those inoculated with bacteria showed a significantly higher dry weight of foliage (Figure 25B).

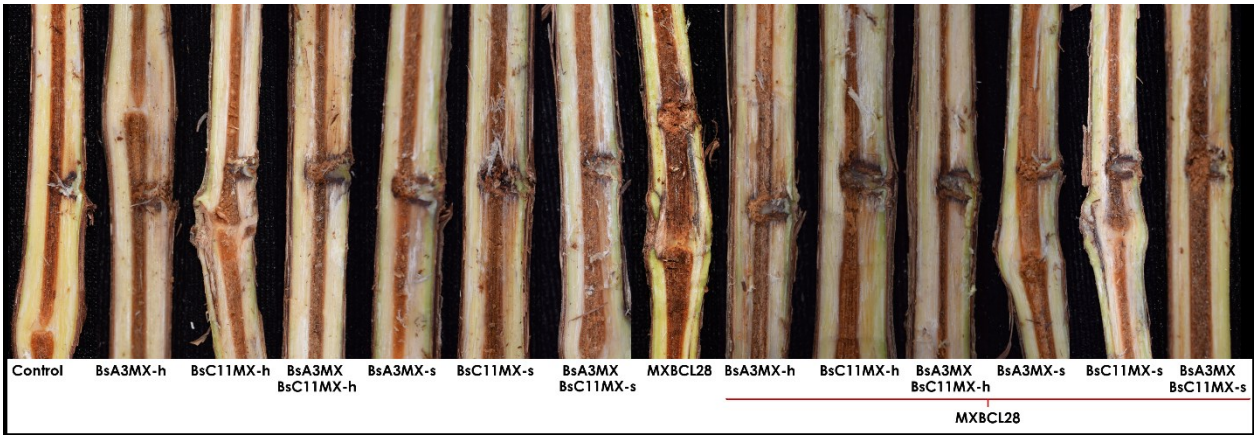

Figure 24. Necrotic lesions 2 mpi observe in grapevine woody shoots inoculated with the fungal pathogen *Lasiodiplodia brasiliensis* MXBCL28 and, *Bacillus amyloliquefaciens* BsA3MX and BsC11MX as a preventive treatment. h: indicates bacteria inoculated into the hole of fresh wound; s: indicates bacteria inoculated into soil at the collar of the plant.

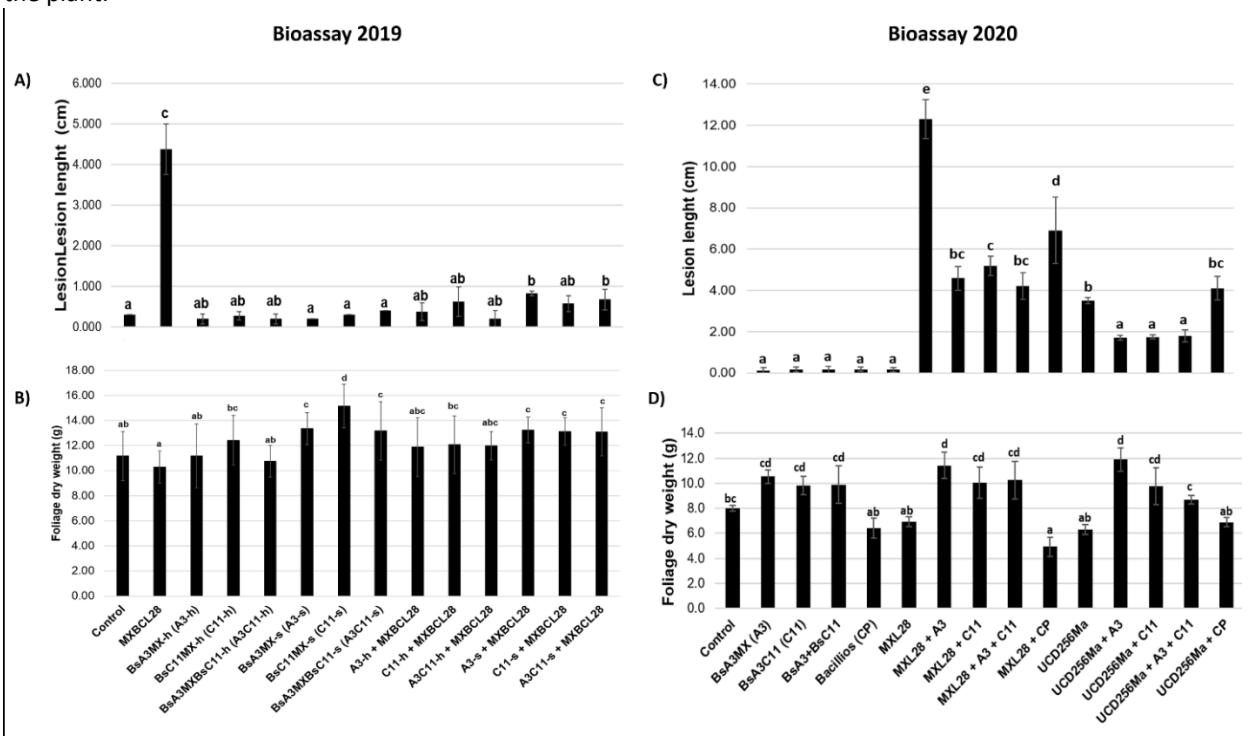

Figure 25. Effects of bacteria in bioassays *in planta*. A) Lesions length in grapevine plants in the 2019 bioassay cause by *Lasiodiplodia brasiliensis* MXBCL28. B) Foliage dry weight of grapevine in the 2019 bioassay. h: indicates bacteria inoculated into the hole of fresh wound; s: indicates bacteria inoculated into soil at the collar of the plant. C) Lesions length in grapevine plants in the 2020 bioassay cause by *Lasiodiplodia brasiliensis* MXBCL28 and *Lasiodiplodia gilanensis* UCD256Ma. D) Foliage dry weight of grapevine plants in the 2020 bioassay. Significance letters were grouped based on Fisher's analysis ($P< 0.05$), the same letter means that there are no statistically significant differences with an $\alpha<0.05$.

5.3.7.2. 2020 biocontrol assay

Plants inoculated with *L. theobromae* MXBCL28 caused lesions up 12 cm in length and *L. gilanensis* UCD256Ma up 8 cm (Figure 25C and 26). BsA3MX and BsC11MX mixed and applied to the soil reduced the necrotic lesions caused by pathogens by around 50%; while using the commercial product, plant lesions were similar to the control plants inoculated only with the pathogen. There were no differences in leaves numbers and the height of plants among treatments. As was observed in the 2019 bioassay, the use of bacteria isolates increased foliage production, even in those inoculated with the pathogens (Figure 25D). Plants with the commercial product had a dry weight of foliage similar to the control plants.

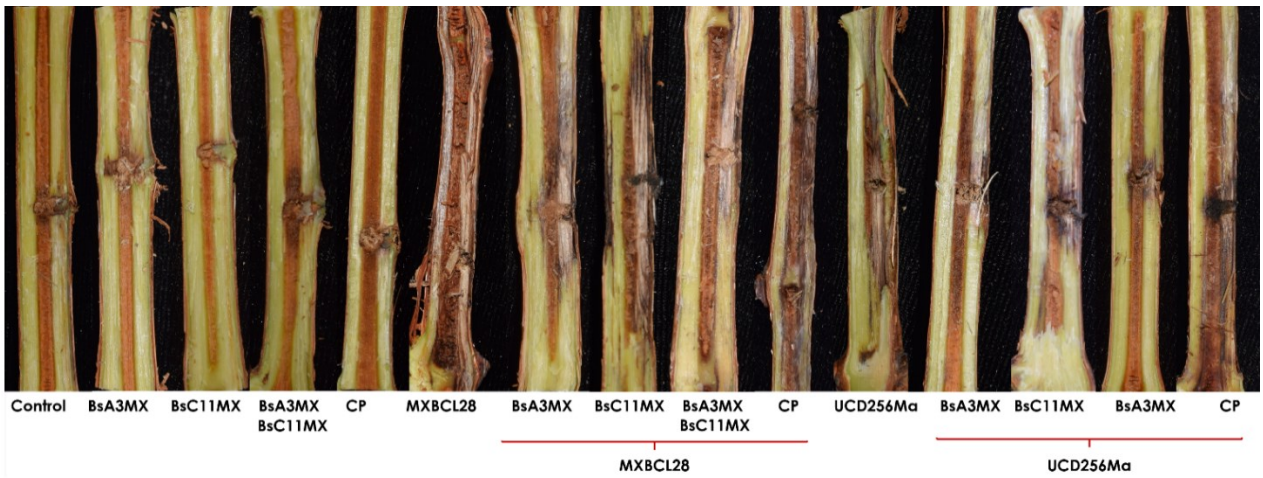

Figure 26. Necrotic lesions 2 mpi observe in grapevine woody shoots inoculated with the fungal pathogens *Lasiodiplodia brasiliensis* MXBCL28 and *Lasiodiplodia gilanensis* UCD256Ma and, *B. amyloliquefaciens* BsA3MX and BsC11MX as a preventive treatment in soil. CP: Comercial product (BACILLIOSS®).

5.4. Discussion

In this study, the isolates of *B. amyloliquefaciens* BsA3MX and BsC11MX inhibited the growth of *Lasiodiplodia* spp. and promoted grapevine growth. Both bacteria produce siderophores and indoleacetic acid, two characteristics in plant growth-promoting bacteria. Siderophores are chelators of Fe^{3+}; their production by beneficial bacteria helps the plant uptake of iron (Radzki et al., 2013). Indole-3-acetic-acid is a common auxin produced by several microorganisms through L- tryptophan metabolism and helps the plant with nutrient uptake and produce longer roots (Datta and Basu, 2000; Mohite, 2013). BsA3MX and BsC11MX bacteria solubilized zinc and phosphorous, which increase the accessibility of these nutrients to the plant**.** Another mechanism of plant growth promotion is the ACC deaminase activity, which decreases

the level of the plant hormone ethylene (Azfal et al., 2016). On the other hand, both bacteria form biofilm, a structure essential for the colonization of the rhizosphere (Anellal et al., 1998).

Bacillus amyloliquefaciens isolates in this study exhibited a high percentage of inhibition of *Lasiodiplodia* spp. in *in vitro* tests, reaching 65% through the production of volatile and non-volatile compounds. The genomes of two isolates of *B. amyloliquefaciens*, FZB42 and GA1, contain several gene clusters involved in synthesizing secondary metabolites to stimulate plant growth and different lipopeptides polyketides with antifungal and antibacterial activity against plant pathogens. Therefore, isolates belonging to this species have enormous potential for biocontrol and to promote plant growth (Chen et al., 2009; Arguelles-Arias et al., 2009). Different *Bacillus* spp. have been evaluated against GTD pathogens, being *Bacillus subtilis* the most studied species reported as efficient in wound protection (Mondello et al., 2018). Ferreira et al., (1991) reported mycelial growth inhibition of *Eutypa lata* (Eutypa dieback agent) by *B. subtilis in vitro* by 91.4 %, while the germination of ascospores was inhibited 100 %. In addition, they observed a malformation in the hyphae of *E. lata* in the presence of extracts of *B. subtilis*. Alfonzo et al. (2009), reported the inhibition of *Lasidiplodia thebromae* (Botryosphaeria dieback agent), *Pheoacremonium aleophilum*, and *Phaeomoniella chlamydospora* (Esca complex agents) under *in vitro* conditions using metabolites from *B. subtilis* AG1. In nursery plants, *B. subtilis* reduces the incidence of fungi of the Esca complex (Halleen et al., 2010). Most of the evaluations of *Bacillus* spp. has been only under *in vitro* conditions as *B. amyloliquefaciens* against *L. mediterraneum* and *F. mediterraneum* with moderately efficient in Italy; *B. firmus*, *B. ginsengihumi*, *B. licheniformis*, *B. pumilus* were evaluated in France against *Pa. chlamydospora* with moderately efficient *in vitro* and with ineffective *in planta* against *N. parvum* (Mondello et al., 2018).

The control of GTDs represents a challenge due to the complexity of the fungi associated. Cultural practices are not sufficient, and the excessive use of chemical compounds can generate resistance in pathogenic organisms and damage the environment (Agustí-Brisach et al., 2015). The use of BCAs is an alternative that does not harm the environment, protects the plant, does not harm the personnel in charge of its application, is long-lasting and can confer benefits to the plant by inducing defense mechanisms; therefore, BCAs are of great interest in viticulture to prevent the spread of GTDs and reduce the use of chemicals (Haider et al., 2016). In this study, the use of BsA3MX and BsC11MX *in planta* as a preventive treatment both, in the wound and applied to the soil, when were applied 15 days before inoculation of the pathogenic fungus, the lesions caused by *L. brasiliensis* MXBCL28 were 80% less in length than when the pathogen was inoculated alone in grapevine plants cv. Merlot. While the use of bacteria as a preventive treatment in soil one week before inoculation of the pathogenic fungus, the lesions caused by

Lasiodiplodia isolates were 50% less in length than when *L. brasiliensis* MXBCL28 and *L. gilanensis* UCD256Ma alone in vine plants cv. Cabernet Sauvignon. Furthermore, compared to the commercial product BACILLIOSS®, based on *B. subtilis* spores, the plants inoculated with the BsA3MX and BsC11MX bacteria showed higher foliage production, even inoculated with the *Lasiodiplodia* isolates, and the necrotic lesions produced by the pathogenic fungi were smaller. Besides, these bacteria presented different characteristics of plant growth promotion, and *in vitro* assays showed that affect the conidia germination of *L. gilanensis*. Thus, we consider that bacteria could be used as a preventive treatment applied to both pruning wounds and soil to reduce the incidence of *Lasiodiplodia* spp. in grapevine plants, nonetheless, field evaluations are necessary to confirm their effectivity against *Lasiodiplodia* spp.

Chapter 6. General discussion

Botryosphaeria dieback is one of the major GTDs worldwide. Since the prohibition of sodium arsenite due to its toxicity, it has become a global problem affecting the productivity and yield of economically important crops, including the grapevine (van Niekerk et al., 2006; Gramaje et al., 2018). In this crop, around 30 species in the Botryosphaeriaceae are associated with Botryosphaeria dieback, including fungi from the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neoscytalidium, Neofussicoccum*, *Phaeobotryosphaeria*, and *Spencermartinsia* (Úrbez-Torres 2011; Rolshausen et al., 2013; Stempien et al., 2017; Gramaje et al., 2018). The main disease symptoms are vascular discoloration and perennial cankers caused by the occlusion of the xylem and phloem; this leads to branches death and eventually of the entire plant.

Despite the importance of Botryosphaeria dieback, information on the biology of the causal agents and the process in which the fungus establishes infection and colonizes the plant is not fully documented (Claverie et al., 2020). These fungi can have an endophytic stage and remain in their host for an extended period without causing symptoms (Slippers and Wingfield, 2007). The transition to the pathogenic phase is related to stress factors such as drought, high temperatures, insufficient nutrient supply, and wounding, which increase the host's host susceptibility (Hrycan et al., 2020). Therefore, the incidence of Botryosphaeriaceae fungi as pathogens has increased due to climate change (Mehl et al., 2017; Batista et al., 2021). The taxonomy of the Botryosphaeriaceae has changed profoundly over the years. First, morphological characteristics were used for taxonomic classification until DNA sequencing appeared, providing tools to determine the phylogenetic relationships. In 2006, Schoch et al. (2006) based on multigene phylogeny, proposed the order Botryosphaeriales with Botryosphaeriaceae as a single-family (Phillips et al. 2013). Later, based on molecular evidence nine families and 33 genera have been recognized in the Botryosphaeriales (Minnis et al., 2012; Wikee et al., 2013; Slippers et al., 2013; Wyka and Broders, 2016; Yang et al., 2017). The most recent phylogenetic revision has reduced to synonymy several previously accepted species of Botryosphaeriaceae (Zhang et al., 2021). The proper identification and classification of Botryosphaeriaceae isolates, and their host range, is important due to the fungal diversity related to the diseases and the differences found in their virulence (Batista et al., 2021). Thus, this information is useful to improve the knowledge of the Botryosphaeriaceae distribution and their host diversity.

In Mexico, the grape production in 2019 was 489,140 tons, being the state of Sonora, the primary producer of table grapes with 318,188 tons, and Baja California of wine with 26,665 tons (SIAP, 2019). Few studies have been done on the impact, incidence, and distribution of fungi associated with GTDs. In the current work, we reported the presence of nine species of the Botryosphaeriaceae family (*B. dothidea*, *D. seriata*, *L. brasiliensis*, *L. crassispora*, *L. exigua*, *L. gilanensis*, *N. australe*, *N. parvum*, and *N. vitifusiforme*) isolated from vineyards in the states of Baja California, Chihuahua, Coahuila, and Sonora. *Lasiodiplodia exigua* was the most prevalent species in Baja California and Sonora, capable of growing up to 40 °C. The isolates of *L. gilanensis*, *L. brasiliensis*, and *N. parvum* presented high virulence in greenhouse pathogenicity assays. Interestingly, *L. gilanensis* was only found in Baja California, while *B. dothidea* was isolated only from samples from Chihuahua and presented lower virulence. *Botryosphaeria dothidea* has been considered a latent pathogen of global importance in woody plants due to its prolonged latent infection or endophytic phase, indicating that this fungus can pass undetected by quarantine systems in traded plants and might represent a future problem for the hosts that it infects, under the appropriate environmental conditions (Marsberg et al., 2017).

The disease management is based on cultural practices that are not efficient, such as the use of disease-free propagation material, performing pruning in dry seasons, applying protective paint on pruning, and removing infected plants (Gramaje et al., 2018). There are no fully efficient fungicides available against GTDs fungi. The increasing consumer demands for more sustainable grapevine production have led to the search for alternatives to control these diseases (Úrbez-Torres et al., 2020). Biological control agents (BCAs) are organisms that antagonist the growth of pathogens through different modes of action such as parasitism, competition for the substrate or nutrients, and the production of toxic compounds (Köhl et al., 2019). For those reasons, the use of BCAs has increased as one of the main alternatives to chemical products. Different species of *Trichoderma* and *Bacillus* have shown potential to control GTDs pathogens (Zhang et al., 2014; Sajitha et al., 2016). In the search for BCAs, the importance of using isolates adapted to the environmental conditions where they will be applied has been emphasized (Köhl et al., 2019). *Bacillus amyloliquefaciens* induces plant growth promotion and has been considered a potential bacterium for biocontrol of different plant pathogens (Chen et al., 2009; Arguelles-Arias et al., 2009). This bacterium has been evaluated only *in vitro* against *Lasiodiplodia mediterranea* and *Fomitipora mediterranea* in Italy (Mondello et al., 2018). The isolates of *B. amyloliquefaciens* reported in this study showed high inhibition against different *Lasiodiplodia* spp., and *in planta* assays, reduced the symptoms of *L. gilanensis* and *L. brasiliensis*. Experiments under field conditions are necessary to confirm their potential as BCAs in grapevine against *Lasiodiplodia*.

In recent years, through the genome and transcriptome analysis of different *Lasiodiplodia* isolates, a higher number of potential virulence factors have been identified. Mainly, genes related to secreted CAZymes, pectin, starch and sucrose metabolism, nutrient uptake, secondary metabolism, transporters, phenolic metabolism, and genes involved in melanin synthesis (Paolinelli-Alfonso et al., 2016; Yan et al., 2018; Félix et al., 2019; Gonçalves et al., 2019; Garcia et al., 2021; Nagel et al., 2021). Furthermore, the expression of some of these gene families has been induced in response to heat stress (Paolinelli-Alfonso et al., 2016; Yan et al., 2018). These findings related to the observations made of the colonization *of L. brasiliensis* in the grapevine can expand our understanding of the biology of these fungi, which will allow for the development of control strategies and disease management.

Chapter 7. General conclusions

- Nine fungal species of the Botryosphaeriaceae family associated with grapevines were identified in the states of Baja California, Chihuahua, Coahuila and Sonora, Mexico.

- *Lasiodiplodia exigua* was the most prevalent in the region of Baja California and Sonora.

- *Lasiodiplodia gilanensis, Lasiodiplodia brasiliense* and *Neofusicoccum parvum* isolates were the most virulent in grapevine plants in greenhouse assays.

- *Lasiodiplodia brasiliense* colonizes the plant by going through the ray parenchyma, overcoming the plant defense barriers and using the plant starch as a carbon source, and it is able to degrade cellulose, hemicellulose and lignin.

- The BsA3MX and BsC11MX isolates of *B. amyloliquefaciens* have potential for the biocontrol of *Lasiodiplodia*.

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Supplementary material

Supplementary 1

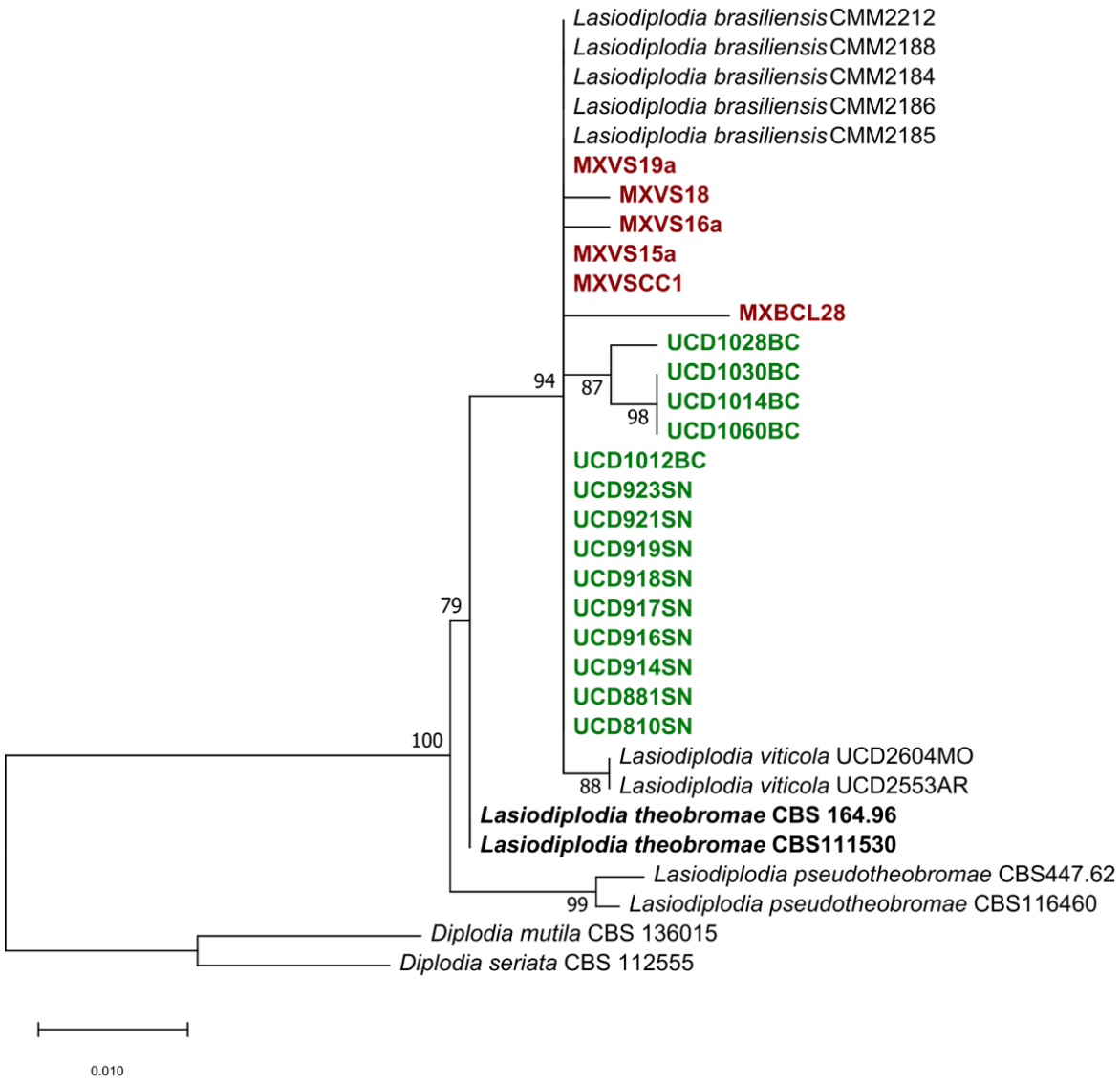

Figure 27. Phylogenetic analyses of *Lasiodiplodia brasiliensis* isolates. Maximum likelihood tree for *Lasiodiplodia brasiliense* with the highest log likelihood (-1361.2) obtained from analysis of ITS, and *tef1* concatenated dataset. Bootstrap values from 1000 replicates greater than 50 are indicated at the nodes. The tree is rooted with *Diplodia mutila* (CBS 136015) and *Diplodia seriata* (CBS 112555). The isolates from this study are indicated in red bold; isolates previously identified as *L. theobromae* are indicated in green bold; and the isolates *L. theobromae sensu stricto* are indicated in black bold.

Supplementary 2

Histological plant techniques

FAA solution to fix tissue

40% Formaldehyde	5 mL
Glacial acetic acid	5 mL
50 % Ethanol	90 mL

Starch staining–Iodine-Potassium Iodide test

Procedure

1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Wash with distilled water
3. Add few drops of IKI solution directly on the sections
4. Incubate for 5 min at room temperature
5. Rinse section in water
6. Mount on slides

10 g of KI in 100 mL of distilled water Add 5 g of iodine into the KI solution Iodine takes time to dissolve completely Store the IKI solution in the dark

Results

Small blue-black bodies indicate the presence of starch granules

Suberin staining–Sudan IV staining

Procedure

1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Transfer sections to a microcentrifuge tube and add 1 mL of Sudan IV solution
3. Incubate for 30 min in stain at 37 °C
4. Rinse in 50% ethanol for 10 min at room temperature.
5. Mount in 60% glycerol and observed

0.001% Sudan IV in 70% ethanol 50% ethanol 60% glycerol

Observe using a TEXAS RED filter (excitation at 542–595 nm, emission at 644 nm) and a DAPI filter (excitation at 330–380 nm, emission at 420 nm). Preparations are not permanent.

Results

Suberin stained with Sudan IV fluoresces in red using TEXAS RED filter, and DAPI filter is used to visualized the plant tissue where suberin is deposited.

Lignin staining–Phloroglucinol

Procedure

1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Transfer sections to a microcentrifuge tube and add 1 mL of Phloroglucinol-HCl solution
3. Incubate for 5 min at room temperature
4. Mount on slide without removing the stain

0.01% Phloroglucinol in 80% ethanol 37% HCl Mix 500 µL of Phloroglucinol solution and 500 µL of HCl

Stain is stable for 30 minutes approximately

Results

The cinnamaldehyde end groups of lignin react with phloroglucinol-HCl, therefore lignin is observed in pink-purple color

Lignin staining–Mäule method

Procedure

1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Wash with distilled water
3. Transfer sections to a microcentrifuge tube and add 1 mL of $KMnO_4$
4. Incubate 2 min and gently mix
5. Draw out the $KMnO_4$ solution and rinse in distilled water until the water solution stays clear
6. Discard the water and add 1 mL of HCl until the deep brown color is discharged (3-5 min)
7. Give a second wash with HCl solution and discard
8. Add 1 mL of concentrated ammonium hydroxide solution
9. Mount on slide using the ammonium hydroxide solution

0.2 g $KMnO_4$ in 40 mL distilled water (store in the dark maximum 7-days) 3.7% HCl (prepare the day of use) Concentated ammonium hydroxide store at 4 °C

Ammonium hydroxide is extremely corrosive, and the solution on the slide dries in 5-10 minutes, therefore images must be taken within that time.

Results

The syringyl lignin units react with the KMnO, therefore lignin is observed in red color

Cellulose staining–Calcofluor white M2R

Procedure

1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Wash with distilled water
3. Transfer sections to a microcentrifuge tube and add 1 mL of Calcofluor
4. Incubate for 5 minutes at room temperature

5. Gently mix and incubate for other 3 min
6. Rinse sections with water three times
7. Mount on slides

0.02% Calcofluor white M2R Storage in dark

Observe using a DAPI filter (excitation at 330–380 nm, emission at 420 nm). Preparations are not permanent.

Results
Cellulose is observed fluorescent.

Cellulose and Hemicellulose staining –Congo Red

Procedure
1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Wash with distilled water
3. Transfer sections to a microcentrifuge tube and add 1 mL of Congo Red
4. Incubate for 10 minutes at room temperature
5. Gently mix and incubate for other 3 min
6. Rinse sections with water three times
7. Mount on slides

0.5% Congo Red in water Storage in dark

Observe using a TEXAS RED filter (excitation at 542–595 nm, emission at 644 nm). Preparations are not permanent.

Results
Cellulose and hemicellulose are observed fluorescent.

Phenolic compounds staining –Toluidine Blue O

Procedure
1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Wash with distilled water
3. Add few drops of TBO directly on the sections and store in 25% ethanol at 4 °C
4. Wait for 5 minutes
5. Rinse sections in water
6. Mount on slides

0.1% Toluidine Blue O (TBO) in distilled water Storage in dark

Results

TBO will react with carboxylated polysaccharides such as pectic acid to give a purplish color, greenish blue or purple with polyphenolic substances, thin-walled parenchyma will be reddish purple; cells with lignified

Melanin staining–Masson-Fontana method

Procedure

1. Use sections of plant tissue fixed in FAA and store in 25% ethanol at 4 °C
2. Wash with distilled water
3. Transfer sections to a microcentrifuge tube and add 1 mL of Masson-Fontana reagent
4. Incubate for 25 min at 56 °C
5. Wash with distilled water
6. Fix in sodium thiosulfate 5% (5 min)
7. Rinse with distilled water
8. Mount on slides

$AgNO_3$ 10 % 50 mL NH_4OH Distilled water 50 mL Add NH_4OH to $AgNO_3$ 10 % solution drop by drop until opalescence appears. Mix with distilled water (same quantity). Filter and store overnight in the dark. Reagent is stable for up to a month stored in the dark.

Results

Melanin reduces ammoniacal silver nitrate solution to metallic silver without using an external reducing agent. Melanized hyphae are observed.

Supplementary 3

Table 10. List of GenBank and culture accession numbers of *Bacillus* used in this study for phylogenetic analysis.

Species	Isolate	Isolation source	Origin	GeneBank accession number
				16S rRNA
B. amyloliquefaciens	NBRC 15535	soil	Japan	NR_112685
B. amyloliquefaciens	W9	marine water sample	India	MH188056
B. amyloliquefaciens	AB-525	rice cake	China	KJ879953
B. amyloliquefaciens	**BsA3MX**	**rhizosphere strawberry**	**Mexico**	**MW651769**
B. amyloliquefaciens	**BsC11MX**	**rhizosphere strawberry**	**Mexico**	**MW651770**
B. axarquiensis	CIP 108772	river-mouth sediments	Spain	DQ993670
B. cereus	ATCC 14579	unknown	unknown	AE016877
B. circulans	IAMI 12462	soil	unknown	D78312
B. coagulans	NBRC 12583	evaporated milk	unknown	AB271752
B. licheniformis	ATCC 14580	unknown	unknown	CP000002
B. mojavensis	IFO 15718	soil	USA	AB021191
B. mycoides	ATCC 6462	soil	unknown	AB021192
B. siamensis	PD-A10	poo-dong	Thailand	GQ281299
B. siamensis	RET2912	landfill soil	India	MN530054
B. siamensis	LFS1715	landfill soil	India	MN519261
B. subtilis	DSM10	unknown	unknown	AJ276351
B. subtilis subsp. *spizizenii*	NBRL B-23049	tunisian desert	Tunisia	AF074970
B. thuringiensis	IAM 12077	mediterranean flour moth	unknown	D16281
B. vallismortis	DSM 11031	soil	USA	AB021198
B. velezensis	CR-502	brackish water	Spain	AY603658
Alicyclobacillus acidocaldarius	DSM 446	acid hot spring	USA	AJ496806

Isolates from this study are highlighted in bold text.