Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Short Notes

Greeneria uvicola associated with dieback in vineyards of Sonora, Mexico

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Summary. The state of Sonora is the main grape production area in Mexico. Grapevine trunk diseases (GTD) are serious disease in this region. During the springs of 2017 and 2018, symptoms of yellow and wilted leaves, and necrotic buds, were observed in several vineyards in Sonora. Affected plants had numerous small, black and bright acervuli. This study aimed to identify the causative agent of this disease. Isolates were obtained from small pieces of damaged plant parts, and were cultured on potato dextrose agar. The developing mycelium was white but turned greyish white after 3 d. Multi-shaped black mucilaginous droplets appeared in the cultures after 2 d, and the agar in Petri dishes was completely covered with mycelium after 7 d, and the colonies had five or six rings with large numbers of conidiomata. Conidia were hyaline or light cream, fusiform, oval or ellipsoid, with truncated bases and narrow almost pointed apices, and were 6–10 mm long and 2–3 mm wide. Phylogenetically analysed concatenated sequences of the DNA from two representative isolates, from the internal transcribed spacer region, and large ribosomal subunit, showed they were in a separate clade which aligned with several strains of *Greeneria uvicola*, confirming the presumptive morphological identity of the isolates. This cosmopolitan ascomycete is responsible for bitter rot of grapes, but the role of this fungus as a cause of grapevine trunk diseases is little known. Pathogenicity tests of the isolates were performed on 1-year-old 'Passion Fire' grapevines plants, one of the new cultivars planted in Sonora. All of strains *G. uvicola* were pathogenic, and the fungus was recovered from the lesions, fulfilling Koch's postulates. This is the first report on the pathogenicity of *G. uvicola* in wood tissues of *Vitis vinifera* in Mexico.

Keywords. *Diaporthales*, *Melanconiellaceae*, multilocus typing, grapevine trunk diseases.

OPEN ACCESS

Citation: J. M. Ávila Salazar, S. F. Moreno Salazar, M. A. Ávila-Luzanía, J. M. Guzmán Ortíz, F. H. Navarro Nava, M. E. Rentería Martínez (2020) *Greeneria uvicola* associated with dieback in vineyards of Sonora, Mexico. *Phytopathologia Mediterranea* 59(3): 605-613. doi: 10.14601/Phyto-11977

Accepted: September 16, 2020

Published: December 30, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Úrbez Torres J.R., Agriculture and Agri-Food Canada, Summerland, British Columbia, Canada.

INTRODUCTION

The state of Sonora is the major grape production region in Mexico. The total production of fresh table, distillate, and raisin grapes was 415,889 tonnes in 2017, with an 18.4% increase compared with that in 2016. Sonora contributed 334,355 tonnes to the total domestic production of grapes (more than 80%), generating 350 to 400 million USD. Eighteen grape varieties recognized in the international market are grown in Sonora for four different markets: table grapes (92.8%), wine grapes (3.7%), raisins (3.3%), and table grapes grown under shade cloth (0.2%). Hermosillo, Caborca, and Guaymas-Empalme are the three main grape-producing regions (SAGARPA, 2018).

Grapevine trunk diseases (GTDs) caused by fungi are considered the most destructive diseases in vineyards (Larignon and Dubos, 1997; Agustí-Brisach and Armengol, 2013; Gramaje *et al*., 2018). Among the main GTD agents are *Phaeomoniella chlamydospora* and *Phaeoacre-monium* sp., which cause for Petri disease (Mostert *et al*., 2006); *Botryosphaeriaceae* species (Úrbez-Torres, 2011), *Phomopsis viticola* (*Diaporthe ampelina*; Úrbez-Torres *et al*., 2013) and *Eutypa lata* (Rudelle *et al.,* 2005), which cause regressive death of branches, and *Cylindrocarpon* sp. which cause blackfoot disease (BFD) (Agustí-Brisach and Armengol, 2013). Some of these pathogens can act independently or synergistically, and have been isolated from old grapevines and from symptomatic and asymptomatic root-stock mother-plants, rootstock cuttings, and young grafted vines (Carlucci *et al.,* 2017). Infected plants may be stunted with reduced loss of vigour, retarded or absent sprouting, biomass reduction, chlorotic and necrotic foliage, shoot mortality, fruit rot, wilting, and death. Necrotic lesions in the roots and reductions in root biomass may also occur (Gramaje and Armengol, 2011).

Castillo-Pando *et al.* (2001) determined that *Botryosphaeria obtusa* (syn. *Diplodia seriata*) was responsible for the regressive death of several vineyards in the Hunter Valley region of Australia. The presence of *Greeneria uvicola* and *Pestalotiopsis* sp. was also observed in the majority of the cases, but the role that these fungi remains uncertain. Subsequently, Abreo *et al.* (2008) found that *G. uvicola*, in addition to *Eutypella vitis* and species of Botryosphaeriaceae, was mainly responsible for GTDs in Uruguayan vineyards.

Although *G. uvicola* can infect grapevine leaves, tendrils, and stems, this fungus primarily attacks the fruit, especially if wet weather conditions persist during harvest (Longland and Sutton, 2008; Wilcox *et al*., 2015; Steel and Greer, 2008).

Greeneria uvicola (syn. *Melanconium fuligineum*; *Diaporthales*) is a cosmopolitan ascomycete responsible for bitter rot of grapes. This fungus reproduces asexually, and the teleomorph is not known. The fungus survives during the winter in stem lesions and mummified berries, and has been shown to attack the fruits of several species of *Vitis*, including *V. aestivalis*, *V. labrusca*, *V. rotundifolia* and *V. vinifera* (Farr *et al*., 2001; Longland and Sutton, 2008).

During April and May of 2017 and 2018, some gravevines in different vineyards in Sonora, located in Hermosillo (Costa de Hermosillo and Pesqueira) and Guaymas-Empalme, showed damage and symptoms of GTDs. Initially, the symptoms were dry leaves, buds, and woody shoots. Cross-sections of affected wood showed triangular black spots in some samples, so these symptoms were associated with the presence of *Lasiodiplodia theobromae*, a fungus responsible for GTD in these Sonora regions. However, microbiological tests revealed the presence of pycnidia similar to those produced by *Botryosphaeriaceae* sp., and conidia like those of *Neofusicoccum* sp. were observed. Nevertheless, an initial molecular analysis amplifying internal transcribed spacer region using ITS1/ITS4 primers indicated a high probability that *G. uvicola* was the causative agent of this disease.

Because *G. uvicola* is considered a pathogen of the grape berries and that it only occurs as a secondary opportunist in GTDs, the present study aimed to confirm presence and identification the fungus using molecular phylogeny, and pathogenicity tests were carried out to determine whether this fungus can be a primary agent capable of infecting vineyards in Sonora, Mexico.

MATERIALS AND METHODS

Sampling and isolation of the causative agent

Fifteen *Vitis vinifera* vineyards were sampled (seven on the Hermosillo Coast, four in the Pesqueira Valley, and four in the Guaymas-Empalme Valleys). The varieties planted in these vineyards were 'Summer Royal', 'Early Sweet', 'Flame Seedless', 'Sweet Celebration', and 'Passion Fire'. Wood was collected from plants with dieback symptoms and the presence of black subepidermal structures in the woody shoots. The samples were separately wrapped in wet paper, placed in plastic bags, labelled and placed on ice for transfer to a laboratory for processing. Each sample was directly examined under a stereoscopic microscope for characteristics and relevant symptoms.

Tissue sections (>5 mm^2) were cut from the margins of necrotic wood and were disinfected in 75% ethanol for 1 min, 1% sodium hypochlorite for 5 min and then

rinsed three times with sterile distilled water. The wood tissue pieces were then placed in Petri dishes containing potato dextrose agar (PDA), which were incubated at 25°C for 7 d. Pure cultures were obtained by transferring a hyphal tip to individual PDA dishes. The resulting grown cultures were stored at 0°C for no longer than 1 month, until further processing.

Morphological analysis

The isolated fungi were characterised for growth rate, and colony shape, consistency, and colour. Preparations were made on microscope slides and examined using a light microscope to determine characteristics of reproductive structures. A microscope stage micrometer (Carl Zeiss Canada Ltd) was used for calibrating the optical systems to determine dimensions of acervuli and conidia. Twenty acervuli and 100 conidia were measured for each isolate.

DNA extraction

Two representative isolates were subjected to molecular analysis. DNA was extracted from mycelia grown in pure cultures on PDA, using the Power Soil DNA Isolation Kit (MoBIO Laboratories). Cell lysis was conducted with a Precellys Evolution homogeniser (Bertin Technologies). DNA integrity was verified on 2% agarose gel. The extracted DNA was quantified using a NanoDrop 1000 (ThermoScientific). Only samples with a 260/280 absorbance ratio between 1.8 and 2 were amplified. The DNA was stored at -20°C until used.

Molecular identification and phylogenetic analysis

DNA amplification reactions were performed for each of the two isolates in a Biorad C1000 Thermal Cycler. Sets of ITS1 and ITS4 primers were used to amplify and sequence the ITS1-5.8-ITS2 region (White *et al*.,1990), and LROR and LR5 primers were used to amplify and sequence the fragment corresponding to the large ribosomal subunit (LSU) gene (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). The amplification products were purified with ExoSap-IT PCR Product Cleanup (Affymetrix). The purified products were separately sequenced in both directions, using the same pair of initiators. The sequencing equipment used was an ABI 3100 Genetic-Analyser (Applied Biosystems). Each sequence was manually checked, and nucleotides in ambiguous positions were corrected using the complementary sequences obtained with both primers, using ChromasPro v2.1.5. Several of the sequences of interest were aligned with existing sequences of different *Melanconiellaceae* family, obtained from the National Center for Biotechnology Information (NCBI) gene database. Evolutionary history was inferred using the neighbour-joining method. *Gaeumannomyces graminis* var. *avenae* CBS 187.65 was used as an external group. In total, 1447 positions were analysed. Evolutionary analyses were performed using MEGA X (Kumar *et al*., 2018).

Pathogenicity tests

Two representative isolates from affected grapevines in the sampling areas were used. Five plants of 'Passion Fire' were grown in polyethylene pots, and were inoculated 1 year after transplanting. Each inoculation was into a 4-month-old shoot or a 1-year-old trunk. A 5 × 2 mm longitudinal incision (1 mm deep) was made iat each inoculation point, and an 8 mm PDA disc with fungal mycelium was then placed in the incision and covered with parafilm to avoid dehydration and contamination (Figures 4A and 4B). This procedure was also performed for five control plants of the same variety, but the inoculations were with mycelia-free culture media.

RESULTS AND DISCUSSION

Field observations

Between 5 and 10% of the plants in each sampled vineyard showed typical GTD symptoms in all varieties. Figure 1A shows symptoms at the beginning of disease development (yellow and dry leaves and black spots in stems). Deformed stems and acervuli were also observed (Figure 1B). Other plants showed more severe symptoms, including dead twisted and deformed branch stems with pronounced cankers, and with acervuli (Figure 1C) and gummosis (Figure 1D).

Morphology of fungal isolates

The initial mycelium of the isolates on PDA was white and then turned greyish-white 3 d later. On the second day, small black droplets with mucilaginous consistency were observed on the mycelium, and these contained bright and shiny black conidiomata of varying shapes (Figure 2A). Initially, the acervuli were aqueous, but they became solid, opaque, and less shiny, with variable shapes as they matured (Figure 2B). Within 1 week,

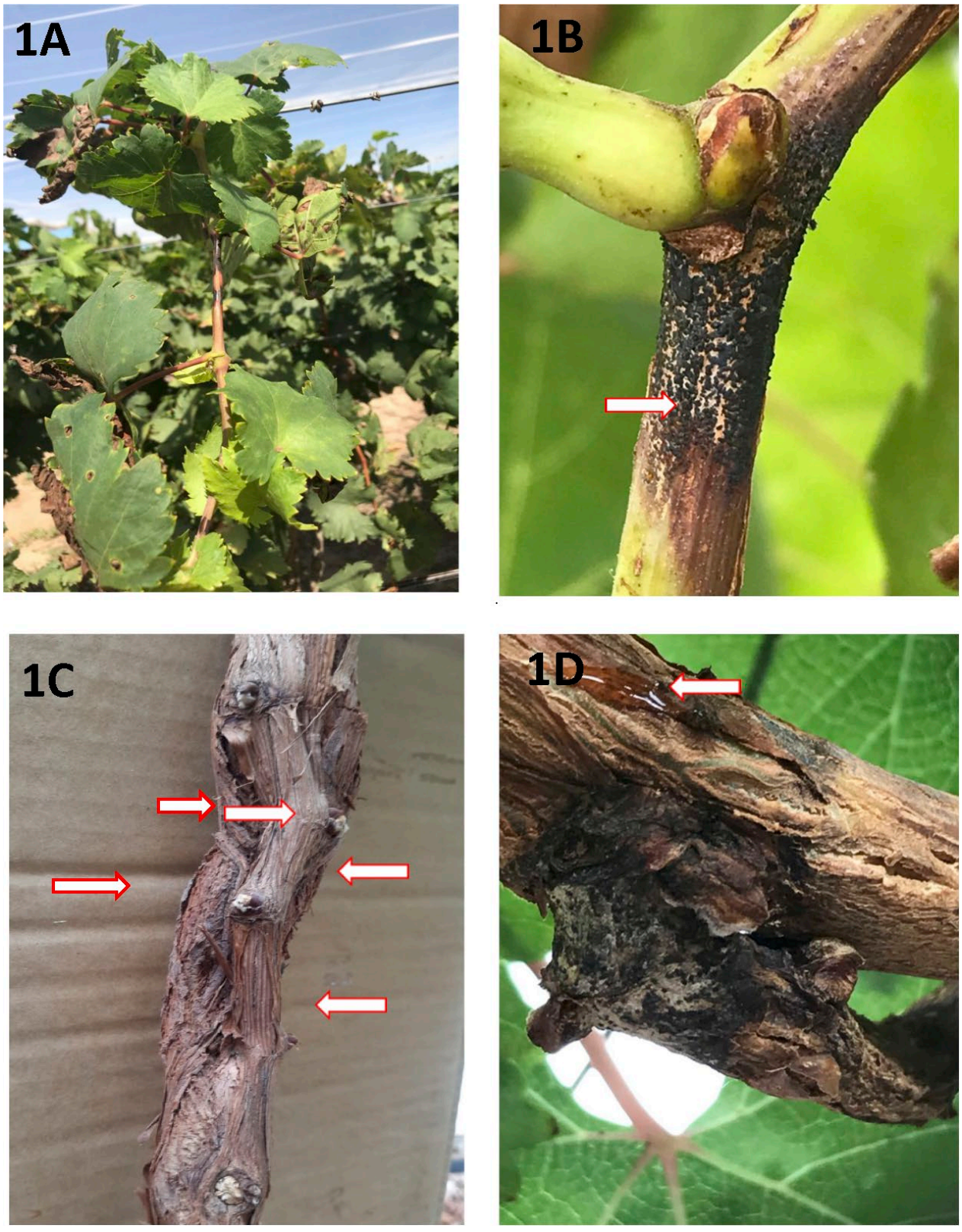

Figure 1. Grapevine trunk diseases caused by *Greeneria uvicola*. The arrows indicate details of the symptoms and damage. 1A: Beginning of the disease in young branches (leaves and stems). 1B; *Greeneria uvicola* acervuli around one shoot. 1C; Dead branch with pronounced canker, twist deformed stem, and presence of acervuli. 1D; Gummosis in dead tissues.

the medium in each Petri dish was completely covered with mycelia, with five to six rings of multiple acervulus-type conidiomata (Figure 2C). These were of dimensions from 400 to 1040 µm (average = 690 µm). Conidia were single-celled, fusiform or ovoid and smooth. The conidiogenic structures inside the acervuli were simple phialides with variable shapes and conidia at their ends. The conidia were hyaline or cream, and fusiform, oval or

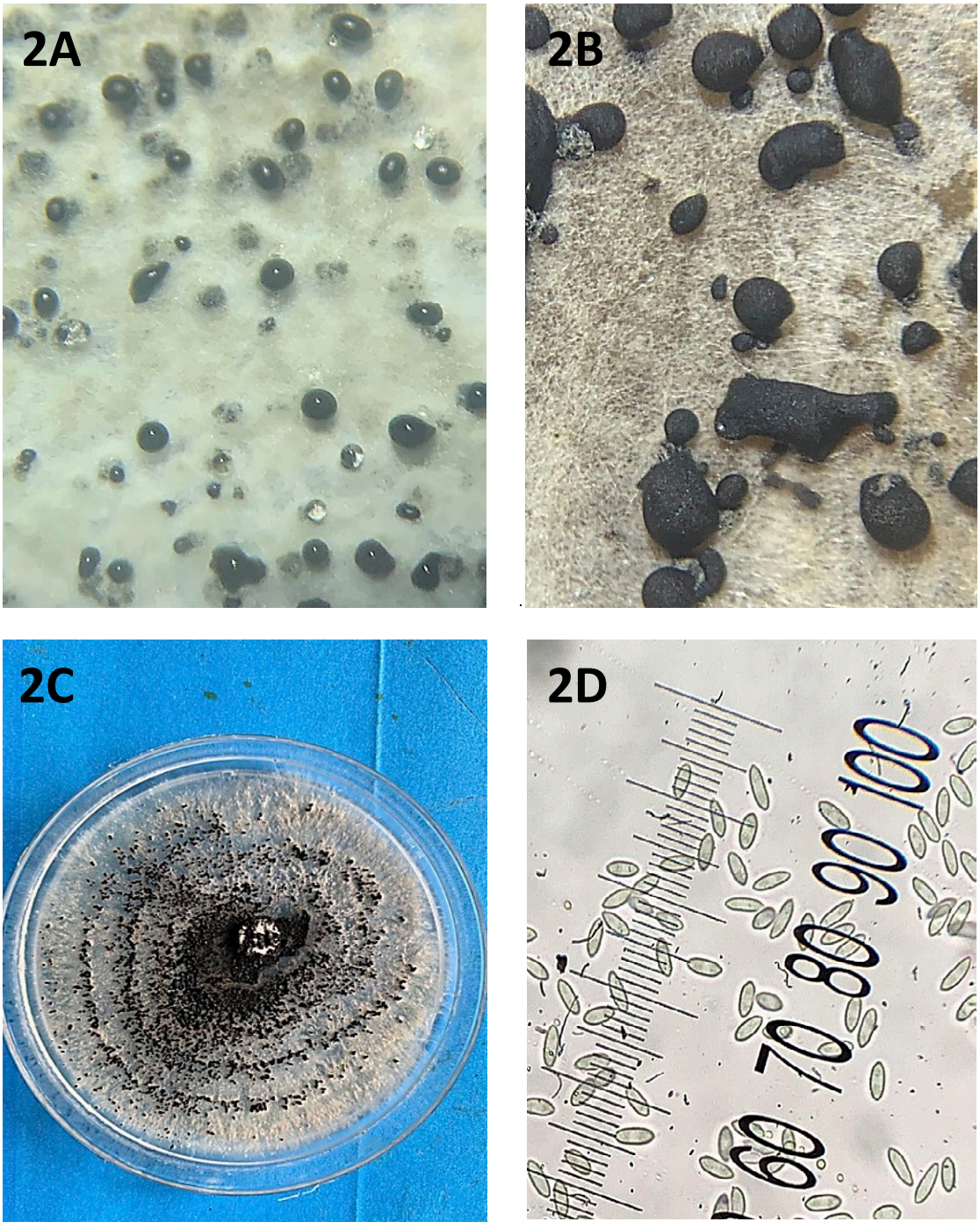

Figure 2. Morphological characteristics of *Greeneria uvicola* cultivated on PDA. 2A: Conidiomata growing over mycelium 2 d after inoculation. 2B: Mature, opaque and deformed acervuli. 2C; Petri dish covered with mycelia, showing concentric rings of multiple conidiomata at 7 d after inoculation. 2D. Morphology of conidia. Scale: each division = 2.5 µm.

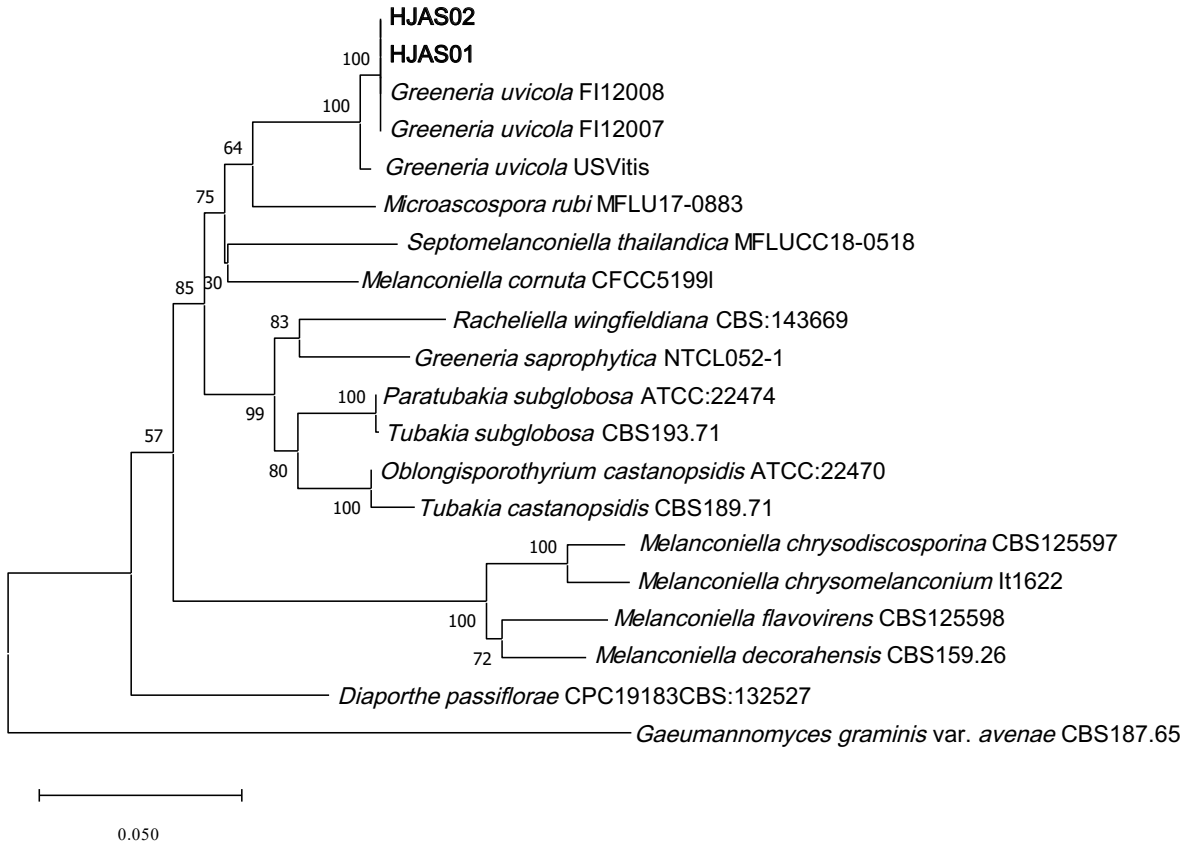

Figure 3. Evolutionary analyses conducted in MEGA X using nucleotide sequences of the *ITS-LSU* genes. Representative isolates of different *Melanconiellaceae* family were included, along with two representative isolates (HJAS01 and HJAS02) from grapevines in the Sonora state of Mexico. This analysis involved 20 nucleotide sequences. The evolutionary history was inferred using the Neighbour-Joining method. A total of 1447 positions were in the final dataset. The scale bar represents a genetic distance of 0.05.

ellipsoid with truncated bases and narrow, almost pointed apices, measuring 6–10 µm (mean = 7.7 ± 2.8 µm) by 2–3 µm (mean = 2.8 ± 0.4) (Figure 2D). These characteristics were consistent with those of *Greeneria*, described by Scribner and Viala (1887).

Molecular identification and phylogenetic analysis

The nucleotide sequences of the ITS and LSU regions of two representative isolates (HJAS01, HJAS02) were deposited in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/), with accession numbers MN611374, MN611375, MN628296, MN628297. Sequences were edited, aligned, and compared with those of fungal species within *Melanconiellaceae*, and were analysed phylogenetically using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980), and are in the units of the number of base substitutions per site. This analysis involved 20 nucleotide sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Phylogenetic analysis showed that isolates HJAS01 and HJAS02, which caused downward death of grapevines in vineyards, belonged to *G. uvicola*, because a well-defined clade (100%) was formed with several isolates of this species from GenBank (Figure 3).

Pathogenicity tests

Three weeks after inoculation, the plants began to show symptoms including general wilting of shoots at the inoculation points, and with chlorosis and leaf necrosis. All shoots and leaves were dried by the 4th week after inoculation (Figure 4D). The non-inoculated plants, which remained healthy (Figure 4C). Longitudinally cut shoots had necrotic interiors and portions

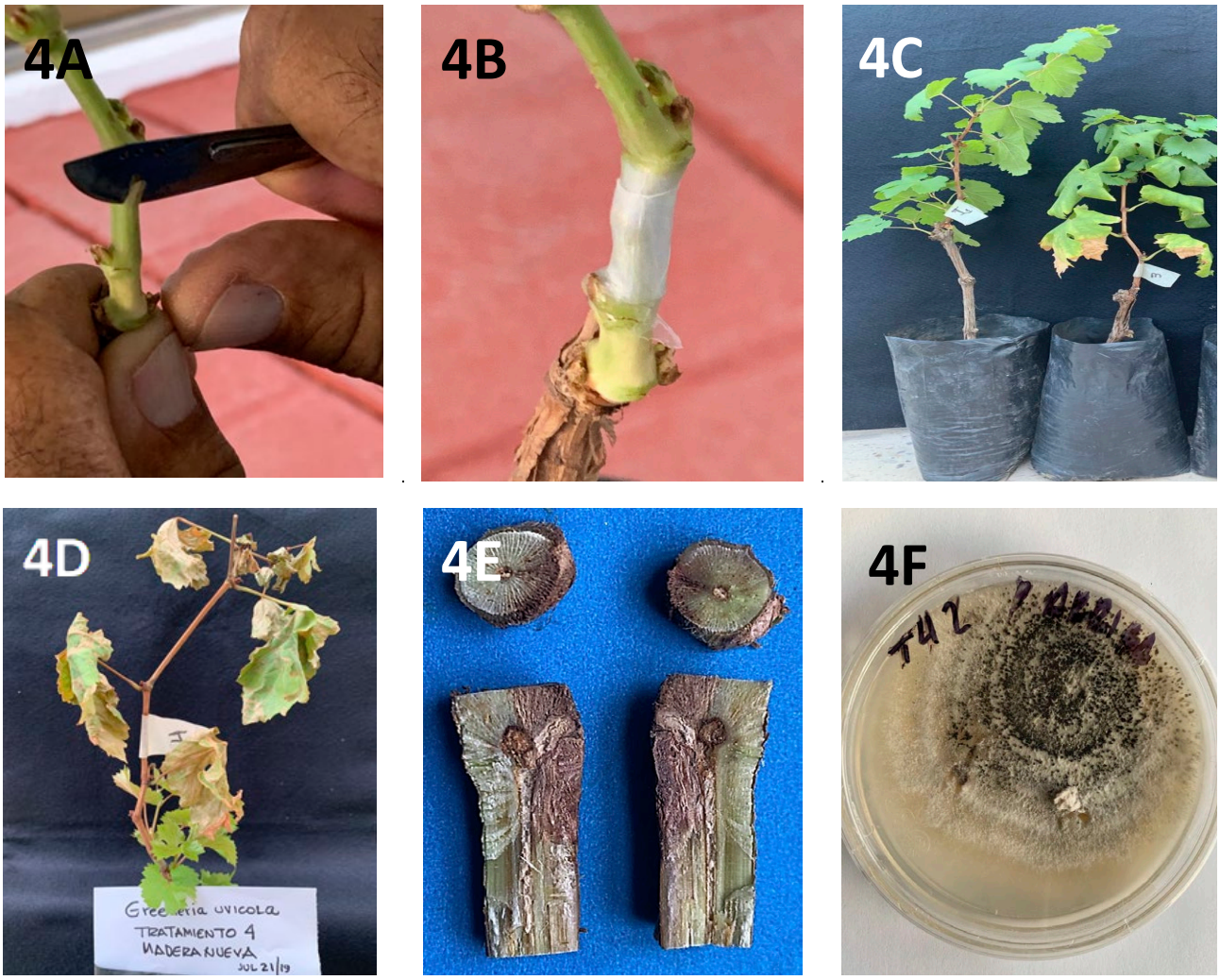

Figure 4. Pathogenicity tests. 4A and 4B; Inoculation. 4C: Healthy plants 30 d after treatment with PDA without *Greeneria uvicola*. 4D: Diseased plants after 30 d of inoculation with *G. uvicola* on PDA. 4E: Transverse and longitudinal cuts through stems of inoculated plants. 4F: *G. uvicola* re-isolated from segments of diseased plant stems.

of dead tissue (Figure 4E). Many acervuli were also observed on the opposite sides and above the inoculation sites. Tissues from these shoots were obtained and cultured in Petri dishes with PDA. *Greeneria uvicola* was isolated and confirmed in all inoculated tissues, fulfilling the Koch's postulates (Figure 4F).

Although *G. uvicola* is typically considered as an ascomycete responsible for the bitter rot of *Vitis vinifera* fruit bunches, some investigations have demonstrated the ability of this fungus to infect host arms and cause their death in vineyards. This species was detected as causal agent of arm dieback in grapevines in Australia (Castillo-Pando *et al.*, 2001) and Uruguay (Abreo *et al.*, 2008).

Because this fungus could be detected from both symptomatic and non-symptomatic plants, Navarrete *et al.* (2011), suggested that it was latent pathogen, capable of producing symptoms when plants are under stress. In the present study, during the field sampling, asymptomatic plants containing *G. uvicola* were detected. After a few weeks, wilt and yellowing were observed on the tips and lateral leaves of the infected shoots, followed by formation of dark rings and general death of leaves on the branches (Figure 1A). The symptoms of *G. uvicola* in Sonora grapevines could be confused with those caused by *Lasiodiplodia theobromae* (Úrbez-Torres *et al.*, 2008). Both fungi produced wedge-shaped staining in cross-section of cordons. However, the presence of numerous visual conidiomata in the affected areas differentiates these two pathogens.

The symptoms of dead arm caused by *G. uvicola* can be confused with those caused by other pathogens, such

as *Eutypa lata* and species of Botryosphaeriaceae (Navarrete *et al*., 2009). Accurate and timely diagnoses are essential for good prevention and control of the disease, and molecular techniques for pathogen detection and identification are very reliable.

In conclusion, the symptoms observed in the field, the morphological characteristics, phylogenetic analysis, and pathogenicity tests carried out for isolates obtained from disease grapevine plants, allowed identification of *G. uvicola* as the causative agent of dieback of arms, bud necrosis, and disease symptoms in shoots and branches in vineyards of Sonora state. This is the first report of the infectious capacity of *G. uvicola* in wood tissues of *Vitis vinifera* plants in Mexico.

ACKNOWLEDGEMENTS

The authors thank Asociación Agrícola Local de Productores de Uva de Mesa, A. C. en Hermosillo, Sonora, México for supporting part of this research.

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