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Research Papers - 12th Special issue on Grapevine Trunk Diseases

Unravelling the colonization mechanism of *Lasiodiplodia brasiliensis* in grapevine plants

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Summary. *Botryosphaeriaceae* cause the degenerative disease Botryosphaeria dieback in many woody hosts, including grapevine. These pathogens penetrate host plants through pruning wounds, and colonize vascular tissues causing necrotic lesions, cankers, and eventually plant death. Colonization processes by *Botryosphaeriaceae* and their interactions with their hosts has been understudied. The colonization mechanisms were examined for *Lasiodiplodia brasiliensis*, a common pathogen causing Botryosphaeria dieback in Mexican vineyards. *Lasiodiplodia brasiliensis* MXBCL28 was inoculated onto grapevine 'Cabernet Sauvignon' plants, and after 2 months, infected tissues were observed with microscopy using histological techniques. *Lasiodiplodia brasiliensis* was also cultured on different carbon sources representing cell walls and non-structural plant components, to complement histology data. The host responded to wounding by developing xylem vessel occlusions with tyloses and deposition of suberin in cambium and ray parenchyma. Infection response also included deposition of suberin in pith tissues, reinforcement of cell walls with phenolic compounds, and lignin deposition in xylem vessels and ray parenchyma. The pathogen could overcome host compartmentalization mechanisms and colonize wood tissue causing extensive necrosis. The fungus was visualized in host cambium, vascular bundles, xylem vessels, and pith, and infected tissues were depleted in starch in the ray parenchyma. Cellulose, hemicellulose, and lignin in cell walls were also degraded, supporting *in vitro* data.

Keywords. Botryosphaeria dieback, plant defense, carbohydrate metabolism.

INTRODUCTION

Grapevine is threatened by many pathogenic microorganisms that reduce yields and fruit quality (Armijo *et al*., 2016). Grapevine Trunk Diseases (GTDs) are considered the most destructive diseases causing significant economic losses to the grape industry (Gubler *et al*., 2005; Kaplan *et al*., 2016; Gispert *et al*., 2020). These diseases are caused by a complex of wood-inhabiting fungi in *Lasiodiplodia, Neofusicoccum*, *Diplodia*, *Eutypa, Eutypella*, *Phaeomoniella*, and *Phaeoacremonium*, among others (Bertsch *et et al*., 2013; Fon-

taine *et al*., 2016; Claverie *et al*., 2020). These fungi form sexual and/or asexual fruiting bodies on infected woody tissues. Spores produced within these structures are released and spread in vineyards by wind and water, and enter plants through pruning wounds. Management of GTDs is mostly achieved by preventative measures that consist of protecting pruning wounds against infections (Rolshausen *et al*., 2010; Kaplan *et al*., 2016; Martínez-Diz *et al*., 2021; Blundell and Eskalen, 2022).

Following wound infection, the GTD pathogens colonize host vascular tissues after spore germination. Most fungi causing GTDs are necrotrophs, degrading structural and non-structural host cell wall components (lignin, cellulose, hemicellulose, starch) for metabolic processes. Imaging data has indicated that GTDs initially invade host xylem vessels before spreading to neighbouring tissues (Amponsah *et al*., 2012; Obrador-Sánchez and Hernandez-Martinez, 2020). The qualitative and quantitative virulence traits (cell wall degrading enzymes, toxins, degradation of anti-fungal plant compounds) are key determinants of the wood colonizing capabilities, symptom types, and aggressiveness of the pathogen (Morales-Cruz *et al*., 2015; Garcia *et al*., 2021). For example, *Eutypa lata* and *Neofusicoccum parvum* develop characteristic wedge-shaped cankers in host woody tissues, and both pathogens share similar hydrolytic enzyme profiles that can break down glucose-rich polymers in secondary cell walls. In contrast, *Phaeomoniella chlamydospora* has limited enzymatic capabilities primarily targeting pectin-rich components, and infections are restricted to xylem elements leading to symptoms concentric rings of dark spots in wood (Rolshausen *et al*., 2008; Morales-Cruz *et al*., 2015; Pouzoulet *et al*., 2017). In addition, the most aggressive *Botryosphaeriaceae* (*Lasiodiplodia* and *Neofusicoccum*) associated with the largest plant lesions also possessed the greatest numbers of protein-coding genes involved in wood degradation and host colonization (Garcia *et al*., 2021).

Host plants respond to invasions by pathogens with constitutive and induced defense mechanisms (Freeman and Beattie 2008; Mithöfer and Boland 2012). Compartmentalization is a general defense response to wounding and pathogen infection to isolate injured tissues (Shigo and Tippett, 1981; Shigo, 1984). The model of compartmentalization of decay in trees (CODIT) describes formation and host reinforcement of anatomical walls to spatially restrict the movement of pathogens. The host impedes longitudinal movement of the pathogen by producing pectin-rich tyloses and gels in the xylem vessels, whereas vertical, radial and tangential movement are restricted by depositing lignin, suberin, and phenolic compounds in ray parenchyma, growth rings and vascular cambium (Shigo and Marx, 1977; Tippett and Shigo 1981; Pearce, 1996; Pearce, 2000). Histological studies have shown reinforcement of cell walls against wood colonizing agents in several pathosystems including those in grapevine (Fleurat-Lessard *et al*., 2013; Pouzoulet *et al*., 2013; Pouzoulet *et al*., 2017; Pouzoulet *et al*., 2022).

Induced defense mechanisms are triggered by effector molecules produced by pathogens, and by host plant cell wall degradation products, and compounds include reactive oxygen species (ROS), enzymes, tannins and phytoalexins (Joshi *et al*., 2021; Kaur *et al*., 2022). Production of phytoalexins (stilbene, resveratrol, flavonoid, viniferin) plays important roles in grapevine defense mechanisms, including plant cell wall reinforcement against biotrophic and necrotrophic pathogens (Adrian *et al*., 2012), and these compounds have been reported in vines infected by GTD pathogens (Spagnolo *et al*., 2014; Magnin-Robert *et al*., 2016; Rusjan *et al*., 2017). Together, this information indicates that phenolic compounds have essential physical and chemical properties that contribute to limiting GTD pathogen colonization, and could affect disease tolerance amongst grapevine cultivars (Rolshausen *et al*., 2008; Gómez *et al*., 2016).

GTD infections lead to host decline and death, because they affect host hydraulic functions by obstructing xylem and phloem translocation of water and nutrients. Infections also affect carbohydrate metabolism by decreasing photosynthesis and depleting carbon storage (Rudelle *et al*., 2005; Fontaine *et al*., 2016; Pouzoulet *et al*., 2017; Batista *et al*., 2021). Some pathogens of the GTD complex have also been reported to live within hosts without causing symptoms (Slippers and Wingfield, 2007; Zhang *et al*., 2021). The transition from commensal to pathogenic phase has been related to stress factors such as drought or heat, which predispose plants and increase their susceptibility (Hrycan *et al*., 2020). The number of reports of diseases caused by *Botryosphaeriaceae* has increased in the past decade, possibly because of climate change (Mehl *et al*., 2017; Batista *et al*., 2021). Transcriptomic and genomic analyses have revealed gene families of cell wall degrading enzymes (CWDE), and carbohydrate metabolism, were 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).

Despite their agricultural importance, studies of *Botryosphaeriaceae* designed to increase understanding of host colonization mechanisms have been limited. *Lasiodiplodia* spp. are among the causal agents of Botryosphaeria dieback, and are aggressive pathogens of grapevine (Úrbez-Torres, 2011). A total of 749 hosts of these fungi have been reported (Batista *et al*., 2021).

In Mexico, in the states of Baja California and Sonora, *Lasiodiplodia brasiliensis* has been frequently isolated from grapevine and has been reported as highly virulent (Rangel-Montoya *et al*., 2021).

The present study aimed to increase understanding of the colonization processes of *L. brasiliensis* in grapevine, and to broaden knowledge of its pathogenesis and subsequent symptom development.

MATERIALS AND METHODS

Fungus and plant materials

Lasiodiplodia brasiliensis MXBCL28 was isolated from a diseased 'Cabernet Sauvignon' grapevine growing in Baja California (Rangel-Montoya *et al*., 2021). This isolate was preserved in 20% glycerol at 4°C, and has been routinely cultivated in Potato Dextrose Agar (PDA; Difco) at 30°C.

One-year-old 'Cabernet Sauvignon' grapevines (the main cultivar in Baja California) were obtained from a local nursery. Plants were inoculated with *L. brasiliensis* MXBCL28 through wounds made in the woody tissues with a drill bit (2 mm diam.) (Úrbez-Torres *et al*., 2010). For each inoculation, a mycelial plug of the fungus was placed inside the wound and was the covered with Parafilm®. Plugs of sterile PDA were used for inoculation control plants. Plants were kept in a greenhouse under semi-controlled conditions at an average temperature of 35°C day and 25°C night, for 2 months during summertime. Ten plants were used per treatment. Subsequently, samples were taken from necrotic lesions. For each sample, a 4 cm long section, including 2 cm above and 2 cm below the inoculation site, was obtained, and was then fixed in FAA solution (Formaldehyde (SIGMA), Acetic acid (Faga Lab), Ethyl Alcohol (Jalmek); 5:5:9) for 24 h at 4°C, and then rinsed and preserved in 80% ethanol at 4°C until used. Part of each sample close to the necrotic tissue was used to re-isolate the inoculated fungus onto PDA to confirm the fungus colonization.

Histology of grapevine plants infected with Lasiodiplodia brasiliensis *MXBCL28*

Longitudinal and transverse sections of fixed tissues of thickness approx. 70 µm were made using a manual microtome. Sections were stained with 0.1% Toluidine B (TBO) (Mallinckrodt Chemical Works) to observe production of phenolic compounds and pectic substances. TBO is a metachromatic cationic dye that interacts with carboxylic groups, staining phenolic compounds purple to greenish-blue or bright blue and polyphenolic substances (e.g. lignin, tannins, pectic acids) purple and reddish-orange (Ling-Lee *et al*., 1977; Ruzin, 1999). For starch staining, iodine-potassium iodide (IKI; Mallinckrodt Chemical Works) was used, containing 5% iodine (Mallinckrodt Chemical Works) and 10% potassium iodide IKI intercalates into starch structure giving making it black (Ruzin, 1999). Suberin deposits were observed using 0.001% Sudan black IV (Chem-Impex) in 70% ethanol. Suberin solubilizes Sudan IV and fluoresces in red (Ruzin, 1999; Yeung, 2015). The presence of lignin in woody tissues was observed using 0.1% Phloroglucinol-HCl (Phl-HCl; Chem-Impex) and Mäule stain (Nakano and Meshitsuka, 1992). The cinnamaldehyde end groups of phenolic compounds react with Phl-HCl, making lignin pink-purple (Adler, 1977; Liljegren *et al*., 2010). The Mäule stain gives colouration to lignin due to the reaction with the syringyl lignin units (Meshitsuka and Nakano, 1977; Yamashita, *et al*., 2016). Calcofluor White M2R 0.02% (Fulka) and Congo Red 0.5% (SIGMA) were used to distinguish cellulose and hemicellulose polysaccharides. Calcofluor White M2R interacts with β-glucans and cellulosic cell walls fluoresce bright blue, while Congo Red interacts with β-glucans and β-xylans and cell walls fluoresce in red (Ruzin, 1999; Kim *et al*., 2008; Mitra and Loqué, 2014). Hyphae of *L. brasiliensis* colonizing the plant tissues were observed using Fontana-Masson stain, which is based on the ability of melanin to reduce ammoniacal silver nitrate solution to metallic silver without using an external reducing agent (Lillie, 1965; Rangel-Montoya *et al*., 2020).

All stained sections were observed using a Nikon Eclipse E200 microscope with an AxioCam HRc camera (Zeiss), and with an AxioVert200 with a RisingCam® U3CMOS camera. Epifluorescence microscopy was carried out using an AxioVert200 microscope supplied with a HBO100 100W mercury lamp with ebq100 power. Cellulose and suberin in the plant tissues were observed using a DAPI filter (excitation at 330–380 nm, emission at 420 nm). A TEXAS RED filter (excitation at 542–595 nm, emission at 644 nm) was used to analyze hemicellulose and suberin. Images were analyzed using AxioVision 4.8.2, RisingView, and ImageJ 1.49v software packages.

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

The ability of *L. brasiliensis* to use different plant components as carbon sources was evaluated. The fungus was grown in Minimal Medium 9 (MM9; 3.0 $g \cdot L^{-1}$ K_2HPO_4 (Jalmek), 3.0 $g \cdot L^{-1}$ KH_2PO_4 (JT Baker), 0.5 $g \cdot L^{-1}$ NaCl (Fermont), 1.0 $g \cdot L^{-1}$ NH_4Cl (SIGMA), 15.0 $g \cdot L^{-1}$ agar (Agarmex)) supplemented as carbon sources with

either glucose (Fermont) 1%, xylose (SIGMA) 1%, lignin (SIGMA) 0.1%, starch (SIGMA) 1%, cellulose SIGMA 1%, xylene (Fermont) 1%, glycerol (Fermont) 1%, pectin (SIGMA) 1%, tannic acid 0.1% (Faga Lab), or ground wood 2% ('Cabernet Sauvignon'). A mycelial plug was placed on the edge of each plate, and the plates were incubated at 30°C for 7 d, during which fungal growth was marked every 24 h. Area of the mycelium covering the agar surface in each Petri dish was calculated using the Integrated density and the fraction area tools of ImageJ software. This experiment was carried out in triplicate.

Fungal biomass was measured by inoculating test tubes containing MM9-liquid supplemented with the different carbon sources listed above, each with a mycelium disk of *L. brasiliensis*. All cultures were incubated at 30°C and 120 rpm for 7 d. Subsequently, the mycelium produced for each growth condition was recovered by filtration on a previously weighed Whatman® filter grade 1, and was dried at 50°C for 3–5 d. The biomass was determined by weighing the dry mycelium obtained from each growth condition, and subtracting the weight of the filter and the mycelium disk used for inoculation. For treatments with grapevine wood, ground wood contained in each sample was carefully removed. The experiment was carried out in triplicate.

Statistical analyses

Assumptions of normality of data from the biomass, growth rate, and area of the mycelium experiments from different carbon sources were confirmed using the Kolmogorov-Smirnov test ($P \geq 0.05$). One-way ANOVA followed by *post hoc* Fisher LSD analyses ($\alpha < 0.05$ for significance) were then carried out for all variables, using STATISTICA 8.0 software.

RESULTS

Colonization of grapevine plants by Lasiodiplodia brasiliensis *MXBCL28*

Two months after inoculation, grapevine plants infected with *L. brasiliensis* showed necrotic lesions along the xylem of mean length 5.5 (+/- 1.2 cm), that were not observed in the control plants (Figure 1A). Fontana-Masson stain allowed observation of melanized mycelium in the inoculation zones, in the vascular cambium of the plants, as well as in the vascular bundles, including those with occlusions (Figure 1 C, D, E and F). No hyphae were observed in the wounded areas or in the pith of the control plants (Figure 1 A).

Cross-sections of control plants stained with iodine-potassium iodide had strong dark colour, indicating the presence of starch in the ray parenchyma and in the wound areas (Figure 2 A). In contrast, cross-sections of infected plants each showed a light brown stain, indicating starch depletion in the ray parenchyma and the pith (Figure 2 B). Under epifluorescence microscopy, control plant tissues stained with Calcofluor White M2R had brownish areas at the wounds, indicating the presence of cellulose (Figure 2 C). Tissues of plants infected with *L. brasiliensis* showed dark zones, indicating depletion of cellulose in the necrotic tissues (Figure 2 D). In sections stained with Congo Red, dark zones were observed in the infected tissues (Figure 2 F) were observed, but not in the control plants (Figure 2 E), indicating the lack of hemicellulose in that area.

Staining with Toluidine Blue O allowed the visualization of phenolic compounds and tyloses in the xylem vessels in both control (Figure 3, A, B and C) and infected plants (Figure 3, D, E and F). In control plants, pectic compounds were observed in the regenerated tissue in reddish-orange and greenish-blue colors (Figure 3A), tyloses in blue-purple dark (Figure 3B), and less abundance of phenolic compounds in the pith (Figure 3C). Observed in purple and greenish-blue colors, in plants inoculated with *L. brasiliensis,* there was also pectin deposition in the occluded xylem vessels (Figure 3E), and phenolic compounds breaking down in the pith (Figure 3F) and in the vascular cambium (Figure 3D).

Infected plants showed lighter pink colour in the lesion areas and in the vascular cambium compared with non-infected tissues (Figure 4, A and B), indicating that the fungus could break down phenolic polymers. Comparison of infected and non-infected wood tissues using the Mäule stain showed that *L. brasiliensis* was able to break down lignin, as indicated by a discolouration in xylem tissues, the ray parenchyma, and the fibres surrounding the occluded vascular bundles (Figure 4 D). Staining with Sudan IV showed red suberin deposits in the control plants, in the cambium cork in the wound areas (Figure 4 E) and in the ray parenchyma (Figure 4 H). In contrast, red suberin was observed in the cambium cork (Figure 4 F), vascular cambium, ray parenchyma (Figure 4 G), and in the pith (Figure 4J), in response to fungal infection. Suberin was not observed in the necrotic rays (Figure 4 K).

Evaluation of carbon sources used for Lasiodiplodia brasiliensis *MXBCL28*

Growth of *L. brasiliensis* MXBCL28 without a carbon source was weak with stunted mycelium (Figure 5

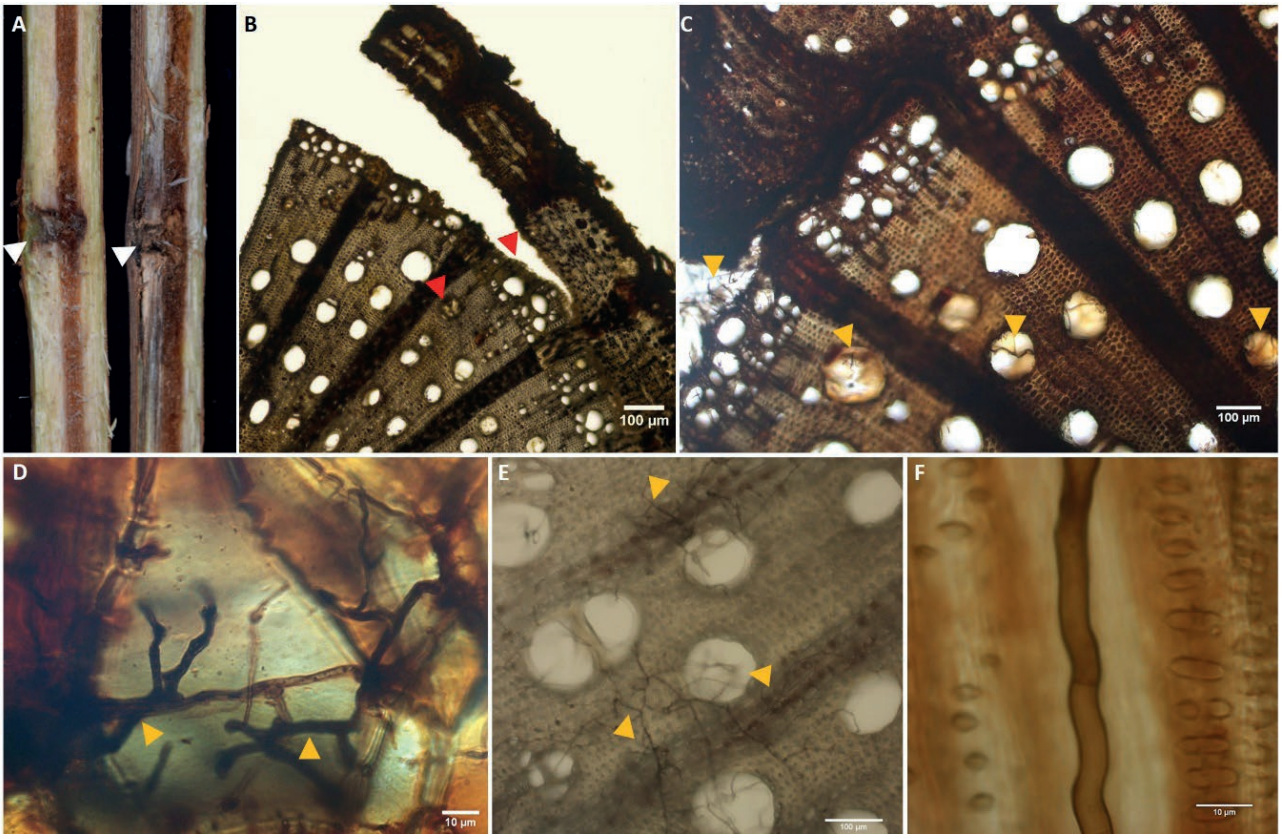

Figure 1. Grapevine plants 'Cabernet Sauvignon' 2 months after inoculation with *Lasiodiplodia brasiliensis* MXLBC28. A, Tissue regeneration in control plants (left), and necrotic lesion caused by *L. brasiliensis* (right); white arrows indicate the wound area. Mycelia of *Lasiodiplodia brasiliensis* growing in grapevine plants 'Cabernet Sauvignon' stained using the Masson-Fontana method. B, Control plants non-inoculated; red arrows indicate the wound area and occlusions. C, Melanized fungus colonizing the vascular cambium and vascular bundles. D, melanized fungus colonizing the plant piths. E, melanized hyphae covering the xylem and ray parenchyma. F, melanized hypha growing in a vascular bundle. Yellow arrows indicate melanized hyphae.

A), and biomass was reduced (Table 1). The fungus was able to utilize pectin, xylan, xylose, and starch as carbon sources (Figure 5 C to F), and glucose (Figure 5 B), producing dense dark gray aerial mycelium (Table 1). *Lasiodiplodia brasiliensis* had a significantly increased growth rate and biomass production using pectin as a carbon source (Figure 5C) or ground grapevine wood (Figure 5 K), since the fungus completely covered the media in Petri dishes in 4 d for these carbon sources. The fungus produced less aerial mycelium with cellulose, lignin and tannic acid as carbon sources, as indicated by the calculations of mycelium area (Figure 5, H to J and Table 1). The reduced biomass and mycelium areas were observed with lignin and tannic acid (Figure 5 H and J) as carbon sources. For tannic acid, although production of aerial mycelium and biomass were low, the fungus covered almost the entire medium surfaces in Petri dishes, with sparse mycelium after 7 days.

DISCUSSION

This study used histology to outline the pathogenesis of *L. brasiliensis* in grapevine, by comparing mechanically wounded plants with those wounded and infected with *L. brasiliensis*. The data obtained have indicated that the physical, constitutive and induced defense responses enacted by the host did not successfully compartmentalize the pathogen.

Wood decay in infected grapevine plants was extensive after 2 months of incubation, indicating that species of *Lasiodiplodia* are highly virulent, as previously shown by Úrbez-Torres and Gubler (2009), Garcia *et al.* (2021) and Rangel-Montoya *et al.* (2021), at least under the local environmental conditions. The high maximum temperatures encountered during the summer months in Mexico are conducive to *L. brasiliensis* pathogenesis (Rangel-Montoya *et al.*, 2021). Melanized hyphae were observed in all infected host woody tissues, including

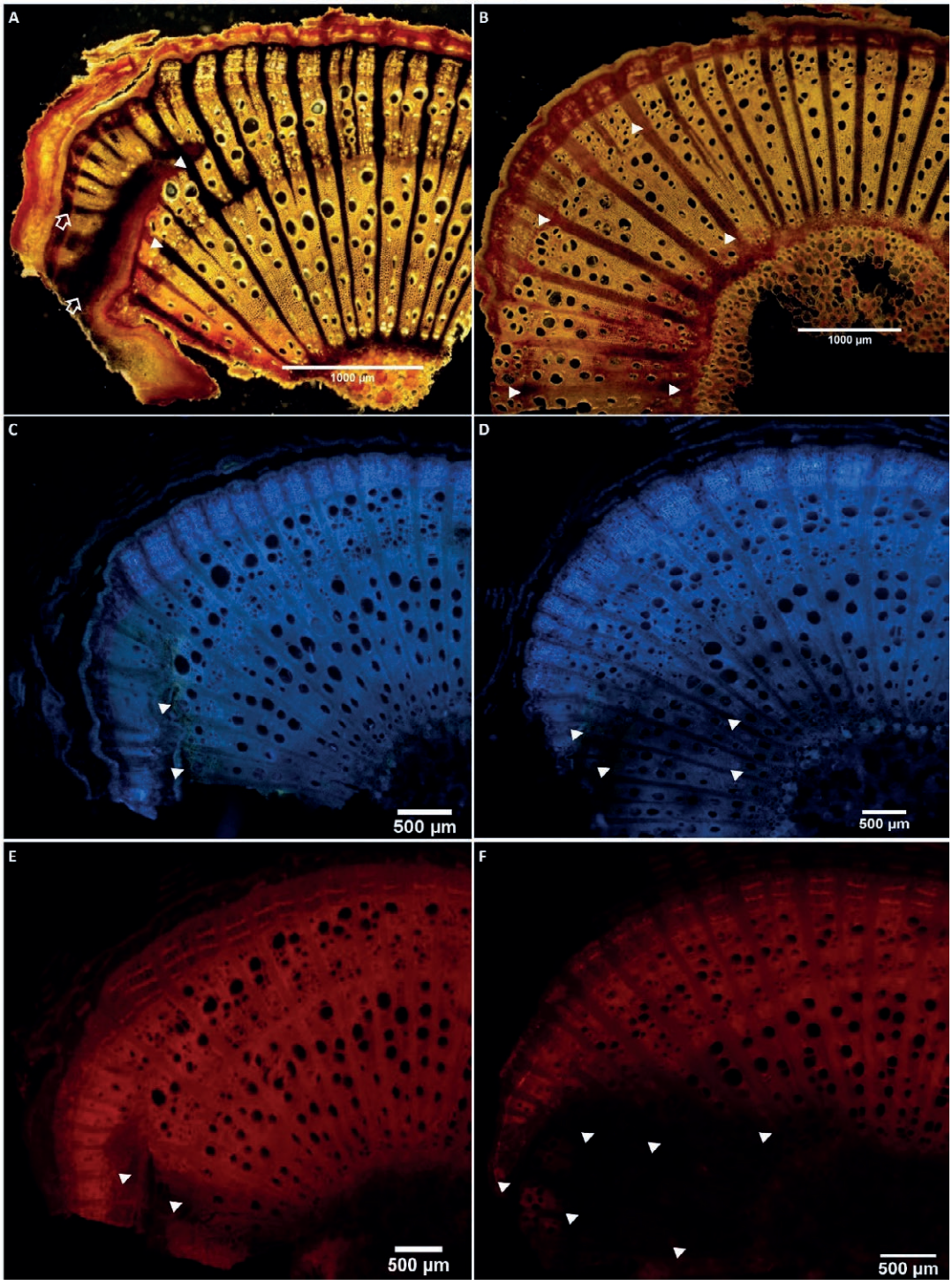

Figure 2. Starch and wood degradation by *Lasiodiplodia brasiliensis* in grapevine plants 'Cabernet Sauvignon' 2 months after inoculation. A, C, and E, non-inoculated plants. B, D, and F, plants inoculated with *L. brasiliensis*. A and B, cross sections stained with iodine-potassium iodide for starch content. A, starch deposition is observed as black zones in the ray parenchyma in control plants; white arrows indicate the wound area and the tissue regeneration, while hollow arrows indicate starch in the newly formed tissue. B, 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. C, D, E and F, epifluorescence microscopy to observe the presence of cellulose and hemicellulose. C and D, sections stained with Calcofluor White M2R, or E and F, Congo Red. Control plants (C and E) show brownish areas in the wound areas. Plants infected with *L. brasiliensis* (D and F) show dark zones without cellulose in the necrotic tissues. White arrows indicate zones without fluorescence where there is no cellulose or hemicellulose.

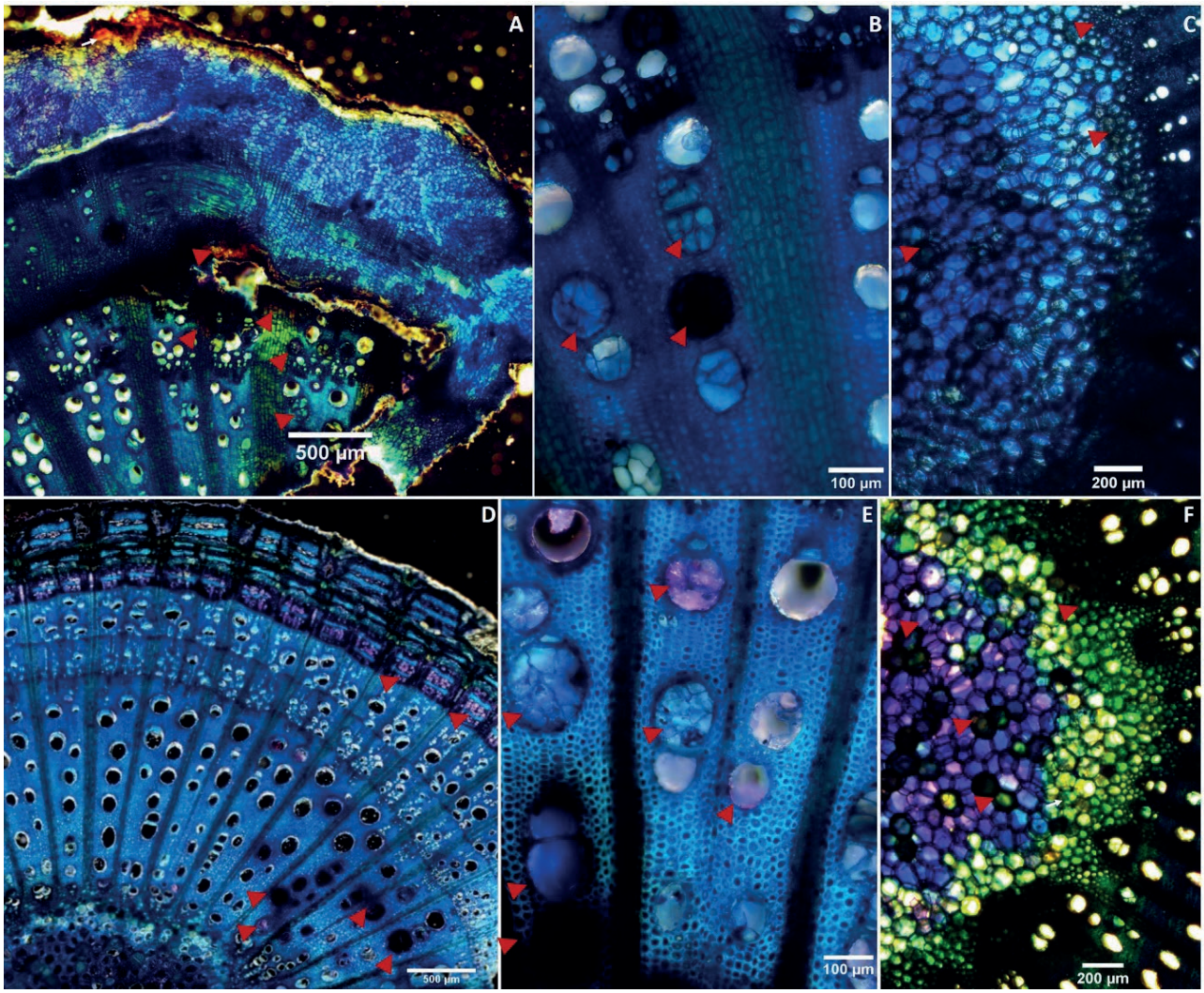

Figure 3. Responses of grapevine plants 'Cabernet Sauvignon' to wounding and infection, producing phenolic and pectic compounds. Cross-sections stained with toluidine blue O. A, B and C, Control plants showing production of phenolic and pectic compounds in the vascular cambium, and in the regenerated tissue. D, E and F, Plants infected with *Lasiodiplodia brasiliensis* showing phenolic compounds in the vascular cambium, phloem (D), vessel occlusions with pectic compounds (E), and the pith (F). Red arrows indicate phenolic compounds stained purple, greenish-blue and blue; and pectic compounds stained purple and reddish-orange in the regenerated tissue and occlusions.

vascular cambium, vascular bundles, and in the pith. Similar observations were made in *L. gilanensis* colonizing grapevine plants one-month post-inoculation (Rangel-Montoya *et al*., 2020).

Melanin is considered to be a virulence factor in several phytopathogenic fungi (Eisenman *et al*., 2020), and is commonly produced by *Botryosphaeriaceae* (Phillips *et al*., 2013; Rangel-Montoya *et al*., 2020). Several *L. gilanensis* genes involved in the synthesis of different melanin pathways were found differentially expressed in the presence of grapevine wood and under heat shock conditions (genes involved in DHN-melanin and pyomelanin pathways), or without heat shock (genes involved in DOPA-melanin pathway) (Paolinelli-Alfonso *et al*., 2016). Melanin protects against UV radiation, enzymatic lysis, and ROS, and *L. gilanensis* can metabolize tyrosine as carbon and nitrogen sources, and uses this amino acid as a DOPA-melanin precursor (Rangel-Montoya *et al*., 2020). Phenylalanine is a precursor of plant lignin and suberin syntheses (Lewis *et al*., 1987), and could be a precursor of DOPA-melanin and pyomelanin pathways, and tyrosine catabolism (Schmaler-Ripcke *et al*., 2009; Eisenman and Casadevall, 2012). We speculate that *Lasiodiplodia* could derail host plant

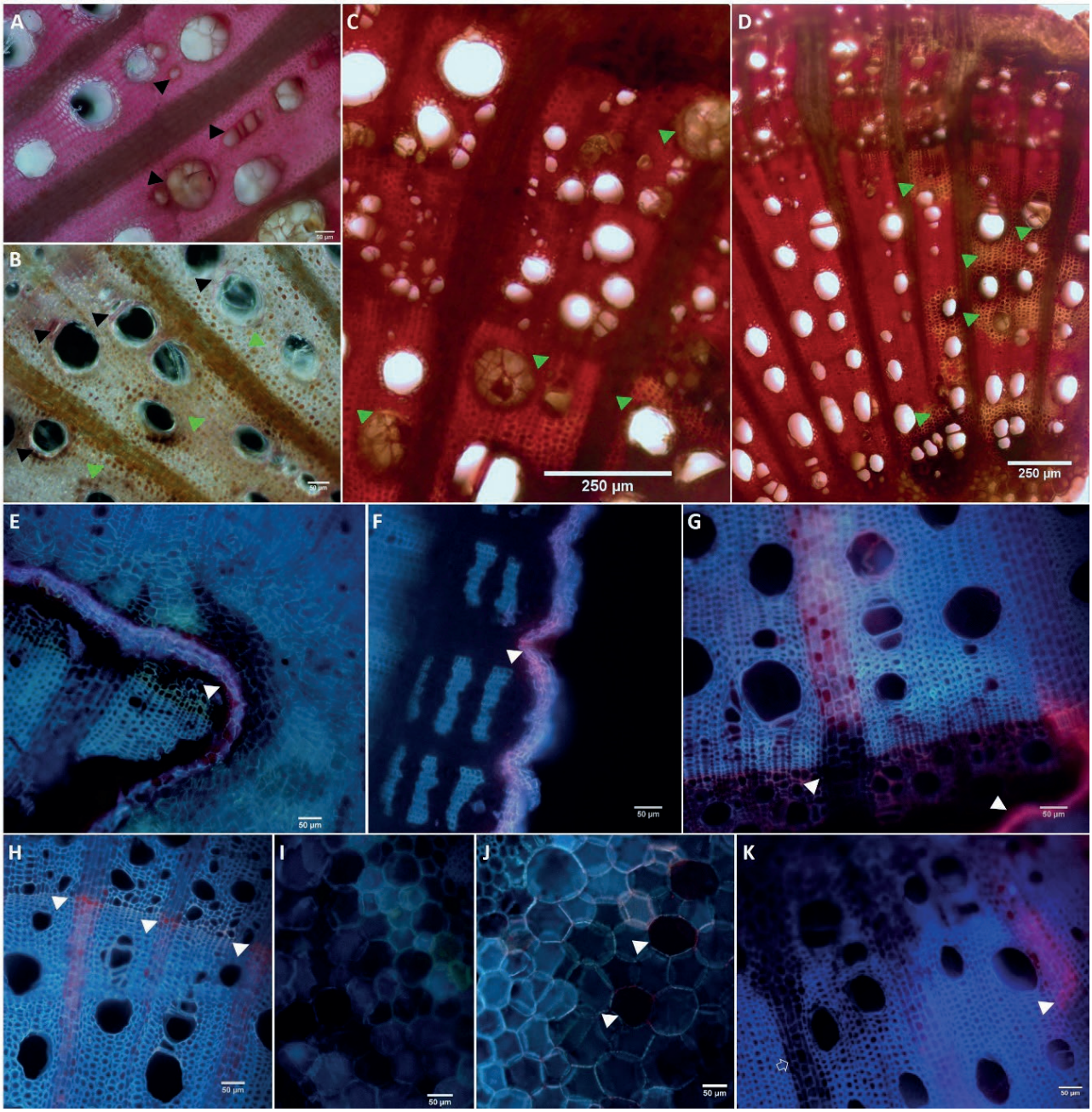

Figure 4. Grapevine plant 'Cabernet Sauvignon' responses of phenolic compounds, lignin, and suberin to *Lasiodiplodia brasiliensis* infections. A and B, cross sections of plant stems stained with Phloroglucinol-HCl show lignin in control plants as intense pink in all tissues (A), while in infected plants lighter pink colouration occurred in the lesion areas and vascular cambium, and phenolic deposits in the fibres surrounding the vascular bundles were observed (B). C to D, cross sections stained with the Mäule method to observe the presence of lignin (red colour). Control plants showed slight discolouration in the tissue surrounding occluded vascular bundles near the wound sites (C), while infected plants showed discolouration in the xylem, ray parenchyma, and fibres surrounding occluded vascular bundles (D). Green arrows indicate zones without lignin, and black arrows indicate lignin surrounding vessels. E to K, Epifluorescence microscopy for suberin localization of grapevine plants 'Cabernet Sauvignon'. Samples were stained with Sudan black IV, and observed under TEXAS RED and DAPI filters. Control plants presenting red suberin in cambium cork (E) and ray parenchyma (H) near the wound area, and without suberin in the pith (I). Infected plants 2 months after inoculation with *L. brasiliensis* showed red suberin deposits in the cork (F), vascular cambium, occlude vessels, and ray parenchyma (G and K), and in the pith (J). White arrows indicate the presence of suberin. The hollow arrow indicates ray parenchyma in the necrotic area without suberin.

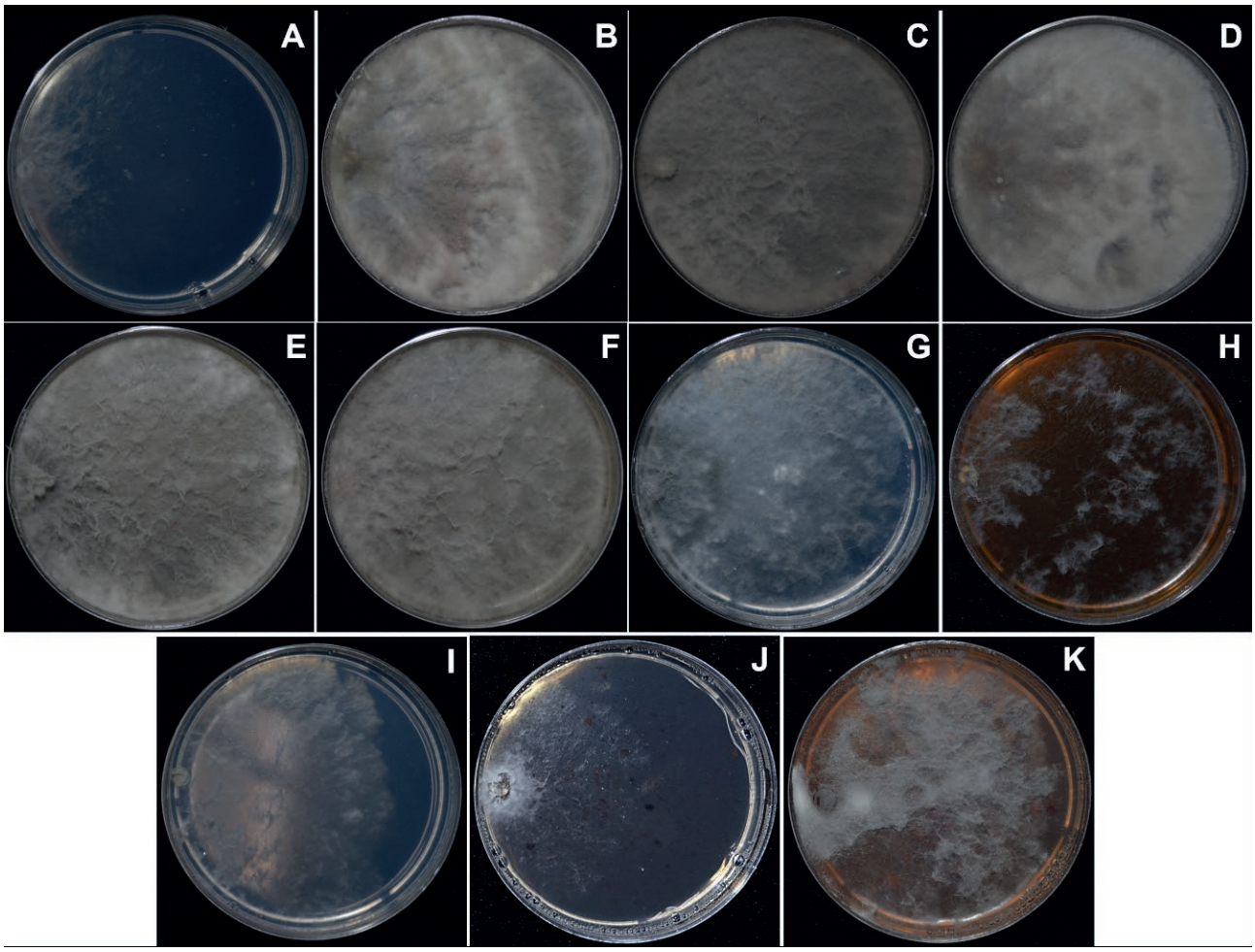

Figure 5. Growth of *Lasiodiplodia brasiliensis* MXBCL28 in Petri dishes containing different carbon sources. A, MM9 without a carbon source (MM9-C). B, MM9 + glucose. C, MM9 + pectin. D, MM9 + xylose. E, MM9 + xylan. F, MM9 + starch. G, MM9 + glycerol. H, MM9 + tannic acid. I, MM9 + cellulose. J, MM9 + lignin. K, MM9 + grapevine wood.

Table 1. Mean weights of biomass, growth rates, and mycelium areas of *Lasiodiplodia brasiliensis* MXBCL28 colonies growing in Petri plates containing PDA amended with different carbon sources.

Carbon source	Mean biomass* (mg)	Mean growth rate* (mm d^{-1})	Mean mycelium area (mm^2)
Grapevine wood	70.3 ± 12.6 a	18.7 ± 1.764 a	5248.8 ± 11.8 ab
Pectin	57.6 ± 3.9 b	17.7 ± 0.8 a	5391.5 ± 62.0 ab
Xylan	47.6 ± 7.7 bc	11.7 ± 0.6 c	5422.6 ± 35.3 a
Glucose	42.3 ± 11.2 cd	14.7 ± 0.5 b	5425.4 ± 39.3 a
Xylose	40.4 ± 2.4 cd	9.3 ± 0.8 d	5424.6 ± 40.7 a
Starch	33.7 ± 6.9 de	9.7 ± 0.2 d	5425.0 ± 39.4 a
Cellulose	28.4 ± 1.6 ef	7.0 ± 0.9 e	4678.6 ± 44.0 c
Glycerol	27.9 ± 9.0 ef	8.4 ± 0.7 de	5273.5 ± 71.1 b
Lignin	21.9 ± 5.4 ef	4.8 ± 0.2 f	3289.3 ± 15.1 d
Tannic acid	19.9 ± 5.7 f	12.6 ± 0.5 c	1998.3 ± 60.8 e
No carbon source	7.2 ± 1.0 g	N/A**	1426.4 ± 10.4 f

metabolic pathways and use phenylalanine or tyrosine as precursors for melanin biosynthesis.

Grapevine responded to *L. brasiliensis* infection by occluding xylem vessels with tyloses and gels, and reinforcing cell walls with phenolic compounds in tyloses, vascular bundles, cambium, and pith. Infections were also accompanied by starch depletion in the ray parenchyma. These results support previous observations from histological studies conducted on several pathogens causing GTDs (Fleurat-Lessard *et al.*, 2013; Pouzoulet *et al.*, 2013; Gómez *et al.*, 2016; Pouzoulet *et al.*, 2017; Obrador-Sánchez and Hernandez-Martinez, 2020), indicating that these are constitutive responses that are enacted by host plants regardless of the type of GTD fungal infection.

In woody plants, non-structural carbohydrate reserves stored mainly as starch in ray parenchyma are

remobilized to fulfill several biological processes, including refilling embolized xylem vessels caused by freeze-thaw cycles or drought, supporting plant vegetative growth after dormancy, and activating host defense systems (Nardini *et al*., 2011; Fontaine *et al*., 2016; Noronha *et al*., 2018; Călugăr *et al*., 2019). Depletion of starch in wood has been observed in many pathogen and grapevine interactions (Rudelle *et al*., 2005; Rolshausen *et al*., 2008; Pouzoulet *et al*., 2017). The present study *in vitro* data also indicated that *L. brasiliensis* can use starch as a carbon source. Genomic and transcriptomic analyses indicated that a putative amylase was induced by *L. gilanensis* in the presence of grapevine wood (Paolinelli-Alfonso *et al*., 2016), and that genes involved in starch metabolism were induced in *L. theobromae* during infection (Yan *et al*., 2018). Together, these results suggest that starch plays a pivotal role as an energy pool accessible for GTD fungi for wood colonization and for grapevine to activate its defense system.

Several fungi causing GTDs can also metabolize structural carbohydrates located in plant cell walls. The hemicellulosic fraction has been shown to be especially degraded during infection by *E. lata*, and aggressive fungi such as *Neofusicoccum* can break down cellulosic material (Rolshausen *et al*., 2008; Stempien *et al*., 2017). Results from the present study showed that cellulose and hemicellulose were depleted in necrotic tissues, and were metabolized *in vitro* by *L. brasiliensis*. Cellulose and hemicellulose are carbohydrate-rich compounds that each constitute 30% of grapevine cells (Rolshausen *et al*., 2008). Genomic information from virulent *Botryosphaeriaceae* (*Neofusicoccum* and *Lasiodiplodia*) demonstrated the high numbers of genes encoding carbohydrate-active enzymes (Garcia *et al*., 2021), indicating that these compounds embedded in cell walls are primary targets for fungal metabolism.

Vessel occlusion was observed in control and inoculated (infected) plants, but the response was much stronger in *L. brasiliensis* infected plants, as was shown in grapevine responses to *P. chlamydospora* inoculations (Pouzoulet *et al*., 2017). Vessel occlusions, such as tyloses, gums, and gels, are formed in vascular bundles to limit the longitudinal spread of the fungus (De Micco *et al*., 2016; Pouzoulet *et al*., 2017). Several *Botryosphaeriaceae* have been shown to first invade xylem vessels before spreading to neighbouring woody tissues (Amponsah *et al*., 2012; Obrador-Sánchez and Hernandez-Martinez, 2020). Tyloses and gels are pectin-rich, and the present study results indicated that *L. brasiliensis* growth was stimulated in the presence of pectin in comparison to other substrates. Expression of a pectate lyase was up-regulated during the first stages of *L. gilanensis* infection in grapevine (Paolinelli-Alfonso, *et al*., 2016). Occlusion of xylem vessels results in loss of hydraulic conductivity, which eventually leads to decreased host physiological functions (Zhao *et al*., 2014).

The present study has shown that suberin accumulated in tyloses walls, and that lignin was deposited near ray parenchyma next to occluded xylem vessels, probably as responses to pathogen tracheid invasion. Suberin deposition was observed in other tissues, including vascular cambium, vascular cork, xylem fibre of vascular bundles, and ray parenchyma. Pouzoulet *et al*. (2013) also noted that grapevine accumulates suberin over lignin, in response to *P. chlamydospora* infections in those tissues. Qualitative and quantitative accumulation of phenolic compounds as physical and chemical barriers has been shown to be a common compartmentalizing mechanism for wood-decay fungi in other pathosystems (Skyba *et al*., 2013; Mounguengui *et al*., 2016). In grapevine, increased cell wall phenolics were found in the wood of the *Eutypa lata* tolerant cultivar Merlot compared with susceptible Cabernet Sauvignon (Rolshausen *et al*., 2008). Increased resveratrol induction was also measured in *N. parvum* tolerant *Vitis sylvestris* compared to *V. vinifera* (Labois *et al*., 2020).

Phenolic compounds in grapevine have also been shown to inhibit the growth of several GTD fungi (Lambert *et al*., 2012; Gómez *et al*., 2016). However, accumulation of phenolics (flavonoids, and stilbenoids) did not effectively wall-off aggressive pathogens, and these compounds are fungistatic rather than fungicidal (Lambert *et al*., 2012; Galarneau *et al*., 2021). The present study did not measure accumulation of phytoalexins. *Lasiodiplodia*, like *Neofusicoccum*, has been reported to display similar virulence factor patterns with respect to detoxification of antimicrobial compounds (Garcia *et al*.,2021). These fungi can degrade phenolic compounds that restrict *in planta* movement. Several genes involved in phenolic metabolism have been found in *Lasiodiplodia* spp. (Paolinelli-Alfonso *et al*., 2016; Yan *et al*., 2018; Gonçalves *et al*., 2019; Garcia *et al*, 2021). These could assist metabolization of phenolic compounds such as salicylic acid and phenylpropanoids produced by plants, to avoid the host defense responses. In addition, results from the present *in vitro* studies showed that *L. brasiliensis* was able to grow in culture media supplemented with glycerol, tannic acid, and lignin, supporting its ability to use these substrates as carbon sources.

Based on the information obtained in this study, and from previously published reports, we propose that once *Lasiodiplodia* conidia penetrate grapevines through wounds, the fungus initially colonizes xylem vessels following germination. The proposed model

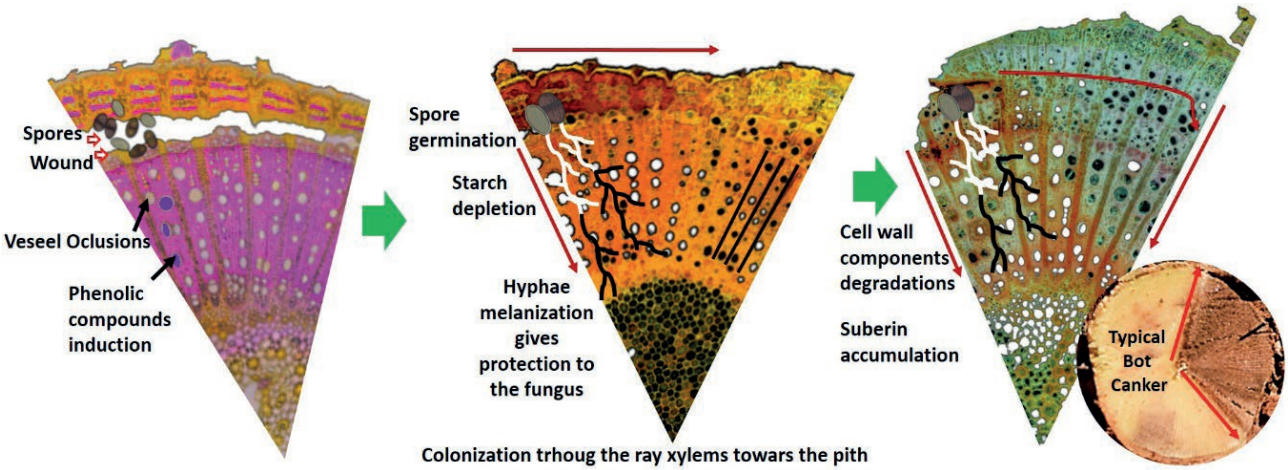

Figure 6. Proposed model of the colonization of grapevine by *Lasiodiplodia brasiliensis*. Spores of *L. brasiliensis* enter a plant wound, germinate and penetrate through xylem vessels. When growing within the host, the melanized mycelium protects the fungus from the host defenses. At the same time, the fungus produces cell wall degrading enzymes, and continues the colonization process using hemicellulose, starch and pectin as carbon sources. This induces vessel occlusions and the production of phenolic compounds. Over time, the fungus degrades lignin and suberin, moving to further xylem rays. This movement induces formation of typical Botryosphaeria cankers.

of the colonization process is illustrated in Figure 6. Upon recognition of the pathogen, the host remobilizes starch resources to trigger defense responses, with rapid xylem occlusion. The pathogen initially metabolizes the readily available starch stored in parenchyma cells and pectin-rich tyloses walls to weaken the intensity of host response and further colonize xylem vessels. Subsequently, the pathogen spreads to neighbouring tissues degrading cell wall structures to gain access to more complex sugars for metabolic processes. Host plant production of suberin, lignin and phytoalexins to compartmentalize the pathogen is probably inadequate against *Lasiodiplodia*, as the pathogen melanin can deactivate their antimicrobial effects and further breaks down phenolics. The pathogen then colonizes host cambium to initiate development of canker symptoms.

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