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The role of melanin in the grapevine trunk disease pathogen *Lasiodiplodia gilanensis*

Edelweiss Airam RANGEL-MONTOYA[1], Marcos PAOLINELLI[2], Philippe ROLSHAUSEN[3], Rufina HERNANDEZ-MARTINEZ[1,*]

[1] *Departamento de Microbiología, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), 22860 Ensenada, Baja California, México*
[2] *Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (INTA), San Martín 3853, 5534, Luján de Cuyo, Mendoza, Argentina. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*
[3] *Department of Botany and Plant Sciences, University of California Riverside, Riverside, 92521, CA, United States of America*
*Corresponding author. E-mail: ruhernan@cicese.mx

Summary. *Lasiodiplodia* (*Botryosphaeriaceae*) includes fungi that are considered among the most aggressive to grapevine, capable of causing cankers and necrotic lesions which eventually lead to death of host plants. A common characteristic of this genus is the presence of melanin in conidia and mycelium. Melanin is produced by the oxidation of phenolic and/or indolic compounds. For some fungi, this pigment is an essential factor for pathogenicity. This study characterized the types and the roles of melanin produced by *Lasiodiplodia gilanensis*. Using specific melanin inhibitors, *L. gilanensis* was shown to synthesize DOPA-melanin, DHN-melanin, and pyomelanin. DOPA-melanin was shown to be involved in production of aerial mycelium and protection against enzymatic lysis and oxidative stress; DHN-melanin to be involved in ramification of mycelium when exposed to nutrient deficiency; and pyomelanin to be related with hyphae development. The fungus used tyrosine as a precursor of DOPA-melanin and as carbon and nitrogen sources, and produced melanin inside the piths of infected plants. Genes involved in melanin synthesis were conserved among the *Botryosphaeriaceae*, highlighting the importance of melanin in this family.

Keywords. Grapevine trunk diseases (GTDs), Botryosphaeria dieback, *Botryosphaeriaceae*, fungal melanin, tyrosine catabolism.

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INTRODUCTION

The *Botryosphaeriaceae* contains several fungal plant pathogens of a wide range of woody plants (Slippers and Wingfield, 2007), with cosmopolitan distribution (Damm *et al*., 2007; Phillips *et al*., 2013). The ability of these fungi to infect multiple hosts increases their economic impacts and ecological risks in many regions (Mehl *et al*., 2017). More than 30 *Botryosphaeriaceae* species from the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neoscytalidium, Neofusicoccum*, *Phaeobotryosphaeria*, and

Spencermartinsia, have been associated with grapevine trunk diseases (GTDs) (Úrbez-Torres, 2011; Gramaje *et al*., 2018). Interactions of these fungi with their hosts have been studied, as well the virulence factors that trigger disease development. Infections of healthy plants occur mainly through pruning wounds, and some of the symptoms caused are leaf spot, fruit rot, dieback, shoot necrosis, vascular discolouration, and perennial cankers (Úrbez-Torres, 2011). In the states of Baja California and Sonora, Mexico, grapevine is one of the most economically important crops, and in these areas, several species of fungi causing GTDs have been reported, including *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl (Úrbez-Torres *et al*., 2008; Candolfi-Arballo *et al*., 2010).

Lasidiplodia theobromae is the type species of *Lasiodiplodia*. This fungus has widespread distribution, but is most common in tropical and subtropical regions, and has been found in more than 500 hosts (Punithalingam, 1976). In grapevine, *L. theobromae* was reported as one of the most aggressive pathogens causing Botryosphaeria dieback (Úrbez-Torres *et al*., 2008). To date, more than eighteen species of *Lasiodiplodia* have been reported, including *L. exigua, L. parva, L. crassispora, L. gilanensis, L. brasiliense, L. laeliocattleyae* (formerly *L. egyptiacae*)*, L. euphorbicola* and, *L. hormozganensis* (Yan *et al*., 2013; Linaldeddu *et al*., 2015; Correia *et al*., 2016; Rodríguez-Gálvez *et al*., 2017). The main differences in these species are the morphology and dimensions of conidia and the morphology of paraphyses (Phillips *et al*., 2013). Conidia are initially hyaline, and later, a single median septum is formed in each conidium. Then, the cell walls become dark brown, and melanin granules are deposited longitudinally on the inner surface, giving a striated appearance (Phillips *et al*., 2013). Since not all conidia become melanized at the same time, it is possible to find hyaline and pigmented conidia in individual pycnidia.

Melanins are macromolecules of high molecular weight, derived from oxidative polymerization of phenolic and/or indolic compounds (Jacobson, 2000). They are usually dark pigments distributed in all biological kingdoms (Butler and Day, 1998; Eisenman and Casadevall, 2012). Melanin is considered a stable, insoluble, and resistant biopolymer due to its complex structure and physicochemical properties (Solano, 2014; Cordero and Casadevall, 2017). Several types of fungal melanin have been described, determined by their precursors; among them, DHN-melanin, DOPA-melanin and pyomelanin are related to pathogenicity or antifungal drug protection (Toledo *et al*., 2017). DHN-melanin is synthesized through a series of dehydration and reduction reactions from 1,3,6,8-tetrahydronaphthalene (1,3,6,8-THN), in which the final product is 1,8-dihydroxynaphthalene (1,8-DHN) that polymerizes to form melanin (Langfelder *et al*., 2003; Eisenman and Casadevall, 2012). DOPA-melanin is synthesized from the oxidation of tyrosine to L-DOPA or directly from L-DOPA to dopaquinone (Langfelder *et al*., 2003; Eisenman and Casadevall, 2012). Pyomelanin is related to tyrosine catabolism and is synthesized from the oxidation of homogentisic acid and its subsequent polymerization (Schmaler-Ripcke *et al*., 2009).

Melanization is not an essential factor for fungus growth, but is involved in a broad spectrum of biological functions (Wu *et al*., 2008). The primary functions of melanin is to protect cells from UV radiation, and from oxidizing agents; melanin interacts with free radicals and other reactive species because of the presence of unpaired electrons (Butler and Day, 1998; Shcherba *et al*., 2000), contributing to the survival of cells under environmental stress conditions (Wu *et al*., 2008; Eisenman *et al*., 2020). Melanin is a virulence factor in some phytopathogenic fungi acting as a non-specific "body-armour" during infection, protecting the fungus against host defense mechanisms and promoting its survival to cause the disease (Hamilton and Gomez, 2002; Nosanchuk and Casadevall, 2003; Eisenamn *et al*., 2020). DHN-melanin has been the most studied melanin in fungi. In the rice pathogen *Magnaporthe oryzae*, production of DHN-melanin is vital in appressorium formation and for the stationary phase of mycelial growth (Howard and Valent, 1996; Nosanchuk and Casadevall, 2003; Eisenman and Casadevall, 2012). In *Colletotrichum lagenarium* (Kubo *et al*., 1982), *C. lindemuthianum* (Wolkow *et al*., 1983), *C. kahawae* (Chen *et al*., 2004), and *Setosphaeria turcica* (Shuangxin *et al*., 2017), DHN-melanin is associated to the appressorium formation. The grapevine trunk disease fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* produce naphthoquinones (scytalone and isosclerone), intermediate metabolites of melanin biosynthesis. These metabolites have been associated with oxidative properties and production of esca disease symptoms in grapevines (Evidente *et al*., 2000; Andolfi *et al*., 2011).

Melanin inhibitors have been used to study the synthesis pathways of melanin in some fungi (Woloshuk and Sisler, 1982; Wheeler and Klich, 1995; Butler *et al*., 2009; Kumar *et al*., 2015). Gonçalves *et al*. (2012) reported that tropolone (DOPA-melanin inhibitor) added to growth media, inhibited the synthesis of melanin in *Aspergillus nidulans*, unlike the use of DHN-melanin inhibitors. Pal *et al*. (2014) used tricyclazole, phthalide, tropolone, and kojic acid in *Aspergillus* spp., and found that in *Aspergillus niger*, *Aspergillus tamarii,* and *Aspergillus flavus*, synthesis of melanin was inhibited

by kojic acid and tropolone, while in *Aspergillus terreus* and *Aspergillus tubingensis* melanin was inhibited in the presence of tricyclazole and phthalide. In the hypersaline yeast *Hortaea werneckii*, which produces DHN-melanin, tricyclazole was used to study the effect of melanin in NaCl tolerance (Kejžar *et al*., 2013). Nitisinone has been primarily used in bacteria, for example, in *Pseudomonas aeruginosa,* in which this compound inhibited the synthesis of pyomelanin (Ketelboeter *et al*., 2014).

In a transcriptomic analysis of *L. gilanensis* UCD-256Ma (formerly *L. theobromae*), genes related to DHN-melanin and pyomelanin pathways were differentially expressed under heat shock and in the presence of grapevine wood. In contrast, genes related to the DOPA-melanin pathway were expressed only in the presence of grapevine wood without heat shock (Paolinelli-Alfonso *et al*., 2016). The present study aimed to identify the types and roles of melanin produced by *L. gilanensis* to increase understanding of this fungus-host interaction.

MATERIALS AND METHODS

Fungus strain and growth conditions

Lasiodiplodia gilanensis UCD256Ma (formerly *L. theobromae*) was isolated from grapevine plants showing Botryosphaeria dieback symptoms in Madera County, California, United States of America (Úrbez-Torres *et al*., 2006), and this isolate was provided by Dr. Douglas Gubler (University of California, Davis) to the laboratory of Phytopathology of CICESE. The stock culture was recovered on potato dextrose agar (PDA) and incubated at 30°C. For the assays described in the following sections, mycelial disks (5 mm diam.) were individually inoculated at one border of Petri dishes containing relevant media, and fungus growth was measured every 24 h. In each experiment, vegetative growth, pigmentation, and formation of aerial mycelium were evaluated, and changes in pigmentation of the fungal colonies were recorded. All experiments were carried out in triplicate and repeated once.

Evaluation of the behaviour of Lasiodiplodia gilanensis *in the presence of melanin inhibitors*

Tropolone and kojic acid, inhibitors of DOPA-melanin; phthalide and tricyclazole, inhibitors of DHN-melanin; and nitisinone, an inhibitor of pyomelanin, were used to perform the following assays. First, the effect of each inhibitor on growth of *L. gilanensis* was evaluated in PDA media supplemented with either 5, 10, 15, 20, 30, 100, or 200 μg mL^{-1} of each inhibitor, as described above. All treatments were incubated at 30°C for up to 4 d. Growth of the fungus was measured in each plate every 24 h. The inhibitors and concentrations in which the fungus showed a decrease in the pigmented dark colour compared to the non-inhibitor experimental controls, were selected for subsequent analyses.

Melanin production in response to environmental stress

Melanin production in response to enzymatic lysis, oxidative stress, and UV radiation was evaluated by combining the environmental stress conditions with the melanin inhibitors that showed effects on melanin production.

Enzymatic lysis stress was assessed using a lyophilized powder of lysing enzymes from *Trichoderma harzianum* (L1412; Sigma) at 10 mg mL^{-1}, and oxidative stress was assessed using 5% v/v hydrogen peroxide (H_2O_2), both added to PDA media. In each plate, a *L. gilanensis* mycelium disk was inoculated at one border, incubated at 30°C, and evaluated as described above. Vegetative growth of the fungus was measured every 24 h up to 4 d.

Effects of UV radiation were assessed using pigmented and hyaline conidia, exposed to UV light using a transilluminator (UVP). The conidia were obtained by inducing the production of pycnidia in Vogel's minimal medium (VMM) supplemented with ground grapevine wood at 5% (w/v). The wood was obtained from 'Cabernet Sauvignon' canes, frozen in liquid nitrogen, ground with a blender (Waring), and then autoclaved. Cultures were incubated under a near-UV electromagnetic radiation lamp for 15 to 20 d. Conidia were then collected under a stereoscopic microscope (Zeigen) using a dissection needle. Hyaline and pigmented conidia were taken individually and suspended in 0.05% Tween 20 (P9416; Sigma). Fifty hyaline or pigmented conidia were placed in PDA, exposed to high-intensity UV radiation of 365 nm for 30 or 60 min, and incubated in darkness at 30°C. The percentage of conidia germination was evaluated after 24 or 48 h, under a stereoscopic microscope (Zeigen).

Evaluation of L-tyrosine metabolism in Lasiodiplodia gilanensis

The ability of *L. gilanensis* to metabolize tyrosine was evaluated as follows. The fungus was grown in Minimal Medium 9 (MM9) (10.0 g L^{-1} glucose, 5.8 g L^{-1} Na_2HPO_4, 3.8 g L^{-1} K_2HPO_4, 0.5 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl) supplemented with 10 mM tyrosine. The use of tyrosine as

carbon, nitrogen, or carbon and nitrogen source was evaluated by preparing the medium without glucose (MM9 -C), without NH_4Cl (MM9 -N) or without both elements (MM9 -CN). After inoculation with the fungus, plates were incubated at 30°C for 7 d. The formation of a halo from decrease in medium opacity indicated catabolic use of tyrosine. Vegetative growth of the fungus was measured every 24 h for each treatment. Experiments were repeated in the presence of previously selected melanin inhibitors.

Evaluation of biomass production in the presence of melanin inhibitors and stress conditions

For each assay, biomass was evaluated as follows. A sterilized cellophane membrane was placed on the top of the medium, inoculated with a mycelium disk of *L. gilanensis*, and plates were incubated at 30°C. In the experiments with melanin inhibitors and stress conditions, membranes were carefully removed after 4 d and weighed. For tyrosine and melanin inhibitors, the membranes were recovered after 7 d. Biomass data were obtained by subtracting the weight of the membrane and the mycelium disk used for the inoculation.

Microscopic observations of Lasiodiplodia gilanensis *colonizing grapevine tissue plants*

For the evaluation of melanin production during colonization by the fungus, one-year-old cuttings of *V. vinifera* 'Cabernet Sauvignon' were used. Plants were drilled in the woody stems and then each inoculated with a 3 mm diam. mycelium plug of a 3-d-old culture of *L. gilanensis*, and the drill wound was covered with parafilm. Control plants were each inoculated with a 3 mm diam. plug of PDA. The plants were left in a growth chamber (PGR15; CONVIRON) for 28 d, with a day/night 30°C/10°C temperature cycle at 50% humidity. Samples (each approx. 2 cm) were then taken from the infection zones (1 cm above and 1 cm below). Lesions were observed under a stereo microscope, and the tissues were fixed in FAA solution (formaldehyde, ethanol, acetic acid; 10%:50%:5%). The samples were then dehydrated in ethanol solutions (25, 50, 80%) and preserved in 80% ethanol at 4°C until analysis. Samples were cut into cross- and longitudinal sections 70 µm thick with a microtome (EMS 5000 Oscillating Tissue Slicer). The sections were stained using the Masson-Fontana technique (modified from Lillie, 1965) to assess for the presence of melanin in the hyphae of *L. gilanensis*. This staining procedure is based on the ability of melanin to reduce ammoniacal silver nitrate solution to metallic silver without using an external reducing agent. Stained samples were analyzed using light microscopy (DM4000; Leica Microsystems).

Comparison of Lasiodiplodia gilanensis *genes involved in melanin synthesis with other* Botryosphaeriaceae *species*

In order to determine if previously reported melanin genes of *L. gilanensis* UCD256Ma (Paolinelli-Alfonso *et al*., 2016) were present in other *Botryosphaeriaceae*, seven genes from three different melanin pathways were compared against the sequenced genomes from the family available in the GenBank database. These were: for the DHN-melanin pathway, short-chain dehydrogenase reductase (*sdr*) and thioesterase (*thr*) genes; for the DOPA-melanin pathway, tyrosinase (*tyr*), and multicopper oxidase (*mco*), and for the pyomelanin pathway, laccase (*lcc*), hydroxyphenylpyruvate dioxygenase (*hppD*) and homogentisate dioxygenase (*hmgD*). Putative gene sequences involved in melanin synthesis were recovered from the transcriptome data of *L. gilanensis* (Paolinelli-Alfonso *et al*., 2016) and a tBLASTx (percent query coverage per hsp: ≥70%, and minimum percent similarity: ≥70%) analysis was performed against *Botryosphaeriaceae* genomes available in the GenBank database to search for sequence similarities (Table S1). In addition, the genome sequenced from *Phaeoacremonium minimum*; another grapevine trunk fungus, was used for comparison.

Data analyses

Statistical analyses were carried out using one-way ANOVA followed by a *post hoc* Fisher analysis, with an $\alpha < 0.05$ for the determination of statistical significance, using STATISTICA 8.0. All the experiments were carried out in triplicate. Graphs of presented data were made with SigmaPlot 11.0 software.

RESULTS

Different melanin inhibitors produce contrasting effects in Lasiodiplodia gilanensis

Kojic acid at concentrations up to 500 µg mL^{-1} did not affect colony pigmentation or mycelial growth. For this reason, Kojic acid was discarded as a DOPA-melanin inhibitor of *L. gilanensis*. Tropolone, another DOPA-melanin inhibitor, diminished the growth and pigmentation at 10 µg mL^{-1}, and colonies had less aerial

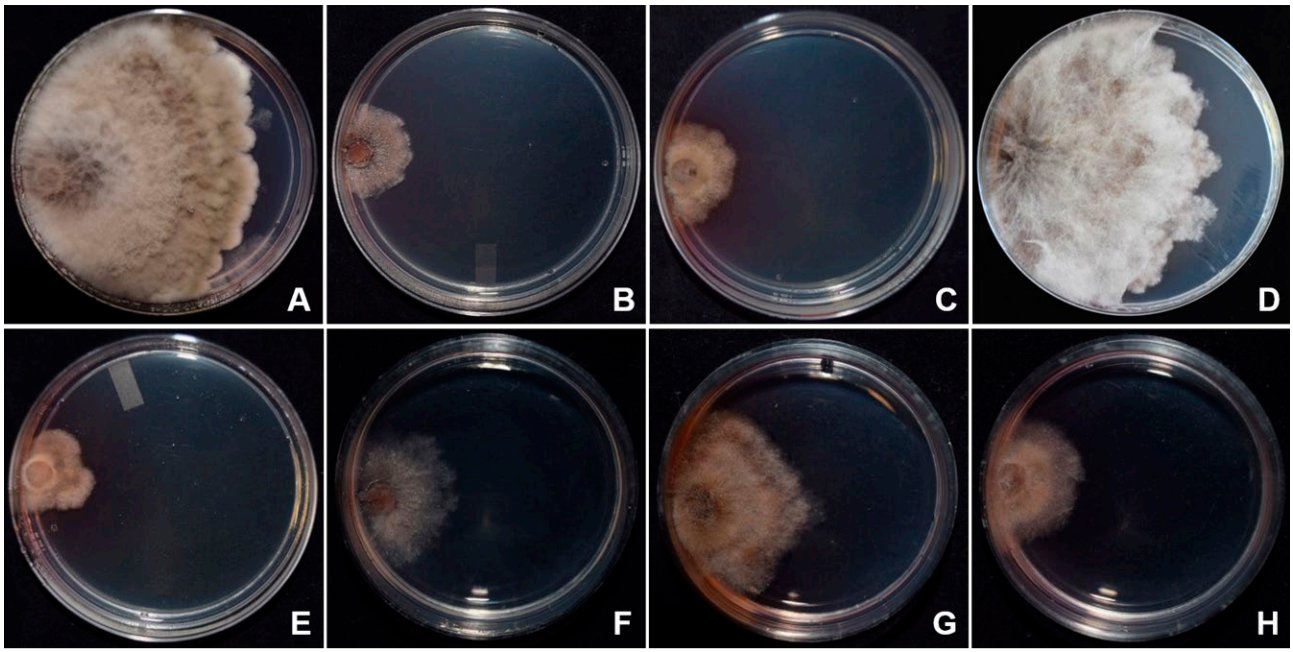

Figure 1. Growth of *Lasiodiplodia gilanensis* on PDA medium with different melanin inhibitors: A) Control. B) PDA + 15 µg mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. C) PDA + 500 µg mL^{-1} phthalide (Ph), DHN-melanin inhibitor. D) PDA + 100 µg mL^{-1} nitisinone (NTBC), pyomelanin inhibitor. E) PDA + Tp + Ph. F) PDA + Tp + NTBC. G) Ph + NTBC. H) Tp + Ph + NTBC.

mycelium in comparison to controls. At 15 µg mL^{-1} flat colonies and the formation of guttation droplets were observed (Figure 1B), and at 20 µg mL^{-1}, the growth of the fungus was inhibited. The concentration of 15 µg mL^{-1} of tropolone was therefore used for subsequent experiments.

For the DHN-melanin inhibitors, the fungus growing in phthalide (100 µg mL^{-1}) developed light-coloured mycelium. At 500 µg mL^{-1}, a noticeable change in morphology, growth inhibition and a slightly brown-orange pigmentation in the colony was observed (Figure 1C). With tricyclazole, at 10 µg mL^{-1}, the colour changed, but the morphology of the fungus was not affected. At 15 and 30 µg mL^{-}, growth inhibition and an orange-brown pigmentation were observed, similar to that observed with phthalide. Since both compounds inhibit the reductase reactions of two hydroxynaphthalene compounds to scytalone and vermelone in the DHN-melanin pathway (Wheeler and Klich, 1995; Suwannarach *et al*., 2019), but phthalide is cheaper, phthalide was selected for subsequent tests.

Nitisinone, a pyomelanin inhibitor, affected the fungus less, since only at 100 µg mL^{-1} was the colony colour lightened and growth slightly diminished (Figure 1D). This concentration was used in subsequent tests.

When phthalide (500 µg mL^{-1}) and tropolone (15 µg mL^{-1}) were used together, less aerial mycelium, slightly orange pigmentation, guttation droplets and growth inhibition were observed (Figure 1E). When nitisinone 100 µg mL^{-1} and tropolone 15 µg mL^{-1} were used together, growth, pigmentation, and formation of aerial mycelium were affected (Figure 1F). The greatest growth inhibition, least formation of aerial mycelium and less colony pigmentation were observed from the combination of phthalide 500 µg mL^{-1}, tropolone 15 µg mL^{-1}, and nitisinone 100 µg mL^{-1} (Figure 1H, 4; Table S2).

Responses of Lasiodiplodia gilanensis *to environmental stress*

Responses to enzymatic lysis

The use of lysing enzymes from *T. harzianum* reduced growth of the fungus, but the formation of aerial mycelium and biomass were similar to the experimental control (Figures 2A and B; 4, and S1; Table S2). The greatest inhibition was observed from the lysing enzymes, with tropolone at 15 µg mL^{-1} (Figures 2D and S1). From phthalide at 500 µg mL^{-1}, mycelium growth was inhibited and a brown-orange colour was observed (Figure 2F). When combining lysing enzymes with tropolone or with phthalide, biomass was similar (Figure 4; Table S2). Nitisinone at 100 µg mL^{-1} reduced growth of *L. gilanensis* compared with the lysing enzymes alone, but not as much as for tropolone (Figures 2H, 2D, and S1).

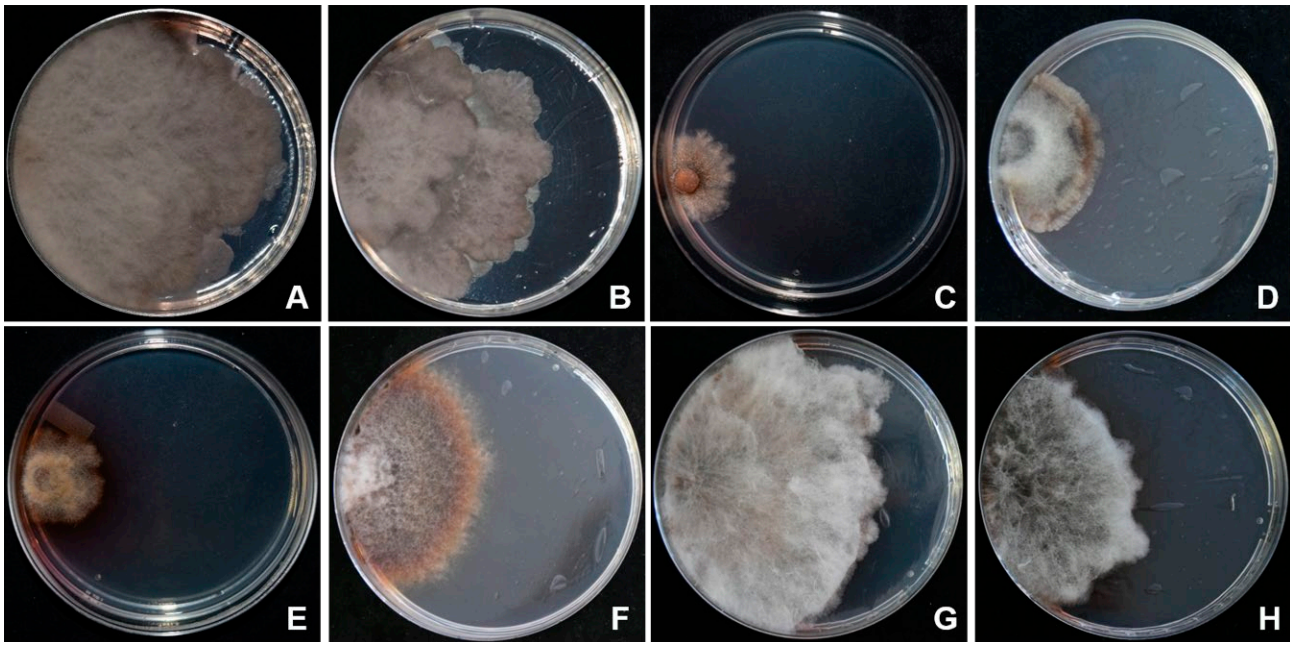

Figure 2. Growth of *Lasiodiplodia gilanensis* under enzymatic stress conditions. A) Control on PDA. B) PDA + 10 mg mL^{-1} lysing enzymes of *Trichoderma harzianum* (LE). C) PDA + 15 µg mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. D) PDA + LE + Tp. E) PDA + 500 µg mL^{-1} phthalide (Ph), DHN-melanin inhibitor. F) PDA + LE + Ph. G) PDA + 100 µg mL^{-1} nitisinone, pyomelanin inhibitor (NTBC). H) PDA + LE + NTBC.

Responses to oxidative stress

Exposure to hydrogen peroxide at 0.5 to 2.5% did not affect vegetative growth of *L. gilanensis*, but at 5%, colony growth was reduced (Figures 3A and B, and S1). When H_2O_2, tropolone and phthalide were combined, decreased tolerance to oxidative stress was observed, since the fungus grew less compared with the experimental control (Figure 3A), with H_2O_2 alone (Figure 3B), or with each inhibitor (Figure 3C-3D). In the combination treatment with nitisinone and H_2O_2, less colony aerial mycelium was observed (Figure 3H), compared with nitisinone alone (Figure 3G). Biomass production was only affected in the presence of H_2O_2 with tropolone (Figure 4; Table S2). The assays of the lysing enzymes and H_2O_2 indicated that DOPA-melanin was the main melanin that protected the fungus from oxidative stress.

Responses to UV radiation

Hyaline and pigmented conidia without exposure to UV radiation both had 100% germination before 24h of incubation. Exposure to UV radiation generally delayed germination and growth of conidia. Hyaline conidia exposed to UV radiation for 30 or 60 min did not germinate after 24h, and at 48h had, respectively, 33% and 20% germination. Melanized conidia exposed to UV for 30 min or 60 min germinated before 24h, reaching, respectively 80% and 40% germination. After 48h, melanized conidia exposed to 30 min of UV radiation reached 83% germination, and 73% of those exposed for 60 min germinated. The mycelia originating from hyaline conidia exposed for 30 or 60 min remained white for longer time than mycelia from melanized conidia (Figure S3). These results indicated that melanin in conidia cell walls enabled the conidia to maintain viability under UV radiation conditions.

Use of tyrosine as carbon and nitrogen sources

The presence of tyrosine 10 mM in MM9 increased growth and biomass of *L. gilanensis* (Figures 5A, 5E, 6 and S2). The fungus without a carbon source (Figure 5B), a nitrogen source (Figure 5C), or without both (Figure 5D) had shallow growth. When tyrosine was added to these treatments (Figure 5F to H), aerial mycelium and colony pigmentation formed, and a pink pigment was secreted and degradation halos developed. This showed that the fungus catabolized tyrosine and used it as carbon and nitrogen sources. When adding the melanin inhibitors in the MM9-CN treatment (Figure 5I to

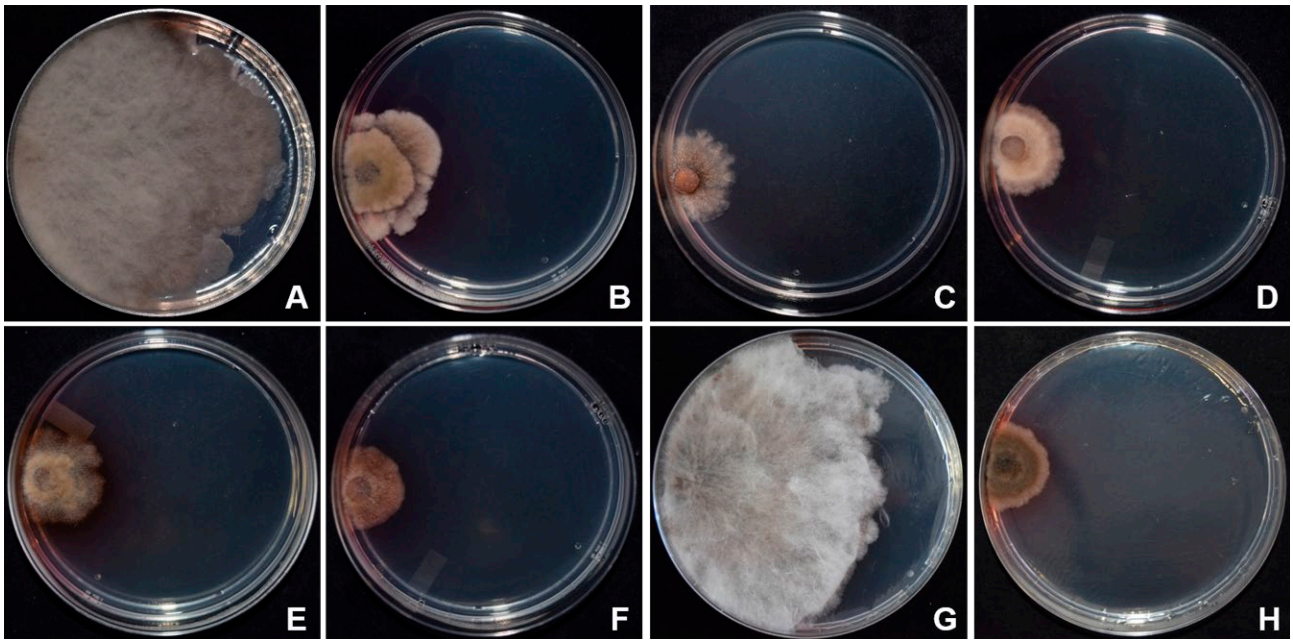

Figure 3. Growth of *Lasiodiplodia gilanensis* under oxidative stress. A) Control. B) PDA + 5% hydrogen peroxide (H_2O_2). C) PDA + 15 μg mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. D) PDA + H_2O_2 + Tp. E) PDA + 500 μg mL^{-1} phthalide (Ph), DHN-melanin inhibitor. F) PDA + H_2O_2 + Ph. G) PDA + 100 μg mL^{-1} nitisinone, pyomelanin inhibitor (NTBC). H) PDA + H_2O_2 + NTBC.

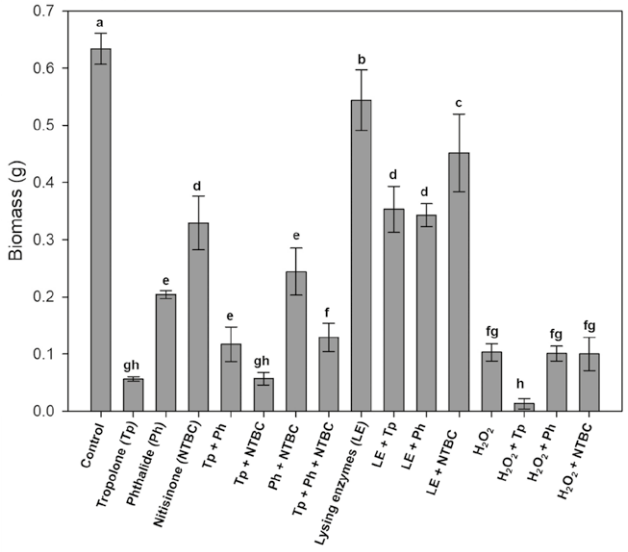

Figure 4. Mean weights of *Lasiodiplodia gilanensis* biomass in the presence of melanin inhibitors and under stress conditions. Bars indicate standard deviations. Means accompanied by the same letters are not significantly different ($\alpha < 0.05$)

L), only in the presence of tropolone was the fungus unable to use tyrosine, since growth was reduced (Figure 5I) and biomass production was greatly reduced (Figure 6; Table S3). The fungus did not grow in the MM9-CN treatment with the three inhibitors of melanin and 10mM tyrosine (Figure 5L).

Melanized mycelium of Lasiodiplodia gilanensis *growing inside grapevine plants*

Lasiodiplodia gilanensis melanized mycelia growing mainly in the pith and vascular bundles of the plants was observed with the use of Masson-Fontana stain (Figure 7). Xylem vessel occlusions were observed in both infected and control plants, probably due to the response of the plant to wounding.

Comparison of Lasiodiplodia gilanensis *genes involved in melanin synthesis with other* Botryosphaeriaceae

The similarity analysis showed that most of the genes were conserved among the *Botryosphaeriaceae*, with similarity percentages greater than 71%. All genes analyzed, previously identified through *L. gilanensis* transcriptome, had over 90% similarity with the genomes of *L. theobromae* CSS-01s and LA-SOL3. Most of the genes involved in the three different melanin pathways (including *sdr*, *thr*, *tyr*, *mco*, *lcc* and, *hmgD*), showed similarity to sequences in *D. seriata* DS831 and F98.1, and *D. corticola* CBS 112549. *Neofusicoccum parvum*

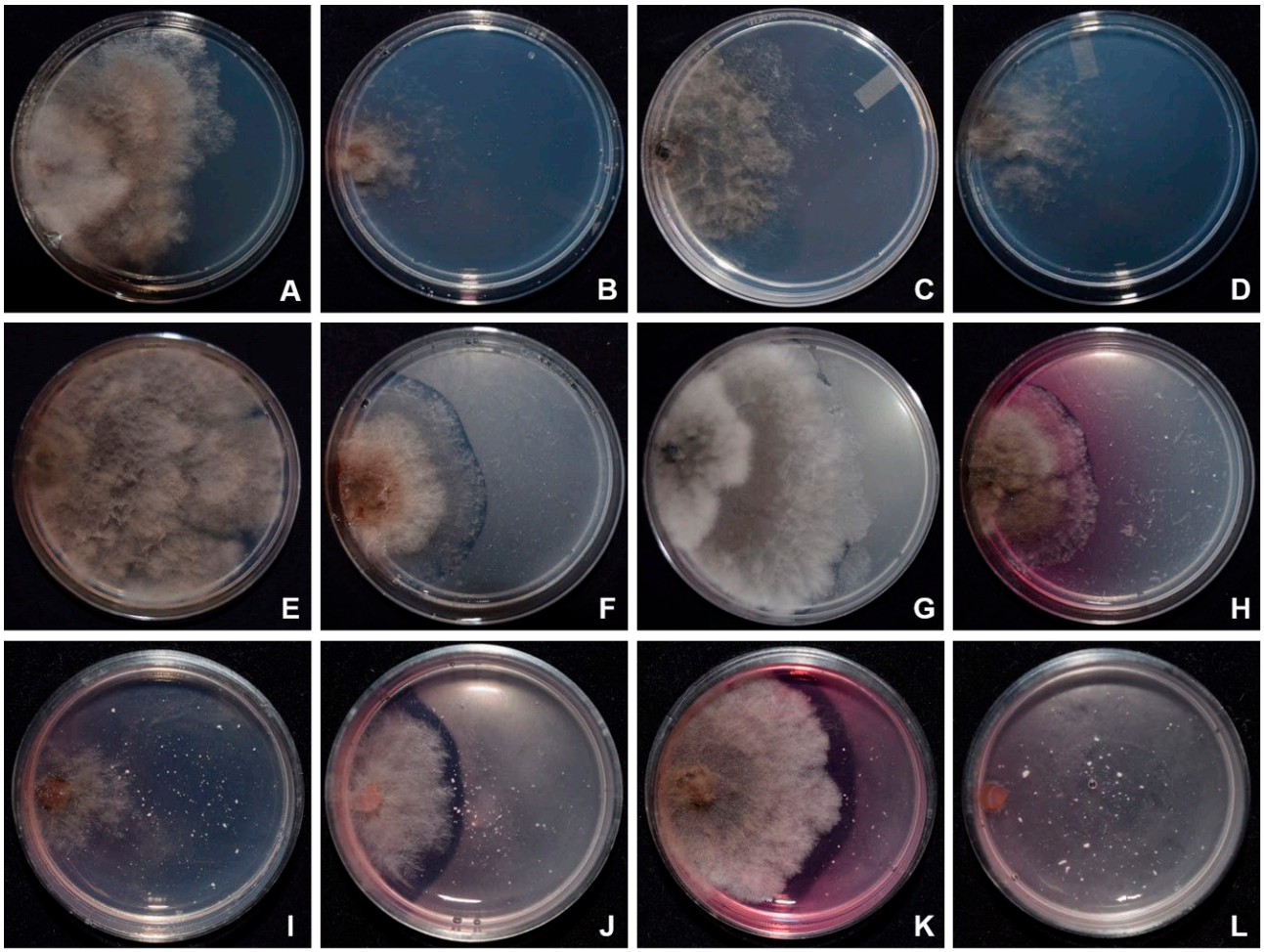

Figure 5. Assessment of effects of tyrosine on growth in *Lasiodiplodia gilanensis*. A) Minimal Medium 9 (MM9). B) MM9 without carbon source (MM9-C). C) MM9 without nitrogen source (MM9-N). D) MM9 without carbon and nitrogen sources (MM9-CN). E) MM9 + 10 mM tyrosine (Tyr). F) MM9-C + Tyr. G) MM9-N + Tyr. H) MM9-CN + Tyr. I) MM9-CN + Tyr + 15 μg·mL^{-1} tropolone (Tp), DOPA-melanin inhibitor. J) MM9-CN + Tyr + 500 μg·mL^{-1} phthalide (Ph), DHN-melanin inhibitor. K) MM9-CN + Tyr + 100 μg·mL^{-1} nitisinone (NTBC), pyomelanin inhibitor. L) MM9-CN + Tyr + Tp + Ph + NTBC.

UCRNP2 had similarity in four genes (*mco*, *lcc*, *hppD* and, *hmgD*), and *Botryosphaeria dothidea* LW030101 only in three genes (*mco*, *lcc* and, *hmg*) (Table 1). The genes *mco*, *lcc* and, *hmgD* were present in all *Botryosphaeriaceae* genomes. Only *hmgD*, which is involved in the tyrosine catabolism and is a precursor in the pyomelanin pathway, was also found in the genome of *P. minimum* with 75% similarity.

DISCUSSION

Melanin inhibitors of three different pathways affected pigmentation, morphology, and the growth of *L. gilanensis*, indicating that this fungus produces DOPA-melanin, DHN-melanin, and pyomelanin. Biosynthesis of three types of melanin has been reported in *S. schenckii* (Romero-Martinez *et al*., 2000; Almeida-Paes *et al*., 2012), *Aspergillus fumigatus* (Schmaler-Ripcke *et al*., 2009; Pal *et al*., 2014), and *Penicillium marneffei* (Liu *et al*., 2014; Boyce *et al*., 2015; Sapmak *et al*., 2015). Ability to synthesize melanin using different pathways may be an adaptation of the fungus to cope with unfavourable conditions, increasing the protection against environmental stress and during host infection (Almeida-Paes *et al*., 2009; Sapmak *et al*., 2015).

Similar gene sequences involved in melanin synthesis are present in the *Botryosphaeriaceae*, and are highly conserved within *Lasiodiplodia*. The genomes from *Diplodia* had high percentages of similarity, for six of

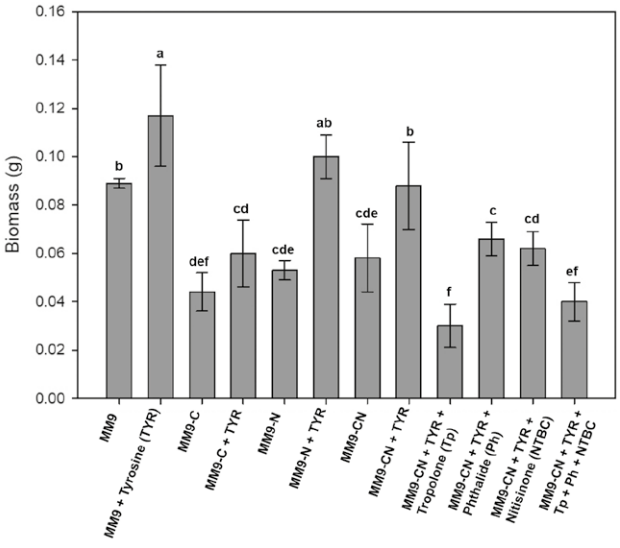

Figure 6. Mean weights of *Lasiodiplodia gilanensis* biomass in the presence of tyrosine and melanin inhibitors. Bars indicate standard deviations. Means accompanied by the same letters are not significantly different ($\alpha < 0.05$).

the seven genes compared, with the *hppD* gene being the only one not found. This is because *Diplodia* is phylogenetically closer to *Lasiodiplodia* than *Neofusicoccum* and *Botryosphaeria*.

A cluster of three genes coding for putative short-chain dehydrogenase/reductases (SDRs) was reported in *N. parvum* (Massonnet *et al*., 2018). The similarity standard was set above 70% in the present study, and these sequences were not so similar with the sequence of the gene *sdr* of *L. gilanensis* (63%). However, this highlights the importance of melanin in the pathogenicity of *Botryosphaeriaceae*.

The homogentisate dioxygenase gene (*hmgD*), which is involved in tyrosine catabolism and is a precursor in the pyomelanin pathway, was detected in all *Botryosphaeriaceae* genomes and in *P. minimum*. This indicates that *hmgD* is essential in the metabolism of fungi causing grapevine trunk diseases.

Aerial mycelium is essential in fungi for the formation and dispersal of reproductive structures (Braun, 2007). For example, a mutant of the *mve1* gene in *Mycosphaerella graminicola* with an albino phenotype produced less aerial mycelium than the wildtype, which indicated a relationship between the lack of melanin and absence of aerial mycelium (Choi and Goodwin, 2011). In *L. gilanensis,* DOPA-melanin is related to the production of aerial mycelium, since the presence of tropolone produced flat colonies. This effect was not due to the interference of any essential pathway. When *Fusarium oxysporum* FFOCR-SQ, and *Trichoderma asperellum* TTORO, isolates with hyaline mycelium from our collection, were grown in the presence of tropolone, no significant effects were observed (Figure S4). Therefore, DOPA-melanin is likely to provide structural strength to the mycelia of *L. gilanensis*, which is necessary for vegetative growth, and DOPA-melanin biosynthesis is likely to be the primary pathway for melanin synthesis in this fungus under *in vitro* conditions. Further genetic studies should confirm this.

The protective role of melanin in enzymatic lysis has been little studied, but melanin-producing mutants of *A. nidulans* (Kuo and Alexander, 1967) and *Aspergillus phoenicis* (Bloomfield and Alexander, 1967) were more susceptible to enzymatic lysis than their respective wild types. In the present study, the presence of cell wall degrading enzymes in the growth media partially inhibited the growth of *L. gilanensis*, but these enzymes did not inhibit formation of aerial mycelium. Instead, when the DOPA-melanin pathway was blocked, growth of the fungus was strongly inhibited. This indicates that the presence of DOPA-melanin helps to maintain cell wall integrity. Since melanin is associated with chitin in fungus cell walls (Nosanchuk *et al*., 2015), as reported for *A. nidulans* (Bull, 1970) and *Cryptococcus neoformans* (Banks *et al*., 2005), this pigment could help conceal target molecules from cell wall degrading enzymes, preventing cell walls from damage and weakening.

DOPA-melanin has been associated with protection against oxidative stress damage in *C. neoformans* (Jacobson and Tinnell, 1993), *Sporothrix* spp., (Almeida-Paes *et al*., 2012), and *A. fumigatus* (Heinekamp *et al*., 2013). In *Inonotus obliquus*, production of DOPA-melanin is one of the primary responses to oxidative stress in the presence of H_2O_2, together with an increment in production of mycelia (Zheng *et al*., 2009). In contrast, in the hypersaline yeast *H. werneckii,* inhibition of DHN-melanin by tricyclazole did not affect survival under H_2O_2 oxidative stress (Kejžar *et al*., 2013), as further evidence that DOPA-melanin is the main melanin involved in protection from oxidative stress. In the present study, the presence of H_2O_2 inhibited growth of *L. gilanensis* but did not affect morphology, pigmentation, or formation of aerial mycelium. However, when the DOPA-melanin pathway was blocked, the fungus did not grow, produced less aerial mycelium, and had different colour, indicating that DOPA-melanin protected the fungal cells against oxidative stress.

Another function of melanin in fungi is the protection against UV radiation (Bell and Wheeler, 1986; Jacobson, 2000; Cordero and Casadevall, 2017). Al-Laaeiby *et al*. (2016) reported differences in the germi-

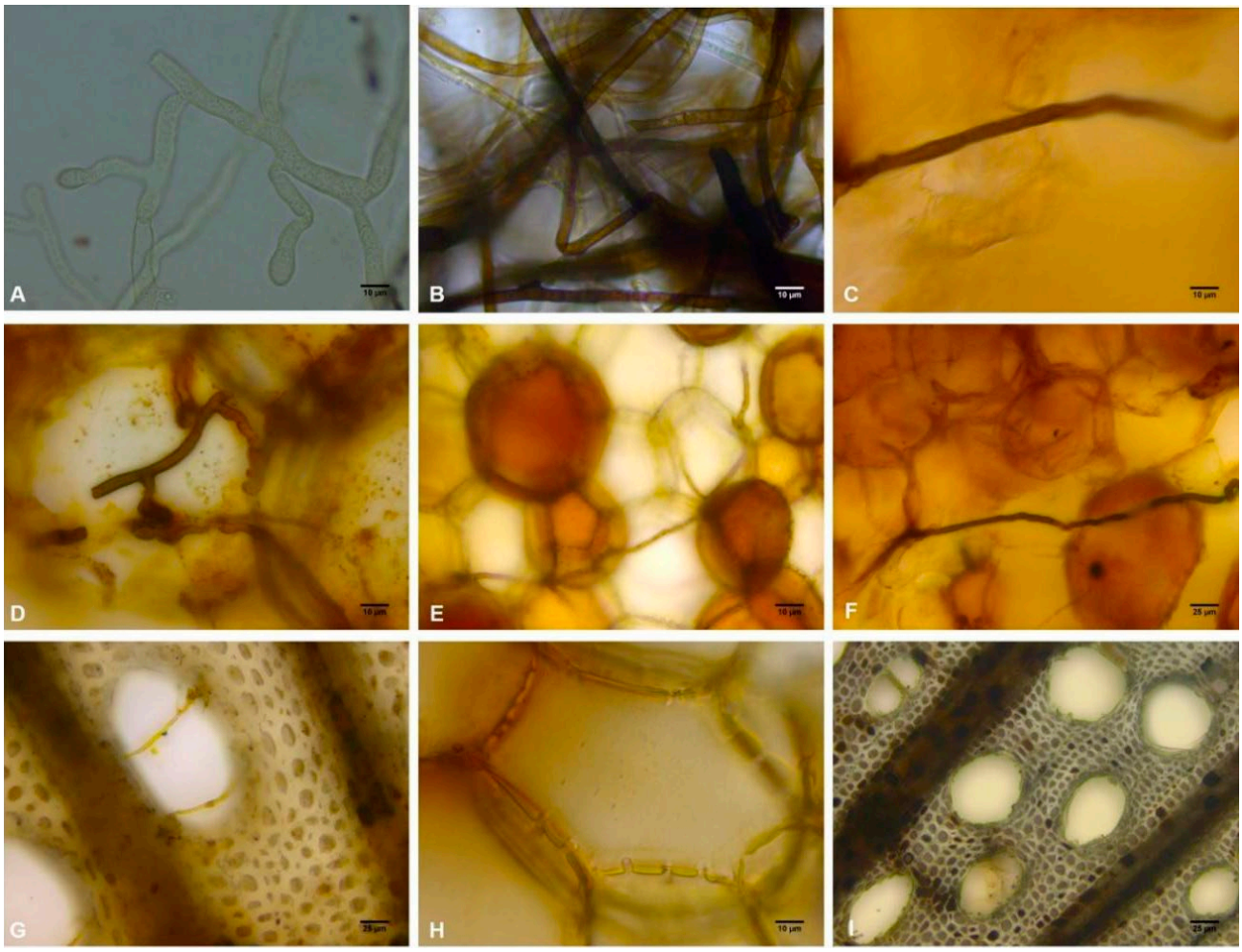

Figure 7. *In planta* melanin staining of *Lasiodiplodia gilanensis* using the Masson-Fontana method. A) Non-melanized fungus on PDA). B) Melanized fungus on PDA). C), D), E), and F) Melanized fungus colonizing the host plant piths. G) Fungus colonizing vascular bundles. H) plant pith in an uninfected plant. I) Vascular bundles in an uninfected plant.

nation of melanized conidia of *Lomentospora prolificans* exposed to 200 mJ cm^{-2} of UV radiation (51% germination) in comparison to conidia deficient in the PKS1 enzyme (28% germination). Hyaline conidia of. *L. gilaniensis* had lower and delayed germination compared with pigmented conidia. Melanin has been reported to absorb UV light, thereby acting as a shield of photons of high energy and protecting cells from damage (Almeida-Paes *et al*., 2012). In the present study, presence of melanin in conidia of *L. gilanensis* gave protection from UV radiation, most probably allowing the conidia to survive under harsh environmental conditions once released from the pycnidia.

Data from the present study also showed that *L. gilanensis* used tyrosine as carbon and nitrogen sources for growth. Use of this amino acid was affected in the presence of tropolone. It was unexpected that nitisinone did not affect growth of the fungus in the presence of tyrosine, since pyomelanin results from tyrosine catabolism. Under *in vitro* conditions, the fungus may use tyrosine mostly for the synthesis of DOPA-melanin, so tyrosine could be the precursor of synthesis of DOPA-melanin, and be essential for growth. As additional evidence, Paolinelli-Alfonso *et al*. (2016) reported induction of *L. gilanensis* genes associated with production of DOPA-melanin, tyrosinase (TYR) and multicopper oxidase (MCO), in the presence of grapevine wood. Phenylalanine is another precursor of DOPA-melanin related to pyomelanin, derived from tyrosine catabolism (Plonka and Grabacka, 2006; Boyce *et al*., 2015). When phenylalanine was evaluated in MM9, growth, aerial mycelium formation and colony pigmentation of the fungus were similar to that observed in the presence of tyrosine. However, the fungus did not use phenylalanine as a car-

Table 1. Comparison of genes involved in the synthesis of melanin among genomes of the *Botryosphaeriaceae*, using tBLASTx analysis of similarity percentages.

Pathway	Gene	Organism	Isolate	Query cover	Similarity Percentage	Accession Number
DHN-melanin	*sdr*	*Diplodia seriata*	DS831	88%	91%	LAQI01000021.1
		Diplodia seriata	F98.1	87%	91%	MSZU01000111.1
		Diplodia corticola	CBS	83%	89%	NW_017971532.1
		Lasiodiplodia theobromae	CSS-01s	100%	94%	KZ107829.1
		Lasiodiplodia theobromae	LA-SOL3	100%	93%	VCHE01000036.1
		Macrophomina phaseolina	MP2_2471002	89%	84%	WHMB01000016.1
	thr	*Diplodia seriata*	DS831	82%	89%	LAQI01000195.1
		Diplodia seriata	F98.1	79%	87%	MSZU01000075.1
		Diplodia corticola	CBS 112549	83%	89%	NW_017971483.1
		Lasiodiplodia theobromae	CSS-01s	96%	98%	KZ107832.1
		Lasiodiplodia theobromae	LA-SOL3	96%	98%	VCHE01000163.1
DOPA-melanin	*tyr*	*Diplodia seriata*	DS831	90%	71%	LAQI01000197.1
		Diplodia seriata	F98.1	90%	71%	MSZU01000087.1
		Diplodia corticola	CBS	89%	73%	NW_017971497.1
		Lasiodiplodia theobromae	CSS-01s	99%	94%	KZ107831.1
		Lasiodiplodia theobromae	LA-SOL3	99%	94%	VCHE01000030.1
	mco	*Neofusicoccum parvum*	UCRNP2	83%	74%	KB915882.1
		Diplodia seriata	DS831	89%	92%	LAQI01000063.1
		Diplodia seriata	F98.1	92%	92%	MSZU01000075.1
		Botryosphaeria dothidea	LW030101	83%	87%	MDSR01000123.1
		Diplodia corticola	CBS 112549	94%	88%	NW_017971485.1
		Lasiodiplodia theobromae	CSS-01s	100%	99%	KZ107832.1
		Lasiodiplodia theobromae	LA-SOL3	100%	99%	VCHE01000017.1
		Macrophomina phaseolina	MP2_2471002	86%	79%	WHMB01000018.1
	lcc	*Neofusicoccum parvum*	UCRNP2	84%	81%	KB916.432.1
		Diplodia seriata	DS831	88%	85%	LAQI01000030.1
		Diplodia seriata	F98.1	88%	85%	MSZU01000080.1
		Botryosphaeria dothidea	LW030101	78%	85%	MDSR01000049.1
		Diplodia corticola	CBS 112549	91%	85%	NW_017971480.1
		Lasiodiplodia theobromae	CSS-01s	98%	97%	KZ107828.1
		Lasiodiplodia theobromae	LA-SOL3	98%	97%	VCHE01000007.1
		Macrophomina phaseolina	MP2_2471002	84%	81%	WHMB01000012.1
Pyomelanin	*hppD*	*Neofusicoccum parvum*	UCRNP2	90%	74%	KB916303.1
		Lasiodiplodia theobromae	CSS-01s	93%	92%	KZ107826.1
		Lasiodiplodia theobromae	LA-SOL3	94%	92%	VCHE01000035.1
	hmgD	*Neofusicoccum parvum*	UCRNP2	82%	93%	KB915800.1
		Diplodia seriata	DS831	95%	95%	LAQI01000171.1
		Diplodia seriata	F98.1	95%	95%	MSZU01000075.1
		Botryosphaeria dothidea	LW030101	85%	94%	MDSR01000002.1
		Diplodia corticola	CBS 112549	93%	95%	NW_017971483.1
		Lasiodiplodia theobromae	CSS-01s	100%	99%	KZ107832.1
		Lasiodiplodia theobromae	LA-SOL3	100%	99%	VCHE01000037.1
		Macrophomina phaseolina	MP2_2471002	87%	95%	WHMB01000018.1
		Phaeoacremonium minimum	UCRPA7	72%	75%	NW_006920969.1

bon or nitrogen source (Figure S5). Thus, phenylalanine did not have an essential role in the melanin pathway of *L. gilanensis*.

For some pathogenic fungi, amino acids are essential sources of carbon and nitrogen (Boyce *et al*., 2015). In plants, tyrosine and phenylalanine give rise to the synthesis of compounds involved in structure and defense, including lignin, suberin, phenylpropanoids, anthocyanins, plastoquinones, isoquinoline alkaloids and flavonoids (Tzin and Galili, 2010; Nelson *et al*., 2013). Some of these compounds are produced near the sites of pathogen infections, and they accumulate in the necrotic tissues, acting in host resistance and defense (Ahuja *et al*., 2012; Nelson *et al*., 2013). Although further *in planta* experiments are required, results of the present study indicate that the ability of *L. gilanensis* to degrade tyrosine confers an advantage during the processes of infection and colonization of grapevine.

It is important to establish how *Lasiodiplodia* infects plants, and the mechanisms these fungi use to counteract host responses to infection. The present study demonstrated that the hyphae of *L. gilanensis* were melanized, mainly among the parenchymal cells in host pith tissues, but not in the vascular bundles. This indicates that the fungus became melanized as host colonization progresses. Although additional study is required using fungus mutants for key melanin genes to fully understand the role of melanin in *L. gilanensis,* results from the present study indicate that melanin protected *L. gilanensis* from adverse environmental conditions, such as the reactive oxygen species, for example, superoxide (O_2^-), or its dismutation product hydrogen peroxide (H_2O_2), which is generated within plants as a primary defense mechanism (Torres *et al*., 2006).

In conclusion, this study demonstrated that *L. gilanensis* synthesized three types of melanin: DOPA-melanin, DHN-melanin, and pyomelanin. DOPA-melanin is the essential melanin, involved in vegetative growth and formation of aerial mycelium. This pathogen utilized tyrosine as a nutrient source, and as a precursor of DOPA-melanin. Melanin protected the fungus against enzymatic lysis and oxidative stress, and conidia from UV radiation. This pathogen also produced melanin within its host as part of the colonization processes.

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