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Research Paper

Microscope observations of Botryosphaeriaceae spp. in the presence of grapevine wood

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Summary. Grapevine trunk diseases (GTD) are important threats to long-term longevity, productivity, and profitability in all grape production systems. Botryosphaeria dieback is caused by at least 32 Botryosphaeriaceae fungi. The main symptoms of this disease on grapevines are wedge-shaped cankers in the trunks, general decline and eventually death of affected plants. Pathogens from this family have broad host ranges and varying virulence. This study aimed to compare, and gain some insight into how, *Lasiodiplodia gilanensis*, *Diplodia seriata,* and *D. corticola* colonize grapevine tissues. *In vitro* studies using pycnidiospores as inoculum, showed that the presence of grapevine wood affected spore germination, hyphal growth and branching, and biomass production, especially in *L. gilanensis*. *In planta,* microscopy revealed the use of xylem vessels as the preferred sites of pathogen entry, and differences in growth and hyphal branching among species. *Lasiodiplodia gilanensis* produced cellulases at the beginning of the pathogen/host interaction to degrade plant tissues and invade parenchymal tissue, while *D. seriata* and *D. corticola* grew endophytically in the first stages of grapevine colonization.

Keywords. Grapevine trunk disease fungi, Botryosphaeria dieback, plant-pathogen interaction.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are a significant challenge for grape production; they threaten grapevine longevity, productivity, and profitability in all grape production systems. To date, there are no effective control methods available for these diseases. GTDs are a group of complex diseases, including Petri disease, Black foot, Esca complex, Eutypa dieback, and Botryosphaeria dieback (Gramaje *et al*., 2018; Mondello *et al*., 2018). The causal agents are fungi, which diminish grape yields, causing gradual deterioration that eventually can lead to the death of grapevines (Siebert, 2001; Gubler *et al*., 2005; Bertsch *et al*., 2013).

The principal point of entry of GTD fungi to host plants is through mechanical damage, pruning wounds, and natural openings (Gubler *et al*.,

2005). Fungi colonize the xylem vessels of plant tissues causing reduction of the flow of nutrients, release of toxins, and degradation of xylem vessel cell walls by release of hydrolytic enzymes. These slowly kill affected vines (Valtaud *et al*., 2009). Plants react to the infections, producing biochemical barriers as accumulations of tyloses and gummosis, causing blockage of the xylem vessels that limit the fungal invasion (Fontaine *et al*., 2016).

From 2000, the role of Botryosphaeriaceae in the GTD black dead arm has been increasingly recognized (Phillips, 2002), and later the disease was renamed as Botryosphaeria dieback (Úrbez-Torres, 2011). To date, at least 30 different species of fungi have been associated with Botryosphaeria dieback, belonging to the genera *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria,* and *Spencermartinsia* (Úrbez-Torres, 2011; Billones-Baaijens *et al*., 2015; Yang *et al.,* 2017). Disease symptoms include wedge-shaped perennial cankers in grapevine trunks, dead arms, sprout decay, bud necrosis, and death of grafts (Úrbez-Torres, 2011).

Botryosphaeriaceae have cosmopolitan distribution (Úrbez-Torres, 2011), although it has been suggested that some genera are more common in different climates, for example, *Diplodia* in temperate regions (Burgess and Wingfield, 2002) and *Lasiodiplodia* in tropical and sub-tropical regions (Mohali *et al*., 2005; Burgess *et al*., 2006). These fungi colonize weak and stressed plants, for example, after drought, hail, wind, or frost (Smith *et al*., 1994; Denman *et al.,* 2000).

The Botryosphaeriaceae have life cycles divided into a sexual phase (teleomorph), which rarely occurs in nature, and an asexual phase (anamorph). Asexual reproduction is through the formation of multilocular ascomata with multilayer walls, which together form in pycnidia (Denman *et al*., 2000). Humidity and rain promote the release of the pycnidiospores which are dispersed mainly by wind (Epstein *et al*., 2008).

In Mexico, five species of Botryosphaeriaceae have been reported, including *Lasiodiplodia theobromae*, *Diplodia seriata, D. corticola, Neofusicoccum vitifusiforme,* and *N. australe* (Úrbez-Torres *et al*., 2008; Candolfi-Arballo *et al*., 2010). Pathogenicity tests showed *L. theobromae* was more virulent than *D. seriata* or *D. corticola*, with *D. corticola* acting as a weak pathogen of *Vitis vinifera* (Úrbez-Torres *et al*., 2009; Varela *et al*., 2011).

Little is known of how Botryosphaeriaceae penetrate and invade host tissues. *Lasiodiplodia theobromae* is also highly aggressive in rubber tree (*Hevea brasiliensis)* (Encinas, 1997), Caribbean pine (*Pinus caribaea)* (Cedeño *et al*., 1996), and cashew *(Anacardium occidentale)* (Muniz *et al*., 2011). In naturally and experimentally infected tissues of Caribbean pine, *L. theobromae* invades all the tissues of sapwood but grows more abundantly in the rays; hyphae invade transversely and longitudinally, degrading the xylem cell walls, quickly invading the vessels, intercellular spaces, and parenchyma (Cedeño *et al*., 1996). On fresh wood of rubber (*H. brasiliensis*) and aspen (*Populus tremula*), after 2 to 3 d, the fungus has fast and abundant growth, with ray and axial parenchyma, and vessels, being the preferred pathways of colonization (Encinas and Daniel, 1997). In 2-month-old cashew plants, *L. theobromae* hyphae are located within the xylem vessels, with extensive development in the secondary xylem tissues (Muniz *et al*., 2011). Differently than in grapevine, *D. corticola* is one of the most aggressive fungal pathogens of *Quercus*, and uses stomata as the host entry points. Strains of this pathogen differ in virulence, inducing different symptoms in infected plants (Félix *et al*., 2017). In peach (*Prunus persica), D. seriata* invades xylem tissues of the superficial xylem layers of the xylem, phloem, meristematic tissues, and parenchyma, causing necrosis (Biggs and Britton, 1988). Conidia of *Neofusicoccum luteum* inoculated onto green tissues were unable to germinate without wounding (Amponsah *et al*., 2012). In apple (*Malus domestica*), *Botryosphaeria dothidea* entered host twigs at the lenticels and grew intercellularly in the exocarps (Guan *et al*., 2015).

Understanding the biology and mechanisms of infection of the Botryosphaeriaceae in grapevine could help with the implementation of disease management methods, which are not able to limit GTDs. In the present study, several microscopy techniques were used to analyze and compare the infection mechanisms of *L. gilaniensis, D. seriata,* and *D. corticola*. Since these fungi are, respectively, of high, intermediate, and low virulence to grapevine (*Vitis vinifera*), the main aim was to study their colonization behaviour during infection of this host.

MATERIALS AND METHODS

Strains used in this study

Diplodia corticola SASII2-3 and *D. seriata* BY06-3 were isolated from grapevines growing in the county of Ensenada, Baja California, Mexico, and these isolates are held in the laboratory of plant pathology of the Center for Scientific Research and Higher Education of Ensenada (CICESE) (Candolfi-Arballo, 2010). *Lasiodiplodia gilaniensis* UCD256Ma, previously isolated and classified as *L. theobromae* (Úrbez-Torres *et al*., 2006), was provided by Dr Douglas W. Gubler from the University of California, Davis. Isolates were identified based on their morphological characteristics and by comparing their ITS

and EF1α with those deposited in the GenBank of the NCBI. All the isolates were grown on potato dextrose agar (PDA; BD Difco™), and were preserved at -80°C in a solution of water and 20% glycerol.

Induction of pycnidia and inoculum preparation

To induce formation of pycnidia, *L. gilaniensis* and *D. seriata* were incubated in Petri plates containing water agar (WA) at 15 g L^{-1}, which was supplemented with 15 μg mL^{-1} chloramphenicol to inhibit bacterial growth. Four to six autoclaved wood toothpicks were placed on the top of the culture medium in each plate, and the plates were kept at 28°C for 3 weeks with a daily photoperiod of 12 h light and 12 h darkness. This procedure gives few pycnidia in *D. corticola*, for pycnidium induction in this fungus, autoclaved vine wood sticks (approx. 3 cm long and 1 cm diam.) were placed on the agar and plates were kept in the above conditions for 4 weeks. Pycnidia were collected with the aid of a dissecting needle. Pycnidiospores were released from pycnidia using a previously sterilized pestle and mortar. Conidia were recovered from the pycnida remains by washing with sterile distilled water and passing twice through Miracloth (475855, EMD Millipore). The final concentration of pycnidiospore suspensions was adjusted to 40 μL^{-1} after counting using a Neubauer chamber.

Evaluation of growth rates of Botryosphaeriaceae in the presence of grapevine wood

Growth rates were each evaluated using 10 μL of pycnidiospore solution of each strain (obtained as above) that were placed at one side of a 9 cm diam. Petri dish, either containing Vogel's Minimum Medium (VMM) or VMM supplemented with grapevine wood (VMM-W). VMM-W was prepared using fresh wood obtained from grapevine plants (approx. 1-y-old). This was washed in sterile purified water, ground in an Osterizer 541® blender, and then sterilized together with VMM medium. The pycniospore-inoculated dishes were placed at 28°C for 72 h. Growth of the fungi was evaluated by measuring the longest hyphae from the inoculation point in each dish.

Comparison of biomass production among Botryosphaeriaceae in the presence of grapevine wood

To evaluate the variation in the biomass production of the three Botryosphaeriaceae in presence of grapevine wood, circles (80 mm diam.) were obtained from a dialysis membrane (Spectrapor membrane tubing, Spectrum Medical Industries, Inc.). The membrane circles were weighed, boiled for 1 h to eliminate salts, and then autoclaved while immersed in distilled water. Once sterilized, they were each placed in the centre of a Petri dish containing either VMM or VMM-W media. Pycnidiospore suspensions (10 μL each) were then individually inoculated on the top of the membrane, and the membrane-containing plates were incubated at 28°C for 60 h. The membranes were then removed from the plates, and the fresh weight of mycelium was determined for each treatment.

Comparison of germination percent and branching in the presence of grapevine wood

Pycnidiospores of Botryosphaeriaceae are released usually in cirri; in *L. gilanensis* and *D. corticola* at the beginning, the pycnidiospores are one-celled and hyaline, but later they become two-celled and pigmented (Úrbez-Torres, 2011). In *D. seriata,* pycnidiospores are initially hyaline but soon became brown before they are released from the pycnidia (Phillips *et al*., 2007). To evaluate the variation in germination of pycnidiospores and the hyphal branches produced for the three Botryosphaeriaceae, the percent of germination and the branching rate were evaluated using the pycnidiospore suspension of each isolate (obtained as above). Ten μL of suspension were placed into each 9 cm diam. Petri dish containing VMM or VMM-W and spread over the medium surface with a glass rod. Plates were incubated at 28°C and observed using an inverted microscope (Zeiss Axiovert) at 2, 4, 6, 8, 12 and 24 h after inoculation, to determine the germination rate, and at 2, 4, 6, and 8 h, to estimate the number of branches produced from each pycnidiospore. For *L. gilaniensis* and *D. corticola*, 60 pycnidiospores were evaluated, half of which were pigmented and half hyaline. For *D. seriata,* 30 pycnidiospores were assessed, since this species only produced pigmented pycnidiospores. The pycnidiospores were each considered as germinated when they presented a hypha of at least half the pycnidiospore length.

Plant inoculation assays to evaluate wood colonization by Botryosphaeriaceae

One-year-old plants of *Vitis vinifera* 'Cabernet Sauvignon', maintained in a greenhouse with temperatures from 15 to 28 °C, were inoculated with spore suspensions, using five plants for each of the tested fungi. Fresh wounds were made by cutting the top of the woody plant stems. After 30 min to avoid exudate

from the plants, 30 µL of the pycnidiospore suspension of each isolate was placed on the wound surface. After 12, 24, and 48 hours post-inoculation, a sample of wood (approx. 1 cm length) from each inoculation site was collected and processed for epifluorescence microscopy and scanning electron microscopy (SEM) observations (see below). For epifluorescence, samples were fixed in FAA solution (90 mL of 50% ethanol, 5 mL of 40% formaldehyde solution, and 5 mL of glacial acetic acid). For SEM, samples were fixed in Karnovsky's reagent (3% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer, pH 7.4). Treated samples were kept in the respective fixatives for 24 h at 4°C.

Epifluorescence microscopy and SEM observations of host colonization

Fixed samples in FAA were stained with the solutions of Calcofluor White stain (Fluka) at 1:1 with 10 % KOH (w/v) and Calcofluor White M2R (Exc/Ems 355/433 nm, Cyanamid) at 1 % (v/v) in 15% KOH (w/v). Each sample was covered with 10 µL of Calcofluor White for 2 min, the excess was then removed, and 10 µL of Calcofluor White M2R was added. After 30 s, the stained samples were observed in an inverted microscope (Zeiss Axiovert) under UV light (340 nm) using a DAPI filter.

For observation with SEM, samples previously fixed with Karnovsky's reagent were washed three times (15 min each) with phosphate buffer (0.2 M, pH 7.2). They were then postfixed under a fume extraction hood with 4% osmium tetraoxide, and incubated for 2 h at 4°C. After another three 15 min washes in the phosphate buffer, samples were dried for 1 week in a desiccator containing silica beads. Dehydrated samples were covered with gold (Ted Pella, 99.99%) using a sputter coater, and were kept in a desiccator until SEM (JEOL JSM-35c) observation.

For epifluorescence and SE microscopy, observations were made in the wound areas of the samples, in longitudinal and transverse stem incisions.

Statistical analyses

Fungus growth, germination, and branching experiments were carried out in triplicate, and data were processed using multiple variance analysis (ANOVA) as a parametric test and by Kruskal-Wallis assessment as a nonparametric analysis. For the isolate biomass data, a T-test analysis was performed. Data were processed using STATISTICA™ software (Hill and Lewicki, 2012).

RESULTS

Effects of grapevine wood on growth and biomass production of L. gilaniensis*,* D. seriata *and* D. corticola

In general, more growth of the three fungi was observed in the presence of grapevine wood (VMM-W) than in VMM. *Lasiodiplodia gilaniensis* reached average colony diameter 6.51 ± 0.15 cm in VMM-W and 5.59 ± 0.13 cm in VMM, with no statistical differences among the treatments. *Diplodia seriata* average colony diameter was 5.58 ± 0.39 cm in VMM-W and 3.67 ± 0.3 cm in VMM; and for *D. corticola,* 3.33 ± 0.3 cm in VMM-W and 1.22 ± 0.09 cm VMM. For both *Diplodia* spp., there were significant differences ($P < 0.05$) among treatments (Figure 1).

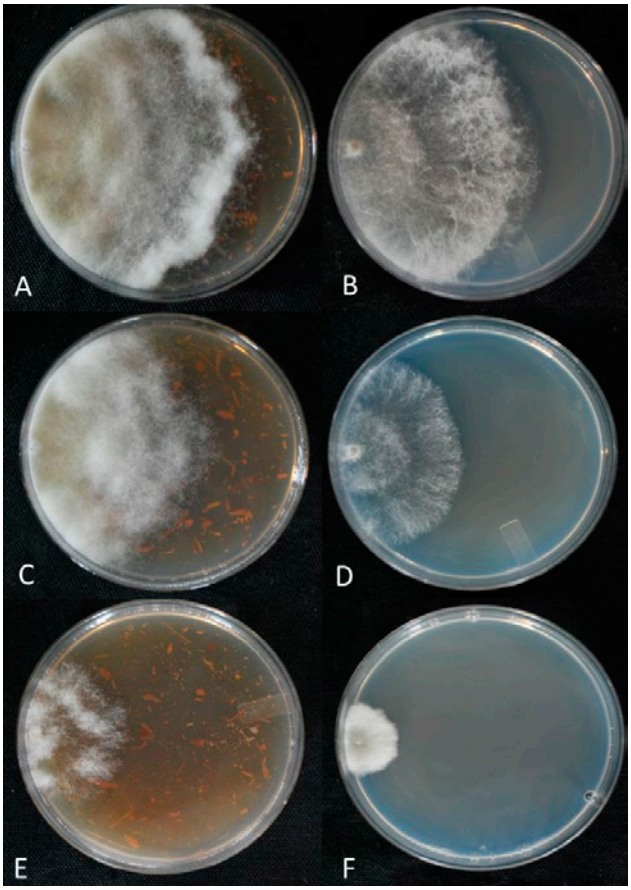

Figure 1. Mean colony diameters for three fungi on Vogel's Minimum Medium with (VMM-W) (A, C, and E) or without (VMM) (B, D, and F) ground grapevine wood. The fungi were *Lasiodiplodia gilaniensis* (A and B), *Diplodia seriata* (C and D), and *D. corticola* (E and F). The fungi were grown for 72 h at 28°C.Visible effects were observed, mainly in *D. corticola* and *D. seriata*.

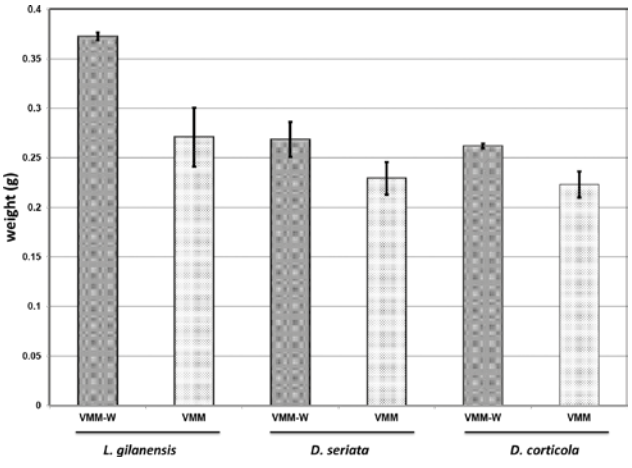

Figure 2. Mean weight of biomass of *Lasiodiplodia gilaniensis*, *Diplodia seriata* or *D. corticola* grown on Vogel's Minimum Medium in the presence (VMM-W) or the absence (VMM) of grapevine wood. There was a strong effect for the presence of grapevine wood for *L. gilaniensis*.

Lasiodiplodia gilaniensis produced the greatest biomass (average weight = 0.372 ± 0.004 g on VMM-W and 0.271 ± 0.029 g on VMM, while for *D. seriata,* the average biomass weight was 0.268 ± 0.018 g on VMM-W and 0.229 ± 0.016 g on VMM. *Diplodia corticola* produced the least biomass in both treatments, with an average weight of 0.262 ± 0.002 g on VMM-W and 0.223 ± 0.013 g on VMM. There were no significant differences ($P > 0.05$) between *D. corticola* and *D. seriata* for both treatments, or with *L. gilaniensis* in the absence of ground grapevine wood in the medium (Figure 2).

Effects of grapevine wood on germination and hyphal branching from pycnidiospores of Botryosphaeriaceae

The presence of wood in the growth medium influenced germination of pycnidiospores. In general, pycnidiospores germinated more rapidly and in greater proportions in the presence of grapevine wood (Figure 3). Two h after inoculation, 70% of *L. gilaniensis* pigmented pycnidiospores germinated on VMM-W (43% on VMM), and reached 90% germination at 4 h in the wood-amended media and 73% on VMM. Six h after inoculation, there were no statistically significant differences observed between the two media (96% on VMM-W and 93% in VMM), because most spores in VMM-W had germinated already at 4 h. Similarly, hyaline pycnidiospores germinated more rapidly and in greater proportions on VMM-W, but germination was less for these pycnidiospores than for pigmented ones (Figure 3A).

In *D. seriata,* germination commenced later (4 h after inoculation) than for the other two fungi, always resulting greater germination in the presence of grapevine wood. However, significant differences were found only at 4 h (53% for VMM-W and 6% for VMM) (Figure 3B).

In *D. corticola*, 60% of pigmented pycnidiospores germinated on VMM-W after 2 h, while there was no germination after the same period on VMM. After 8 h, 90% germination was recorded on the wood-amended media, compared with 46% on VMM. Similarly, hyaline pycnidiospores germinated more rapidly and in greater proportions on VMM-W, but always at less proportions than pigmented pycnidiospores (Figure 3C).

Lasiodiplodia gilaniensis exhibited the greatest hyphal branching on VMM-W (ten branches after 8 h), while *D. seriata* had one branch and *D. corticola* two

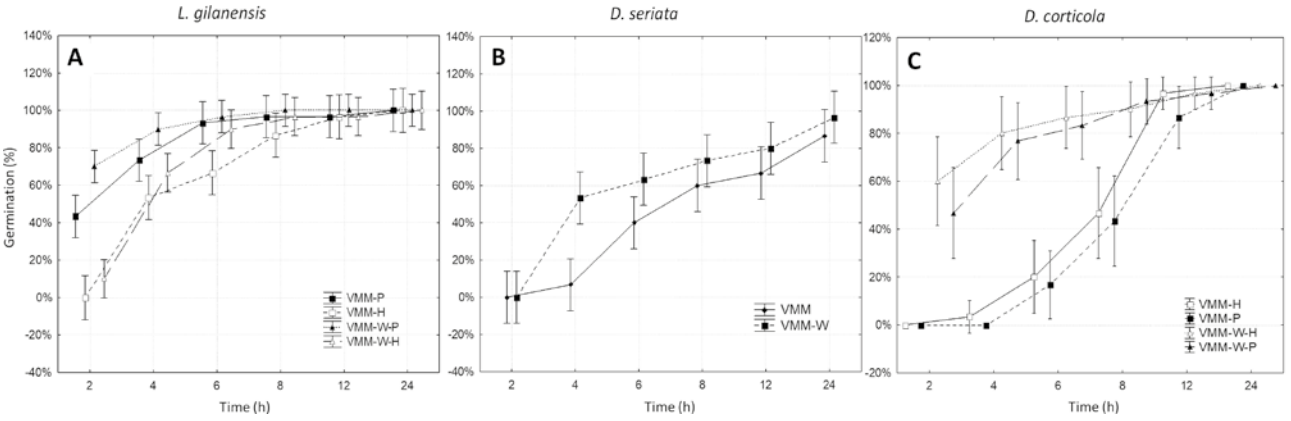

Figure 3. Mean germination proportions for pigmented (P) and hyaline (H) pycnidiospores of *Lasiodiplodia gilaniensis* (A), *Diplodia corticola* (B), and *D. seriata* (C), grown in Vogel's Minimum Medium (VMM) or VMM supplemented with grapevine wood (VMM-W). The presence of grapevine wood in the medium mostly affected germination of *D. corticola*.

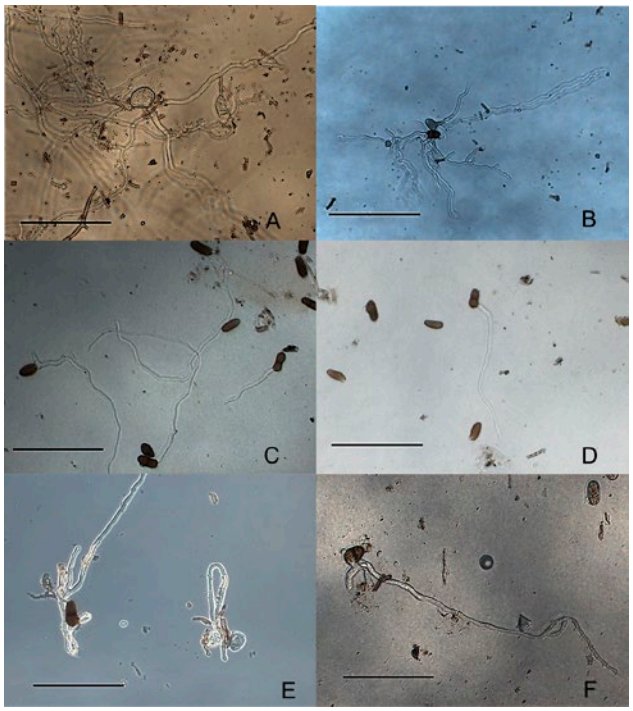

Figure 4. Hyphal branches observed in the fungi *Lasiodiplodia gilaniensis* (A and B), *Diplodia seriata,* (C and D) and *D. corticola* (E and F), after 8 h growth on Vogel's Minimum Medium supplemented with (A, C, and E) or without grapevine wood (B, D, and F). Increased numbers of hyphal branches were observed in the media containing wood. Bars = 50 µm.

branches after the same period) (Figure 4). The presence of grapevine wood in the media did not affect the branching in *L. gilaniensis*, but there were differences among hyaline and pigmented pycnidiospores, with a greater number of branches observed in pigmented than hyaline pycnidiospores (Figure 5A). In *D. seriata,* the presence of wood stimulated hyphal branching, although with no statistical significance (Figure 5B). In *D. corticola,* the number of hyphal branches was greater in the treatments with wood for both hyaline and pigmented pycnidiospores (Figure 5C).

Colonization of grapevine woody tissues by three Botryospaeriaceae *pathogens*

Microscope observations showed no germinated pycnidiospores of *L. gilaniensis, D. seriata* or *D. corticola* in grapevine wood 12 h after inoculation (Figures 6A, 6D, and 6G). In contrast, after 24 h, germination was abundant observed (Figures 6B, 6E, and 6H), especially in *L. gilaniensis*. None of the fungi formed appressoria.

At 24 h after inoculation, all the pycnidiospores of *L. gilaniensis* had germinated. Germ tubes emerged from the central parts of the spores, and the resulting hyphal branches showed directional growth to xylem vessels, tracheids, and phloem. The xylem vessels were the most frequent site of pathogen entry (Figure 6B). In SEM, the entrance through xylem vessels and tracheids was more evident, as was collapse of some spores (Figures 7A, 7B).

Also at 24 h after inoculation, most of the pycnidiospores of *D. seriata* had germinated, developing two to three branches with linear growth of the hyphae until finding openings to penetrate host tissues (Figure 6E). Similar to *L. gilaniensis*, germination hyphae came out from the central parts of the pycnidiospore towards xylem vessels and tracheids, with the xylem vessels being the most common host entry sites (Figure 6E). In SEM,

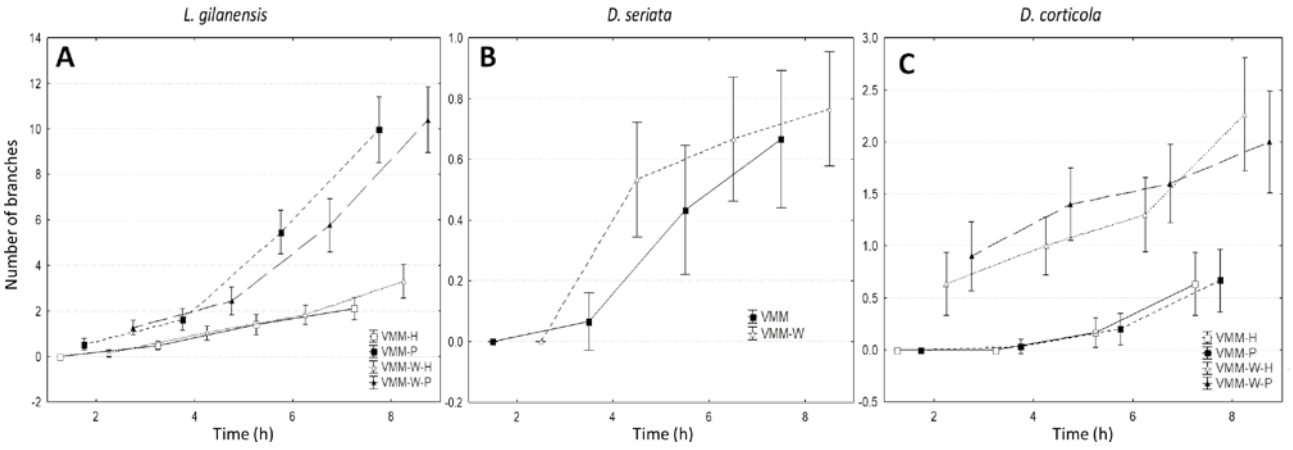

Figure 5. Mean numbers of hyphal branches produced by pigmented (P) or hyaline (H) pycnidiospores of *Lasiodiplodia gilaniensis* (A), *Diplodia seriata* (B), or *D. corticola* (C), in the absence (VMM) or presence of grapevine wood (VMM-W) in the growth medium. The presence of grapevine wood affected the branching of *D. corticola*.

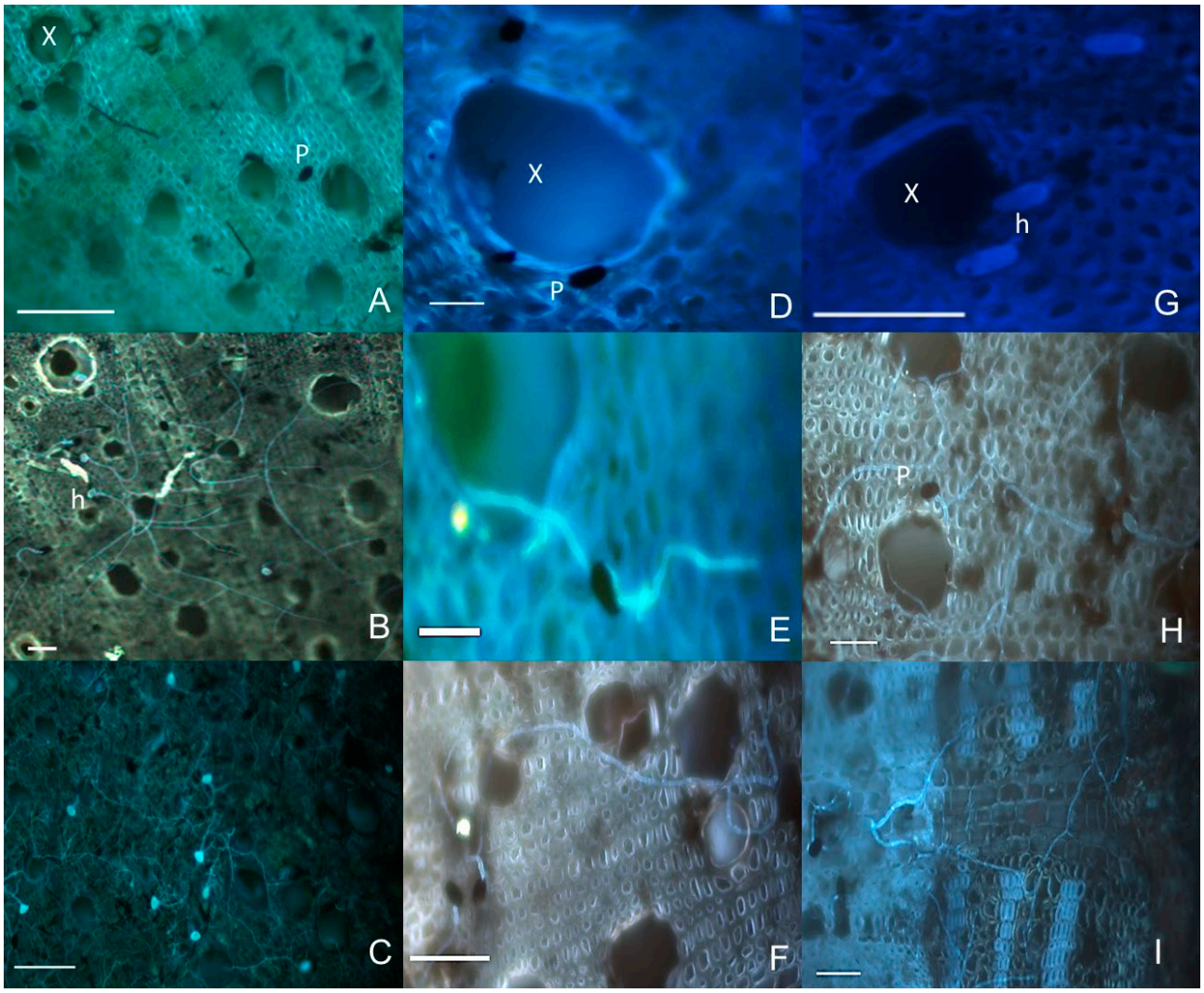

Figure 6. Transverse sections of grapevine stems inoculated with pycnidiospores of *Lasiodiplodia gilanensis* (A, B and C), *Diplodia seriata* (D, E, and F) or *D. corticola* (G, H and I), observed under epifluorescence microscopy at 12 h (A, D and G), 24 h (B, E and H) or 48 h (C, F and I) after inoculation with these fungi. Greater hyphal branching and colonization was observed in stems inoculated with *L. gilanensis.* X) xylem vessel; h) hyaline pycnidiospores; p) pigmented pycnidiospores. Bars = 50 µm.

D. seriata grew in and out of the xylem vessels, and in some cases, hyphae and conidia were distorted (Figures 7C and 7D).

In contrast, fewer pycnidiospores of *D. corticola* germinated and showed few ramifications and septa at 24 h (Figures 6H). As in the other species, hyphae came out from the central parts of the spores and entered the host xylem vessels, tracheids, and phloem, with the xylem vessels being the most common site of entry (Figures 6H, 7E, 7F). In pycnidiospores close or inside the xylem vessels, several mycelium branches were observed (Figures 7E and 7F).

At 48 h after inoculation for the grapevines inoculated with *L. gilaniensis,* extensive mycelium growth was observed invading the host wood, as highly branched hyphae of different diameters. These entered through xylem vessels, tracheids, and phloem (Figure 6C). In longitudinal stem sections, the fungus colonized the xylem vessels, degrading and thickening the cell walls of the pits, and moved to the adjacent tissue (Figures 8A, 8B). More hyphae were observed entering the xylem vessels and tracheids than the phloem tissues. In transverse stem sections, *L. gilaniensis* was growing on medullary and radial parenchymal tissues, but growth was mainly observed in the secondary xylem. In the xylem vessels, formation of tyloses was evident but without obvious tissue damage (Figures 9A, 9B).

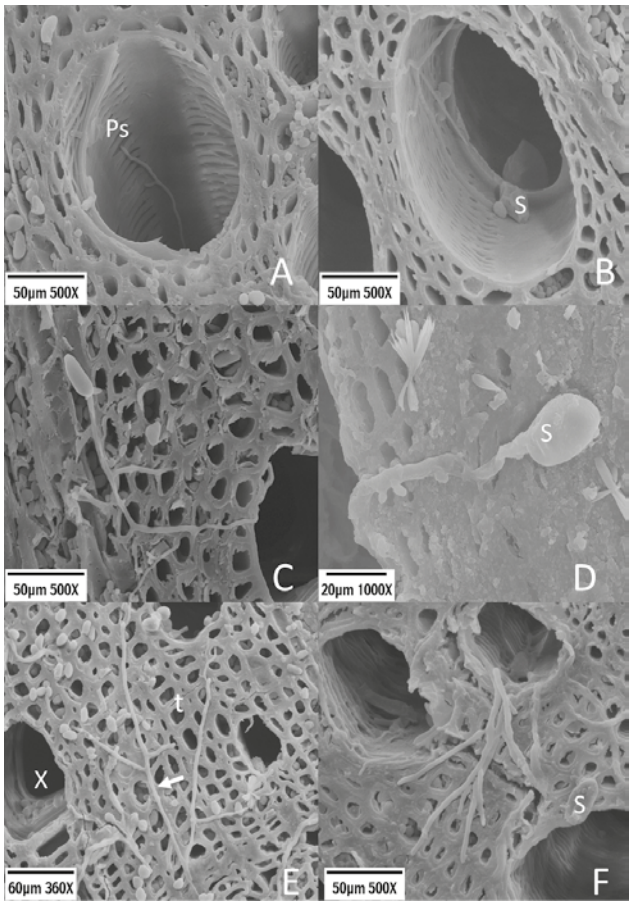

Figure 7. SEM of cross-sections of grapevine stems inoculated with spores of *Lasiodiplodia gilaniensis* (A and B), *Diplodia seriata* (C and D) or *D. corticola* (E and F), at 24 h after inoculation. A, hyphae entering the pits to colonize adjacent tissue. B, spores and hyphae growing in and out of a xylem vessel. C and D, hyphae entering the xylem vessels and tracheids. E, hyphae entering tracheids. F, germinated spore with hyphae entering and branching in and out of a xylem vessel. X) xylem vessel; t) tracheids; S) spore; Ps) pits.

The germinated spores of *D. seriata* also developed four to six branches, moving towards of the phloem and xylem vessels (Figures 6F, 9D). The formation of tyloses was also observed (Figure 9C). In longitudinal stem sections, *D. seriata* colonized the xylem vessels and the medullar and radial parenchymal tissues, without showing symptoms of degradation of the tissues but using the pits as anchorage sites and possibly for the colonization of the adjacent cells (Figure 8B).

Also at 48 h after inoculation with *D. corticola,* most pycnidiospores were germinated, and hyphae were entering the xylem vessels (Figure 6I). In longitudinal sections, hyphae were colonizing the xylem vessels; however, no degradation of the xylem walls or colonization of the adjacent tissues was observed (Figure 8C). Mycelium

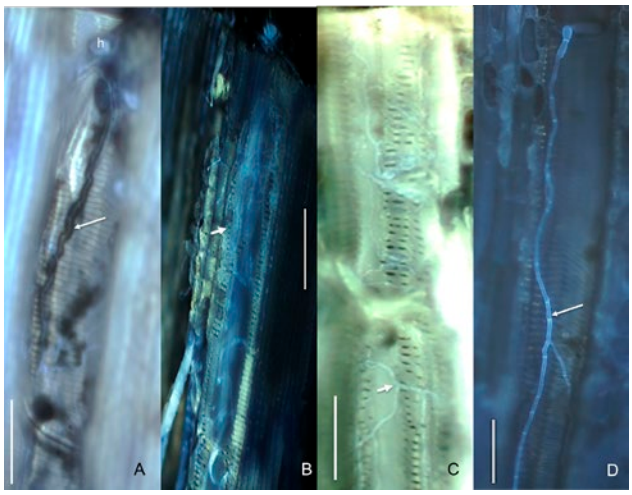

Figure 8. Longitudinal sections of grapevine stems inoculated with pycnidiospores of *Lasiodiplodia gilaniensis* (A and B), *Diplodia seriata* (C) or *D. corticola* (D), observed using epifluorescence microscopy 48 h after inoculation. In *L. gilaniensis*, degradation of the cell wall of a vessel (A) and colonization of the xylem vessels (B) were observed. C, Hyphal branches inside the trachea colonizing a vessel and using pits as anchorage sites. D, Hyphal branches growing along the trachea. X) xylem vessel; h) hyaline pycnidiospores, arrow) hypha. Bars = 50 µm.

was growing on medullary and radial parenchymal tissues of the vessels, and the hyphal branches were using the pits as anchoring sites, possibly for colonization of adjacent tissues (Figures 9E and 9F).

DISCUSSION

The initial state for the establishment of some fungi in host plants is germination of spores, and the substrate to which the spore attaches regulates germ tube directional growth, branching, and appressorium formation (Nicholson and Epstein, 1991). The purpose of the present investigation was to determine the influence of grapevine wood upon these infection stages in three Botryosphaeriaceae species.

Pathogen growth rates were affected by the presence of grapevine wood, especially in *D. seriata* and *D. corticola*. For *L. gilanensis,* this effect was less, possibly due to rapid growth, since the most rapid growth was observed in *L. gilanensis*, followed by *D. seriata*, which had medium growth, and *D. corticola* with the least. Pathogenicity tests in grapevine have shown that *Lasiodiplodia* spp. are highly virulent pathogens, causing pronounced tissue damage in comparison with other species of the Botryosphaeriaceae (Úrbez-Torres and Gubler, 2009; Candolfi-Arballo *et al.*, 2010). This obser-

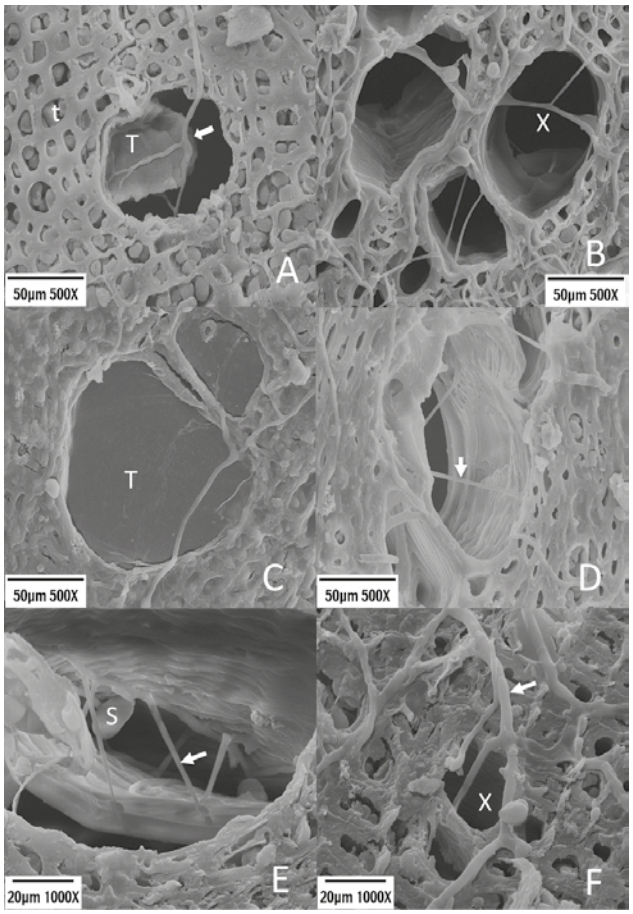

Figure 9. SEM cross-sections of grapevine stems inoculated with pycnidiospores of *Lasiodiplodia gilaniensis* (A and B), *Diplodia seriata* (C and D) or *D. corticola* (E and F), at 48 h after inoculation. A, tylosis formation in a xylem vessel, and hyphae entering the vessel. B, hyphae entering a xylem vessel. A large amount of mycelium is present. C, hyphal entrance blocked by a tylosis. D, hyphae entering xylem vessels, using pits as anchoring sites for colonization of the xylem vessel cell walls (arrow). E, pycnidiospore inside a trachea, colonizing a xylem vessel and using the pits as anchor sites. The cell wall is not damaged. D, hyphae entering a xylem vessel. Arrows indicate hyphae. X) xylem vessel; T) tyloses; t) tracheids; S) pycnidiospore.

vation is similar to that for the present study, where *L. gilaniensis* had more rapid growth on grapevine stems that the two *Diplodia* spp. This rapid growth is likely to assist rapid colonization of grapevine by *L. gilaniensis*.

The presence of wood also increased the germination of spores, especially in pigmented pycnidiospores of *L. gilanensis*, which had the highest percentage of germination in the shortest time. Differently, in previous work, hyaline spores of *L. theobromae* had a higher percentage of germination than pigmented spores, and a faster germination rate in PDA, a nutritionally rich media (Úrbez-Torres *et al*., 2010). Here, minimal media was used; thus, the observed differences probably are due to the isolate and the composition of the culture medium (Arauz and Sutton, 1989).

Pycnidiospores germinated more rapidly in culture media compared with *in planta*. *In vitro,* after a period of 12 h in the presence of grinded grapevine wood, up to 80% of the spores of the three species had germinated. Similarly, up to 90% of spores of *B. dothidea* germinated at 4 h in PDA at 25°C (Úrbez-Torres *et al*., 2010); while in apple after 9 h at 25°C these spores had not completely germinated (Kim *et al.,* 1999). Since autoclaving wood increases soluble sugars (Hulme and Shields, 1970), the media supplemented with grapevine wood probably enhanced spore germination, while *in planta*, the lack of appropriate conditions for germination, such as temperature, water availability and the presence of nutrients transferred from the host into the water, may have slowed the spore germination (Barkai-Golan, 2001).

Formation of appressoria in Botryosphaeriaceae has been previously observed only in *B. dothidea* growing in apple fruit (Kim *et al*., 1999). Similarly, none of the three pathogens studied here formed appressoria. Instead, after germination, the hyphae entered the closest traqueid or xylem vessels.

Greater biomass was produced in the three fungal species in the presence of grapevine wood than without, especially in *L. gilanensis*. Also in this fungus, when growing *in planta,* hyphae were surrounded by dark haloes, and the invasion of parenchyma cells was observed. Since calcofluor binds to chitin and cellulose (Harrington and Hageage, 2003), the halo indicates the production of cellulases and the degradation of cell walls of xylem vessels. For the isolate of *L. gilanensis* used here (formerly named *L. theobromae*), several plant cell wall-degrading enzymes involved in cellulose and hemicellulose degradation have been found to be up-regulated in response to grapevine wood (Paolinelli-Alfonso *et al*., 2016). This fungus may therefore be using the cell wall components for growth from the beginning of the fungus-plant interaction. In contrast, tissue degradation was not observed in *D. seriata* or *D. corticola*. This supports the likelihood that both these species can penetrate and survive in plants without causing damage, while having the capacity to become pathogens. However, both of these *Diplodia* spp. are less pathogenic to grapevine than *L. theobromae* (Taylor *et al*., 2005; Candolfi-Arballo *et al*., 2010; Úrbez-Torres, 2011).

There was a marked tendency for the three species to use xylem vessels as a preferential site of entry to grapevine stems, with tracheids as a secondary entrance point, and phloem very rarely. These observations are consistent with those for other members of Botryosphaeriaceae,

such as *Neofusicoccum australe*, *N. luteum*, *N. parvum,* and *D. mutila* infecting grapevine (Amponsah *et al*., 2012); *L. theobromae* infecting *Pinus caribaea* (Cedeño *et al*., 1996), *Populus tremula*, *Hevea brasiliensis* (Encinas and Daniel, 1997) and *Anacardium occidentale* (Muniz *et al*., 2011); and for *Diplodia seriata* and *B. dothidea* in *Prunus persica* (Biggs and Britton, 1988).

SEM observations confirmed the tracheae (vessel elements) were a preferred site for grapevine wood colonization in all three pathogen species evaluated. The three Botryosphaeriaceae interacted differently with parenchyma tissue, where degradation and invasion were detected during *L. gilanensis* colonization, while no colonization by *D. seriata* and *D. corticola* was observed. This agrees with the behaviour of *D. seriata* observed in peach, where xylem vessel colonization occurred 7 d after inoculation, while the colonization of parenchymal tissue occurred after 28 d (Biggs and Britton,1988). The present study also confirmed that *L. gilaniensis* rapidly colonizes grapevine tissues.

The formation of tyloses in response to the colonization by the three fungi agrees with observations of *L. theobromae* (Al-Saadoon, 2012). SEM also indicated the ability of *L. gilaniensis* to use the xylem pits to colonize adjacent tissues, but this was not observed in *D. seriata* or *D. corticola*. The same behaviour was reported for *Neofusicoccum luteum* which is another highly virulent pathogen of grapevine, and colonizes cashew (Muniz *et al*., 2011), and grapevine (Amposah *et al*., 2012; (Úrbez-Torres, 2011). The ability of these species to use xylem pits may confer ability to efficiently colonize hosts and increase virulence.

This is the first study to compare effects of grapevine wood on germination kinetics and colonization of three botryosphaeriaceous fungi, indicating that their behaviour *in planta* correlates with their relative levels of pathogenicity.

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