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Investigation of the role of the L-DOPA pathway in the production of melanin in *Monilinia* spp. and its relationship with fungal development

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Abstract *Monilinia* spp., the causal agent of brown rot in stone fruits, relies on melanin biosynthesis for development, survival, and longevity. In this study, we identified for the first time the presence of the L-3,4-dihydroxyphenylalanine (L-DOPA) pathway in *Monilinia* spp. (*M. laxa*, *M. fructicola*, and *M. fructigena*) through BLAST analysis, and detected L-DOPA-derived pigments using UHPLC-MS/MS. The pathway was evaluated under control conditions, upon inhibition of the dihydroxynaphthalene (DHN)-melanin pathway by tricyclazole, and in the presence of kojic acid. Our results indicate that the L-DOPA pathway may remain active under all tested conditions in darkness. Tricyclazole induced phenotypic and transcriptional changes, whereas kojic acid did not inhibit the L-DOPA pathway. Moreover, developmental analyses showed that L-DOPA-derived pigments were sufficient to sustain normal fungal development (i.e., conidiation and viability) when the DHN pathway was blocked. However, in *M. laxa*, the absence of DHN affected the germination capacity. Although these differences were small, the results indicate that L-DOPA–derived pigments may contribute to proper development across the three species, without providing conclusive evidence of a causal role. Importantly, these findings provide the first evidence that *Monilinia* spp. can utilize more than one melanin biosynthetic pathway to support survival and development, underscoring the need for further investigation into the environmental factors that modulate their activation.

Keywords Eumelanin · Fungal pigments · Kojic acid · Melanin biosynthesis · Pheomelanin · Tricyclazole

Introduction

Fungi produce a wide variety of natural products, especially under stress conditions. They are called secondary metabolites, and among them, melanin is one of the key pigments due to its multifunctional properties (Cordero & Casadevall, 2017). In the fungal kingdom, melanisation is observed in all phyla (Cordero & Casadevall, 2017), contributing to the adaptability and survival of fungi to improve their competitive capabilities under stress conditions (Schumacher, 2016).

This pigment is defined as a stable, insoluble and resistant biochemical compound, product of the polymerization of phenolic compounds (Riley, 1997). Melanin macromolecules show a wide diversity and

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are composed of many different types of monomeric units that are connected through strong carbon–carbon bonds (Wakamatsu & Ito, 2002). Consequently, this pigment can only be decomposed by acid and alkaline oxidation (Eisenman & Casadevall, 2012).

Fungal melanin derived from the precursor molecule DHN, that finally produces melanin through a polyketide synthase (PKS), resides mainly in ascomycetes (Breitenbach et al., 2022; Butler et al., 2009), such as *Botrytis cinerea* and *Monilinia* spp. (Schumacher, 2016; Verde-Yáñez et al., 2023a). However, human pathogens (Eisenman & Casadevall, 2012) or basidiomycetes (Ribera et al., 2019) usually synthesize melanin through the L-tyrosine or L-DOPA metabolic pathways, in which the compound dopaquinone is generated and subsequent steps occur through a series of spontaneous reactions that finally lead to the polymerization of these pigment.

Currently, melanin pigments can be classified into five types based on their structural monomers: eumelanin, pheomelanin, allomelanin, and to a lesser extent, pyomelanin and GHB-melanin or PAP-melanin (Cao et al., 2021; Liu et al., 2022; Singh et al., 2021). Eumelanin is composed of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and 5,6-dihydroxyindole (DHI) units, and pheomelanin is composed of benzothiazole and benzothiazine units, being both pigments products of the oxidation of the L-DOPA pathway. On the other hand, allomelanin derives from the oxidation of nitrogen-free diphenols from the DHN pathway (Eisenman & Casadevall, 2012), while pyomelanin is derived from tyrosine (Singh et al., 2021). Regarding their coloration, pyomelanin is associated with a black colour, while eumelanin and allomelanin show a range from black to brown. In the case of pheomelanin, it provides a red to yellow pigmentation (Eisenman & Casadevall, 2012; Singh et al., 2021). Finally, the GHB-melanin or PAP-melanin pigment, that gives a brown coloration, is derived from γ-glutaminyl-3,4-dihydroxybenzene (GHB), that is oxidized and polymerized to form the final pigment (Liu et al., 2022).

The importance of DHN and L-DOPA pathways in the biosynthesis of these pigments is still unclear and controversial. In some fungi, such as *Cryomyces antarcticus* (Pacelli et al., 2020), *Sporothrix schenckii* (Almeida-Paes et al., 2017) and the genus *Aspergillus* spp. (Pal et al., 2014), it has been revealed the use of the two melanin biosynthetic pathways, which are activated depending on the conditions in which the fungi are grown. To determine the use of the melanin pathways (DHN and L-DOPA), there exists specific inhibitors for both pathways. Among the different specific inhibitors of the DHN pathway, such as tricyclazole, pyroquilon, chlobenthiazone and coumarin (Wheeler & Kuch, 1995), tricyclazole is one of the most widely used. Its specific action relies on the inhibition of THN reductases (THNR), enzymes involved in two reduction steps (T4HN to scytalone and T3HN to vermelone) (Schumacher, 2016). This inhibition results in an orange pigmentation due to the accumulation of T4HN and T3HN compounds, and their autoxidation products, flaviolin and 2-hydroxyjuglone (2-HJ) (Franzen et al., 2006). By using the inhibitor tricyclazole, it has been demonstrated in *B. cinerea* that the DHN pathway is active during the different stages of the life cycle of the fungus, and specifically, in the production of melanin in conidiophores, conidia and sclerotia (Schumacher, 2016). In the case of *Monilinia* spp., the effect of tricyclazole has been determined on the pigmentation and growth rate of *M. laxa* (De Cal & Melgarejo, 1994), although more recently, the presence of the DHN biosynthetic machinery has been demonstrated in *M. laxa*, *M. fructicola* and *M. fructigena* (De Miccolis Angelini et al., 2022), as well as its transcriptional regulation when grown under darkness (Verde-Yáñez et al., 2023a) and under different light wavelengths (Verde-Yáñez et al., 2023b). In the case of the L-DOPA pathway, different inhibitors can be used such as niacin, hydroquinone and tropolone, although in ascomycete the most used inhibitor is kojic acid (Lee et al., 2003; Pal et al., 2014). Unlike the DHN pathway, and to consider effective the inhibition of the L-DOPA pathway, discoloration must occur in the fungi (Pal et al., 2014). However, the existence and the use of the L-DOPA pathway in *Monilinia* spp. is still unknown.

This study describes, for the first time, the existence of the L-DOPA melanin biosynthetic pathway through gene identification, the use of the specific inhibitor of DHN (tricyclazole) and L-DOPA (kojic acid) pathway, as well as the identification of the L-DOPA-derived melanin pigments in *Monilinia* spp. In addition, several developmental parameters of *Monilinia* spp. were evaluated to unravel the importance of these melanin biosynthetic pathways in its development and survival.

Materials and methods

Monilinia strains and culture conditions

In this study, three *Monilinia* species were used: *Monilinia laxa* (ML8L), *M. fructicola* (CPMC6), and *M. fructigena* (GENA6). *M. laxa*, *M. fructicola* and *M. fructigena* strains are deposited in the Spanish Culture Type Collection (CECT 21100, CECT 21105 and CECT 21206, respectively). Currently, the genomes of the three species of *Monilinia* (*M. laxa*, *M. fructicola* and *M. fructigena*) are available (De Miccolis Angelini et al., 2019; Landi et al., 2018, 2020; Marcet-Houben et al., 2021; Rivera et al., 2018; Vilanova et al., 2021).

Conidial suspensions were prepared from 7 to 9-day-old cultures grown on PDA supplemented with 25% tomato pulp (PDA-T). For both *M. laxa* and *M. fructicola*, inoculum was prepared and filtered through two layers of sterile cheesecloth to minimize the presence of mycelium fragments as described by Baró-Montel et al. (2019). Due to the inability of *M. fructigena* to produce conidia, this species was prepared as described by Verde-Yáñez et al. (2023b).

To inhibit the melanin pathways, the inhibitors tricyclazole (Sigma, Schnelldorf, Germany) and kojic acid (Sigma, Schnelldorf, Germany) were used. Kojic acid and tricyclazole concentrations were selected based on previous reports in phytopathogenic fungi such as *B. cinerea* (Schumacher, 2016), *Exophiala mesophila* (Medina-Armijo et al., 2024), and *Aspergillus spp.* (Pal et al., 2014). The selected tested concentration for all the assays was 50 µg mL^{-1} and 150 µg mL^{-1}, respectively. Both inhibitors, tricyclazole and kojic acid, were dissolved in sterile water.

To assess the effect of tricyclazole and kojic acid, one drop (10 µL) of fungal suspensions of *Monilinia* spp. (1×10^5 conidia mL^{-1}) were placed on PDA-T (control) and PDA-T medium containing the inhibitors and incubated under darkness for 7 days at 20 °C.

After the incubation period, a visual inspection of the colonies, and a characterization of the development of the three *Monilinia* species was assessed. On the other hand, fungal samples of the three *Monilinia* spp. grown under the different conditions (control, tricyclazole and kojic acid) were frozen for gene expression analysis and for the identification and quantification of L-DOPA-derived melanin pigments by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). All experiments were performed twice using three replicates per condition.

Colony morphology and conidia characterization

A visual inspection of colony features was performed according to EPPO standard PM 7/18 (3). (Oepp, 2020).

Growth rate

Colony growth rate (cm day^{-1}) for each *Monilinia* species and conditions (control, tricyclazole and kojic acid) was determined as the slope of the linear equation obtained from individual measurements of mean colony diameter (cm) in two perpendicular directions of the Petri dish against the time in which the measurements were performed (days).

Conidial quantification

Quantification of total conidia was performed by preparing the conidial suspensions as described in Sect. 2.1. The conidia concentration was represented in $\log_{10}$ (conidia mL^{-1}) for each *Monilinia* species and conditions (control and tricyclazole).

Cell viability

Cell viability for each species and conditions (control and tricyclazole) was evaluated as described by Verde-Yáñez *et* al. (2023b) and expressed as conidia in $\log_{10}$ (conidia mL^{-1}).

Germination of conidia

The percentage (%) of germinated conidia was studied under light microscopy. Drops (10 µL) of each conidial suspension (1×10^5 conidia mL^{-1}) were incubated for 7 days at 20 °C under darkness without or in the presence of tricyclazole in PDA medium plates.

Sampling was performed every 30 min for 6 h, by stopping the germination process with 1 mL of 25% ammonia added to a filter paper placed on the lid of the Petri dish. Germinated conidia were considered those showing a deformation of the cell wall, forming

a germ tube equal to or longer than the smallest diameter of conidia.

Fungal RNA extraction and qPCR analysis

RNA was extracted from cultures of *Monilinia* spp. (control and tricyclazole), using TRI reagent (Sigma, MO, USA) as described by Baró-Montel et al. (2019), using 3 biological replicates for each condition and species.

cDNA synthesis was performed from 2 µg of DNase-treated RNA samples using the commercial Superscript IV First-Strand reverse transcriptase cDNA Synthesis Reaction kit (Invitrogen, CA, USA).

Gene expression analysis was performed as described by Baró-Montel et al. (2019). A total of 5 genes belonging to the DHN pathway were selected (*PKS12*, *YGH1*, *BRN1*, *BRN2* and *SCD1*), which act upstream and downstream of the inhibition site of tricyclazole. Primers for these genes were retrieved from Verde-Yáñez et al.(2023a). For each selected gene, primer sequences were common for the three *Monilinia* species. *ELONGATION FACTOR 1A* (*EF1-α*) was selected as reference gene based on its constant expression among conditions. Primer efficiency was determined using threefold cDNA dilutions in triplicate, and primer specificity was checked by analysing the melting curves at temperatures ranging from 60 to 95 °C. A non-template control (NTC) was included using water instead of cDNA. Relative gene expression was expressed as Mean Normalized Expression (MNE) and calculated using the method described by Muller et al.(2002).

Identification of L-DOPA biosynthetic and regulatory genes in *Monilinia* spp.

A BLAST analysis was performed to identify the melanogenic genes of the L-DOPA biosynthetic pathway in the genome of the three *Monilinia* species using *B. cinerea* as the reference genome. Candidate genes from *B. cinerea* were used as query sequences for a BLAST analysis to search for homologies within *M. laxa* (ML8L) (Naranjo-Ortíz et al., 2018), *M. fructicola* (CPMC6) (Vilanova et al., 2021), and *M. fructigena* (GENA6) (Marcet-Houben et al., 2021) genomes using NCBI Genome Workbench software v. 2.11.10 (https://www.ncbi.nlm.nih.gov/tools/gbench/), and the BLAST tool implemented therein, setting an expect (E) value of 10^3. The identity (> 60%) and the fraction of query sequences covered by the match region (> 50%) were used as filter criteria to select only reliable hits. Results obtained were checked by carrying out BLATSX, BLASTN, and TBLASTN analyses. The coding sequences of the candidate genes from *B. cinerea* and those from *M. laxa*, *M. fructicola*, and *M. fructigena* obtained from the BLAST analysis were aligned in Geneious Prime using the MUSCLE alignment plugin.

Analysis of melanin pigments

To evaluate the possible production of melanin by the L-DOPA pathway upon the inhibition of the DHN pathway in *Monilinia* spp., eumelanin and pheomelanin polymeric pigments were submitted to an alkaline oxidation as further described. After that, pyrrole-2,3-dicarboxylic acid (PDCA) for eumelanin and thiazole-4,5-dicarboxylic acid (TDCA) for pheomelanin were analysed by UHPLC-MS/MS. To assess the detection and possible production of the DHN pathway-derived compound DHN after inhibition in *Monilinia* spp., an acidic oxidation was performed for further analysis by UHPLC-MS/MS as described below. These studies were performed using four replicates per condition (control and tricyclazole) and species.

Extraction and alkaline oxidation of Monilinia spp. melanin

Samples of *Monilinia* spp. were weighed (200 mg). Then, an alkaline oxidation was carried out using hydrogen peroxide (H_2O_2) (Fisher Scientific, Madrid, Spain) as described by Affenzeller et al. (2019), with some modifications. Briefly, the oxidation reaction of each sample was carried out for 20 h at 25 °C under vigorous stirring using (100 µL) H_2O (Sigma-Aldrich, Darmstadt, Germany), (375 µL) 1 M K_2CO_3 (Sigma-Aldrich, Darmstadt, Germany), and (25 µL) 30% H_2O_2 (Fisher Scientific). The remaining H_2O_2 was decomposed by adding 50 µL of 10% Na_2SO_3 (Sigma-Aldrich, Darmstadt, Germany), and the mixture was acidified with 140 µL 6 M HCl (Sigma-Aldrich, Darmstadt, Germany). Then, the samples were centrifuged (Hettich® MIKRO 200/200R, Sigma-Aldrich, Darmstadt, Germany) at 8000 rpm for 5 min and the supernatant was filtered through a 0.22 µm hydrophilic PTFE filter (Clarify syringe

filters™, LS Scientific limited, Kent, United Kingdom) for UHPLC-MS/MS analysis.

Extraction and acid oxidation of Monilinia spp. melanin

Samples of *Monilinia* spp. were weighed (200 mg). First, an acid oxidation was carried out using 1 mL of methanol (CH_3OH) (Sigma, Schnelldorf, Germany). Secondly, the mixture was shaken vigorously for 10 min. Then, the samples were centrifuged for 12 min at 4 ºC at 10.000 rpm. Finally, the supernatant was recovered and filtered with a 0.22 µm hydrophilic PTFE filter (Clarify syringe filters™, LS Scientific limited, Kent, United Kingdom) for UHPLC-MS/MS analysis.

UHPLC-MS/MS analysis

The melanin standards were pyrrole-2,3-dicarboxylic acid (PDCA) monohydrate (CAS number 1329802–41-6), purchased from Santa Cruz Biotechnology (Dallas, Texas) for eumelanin pigment identification, thiazole-4,5-dicarboxylic acid (TDCA) (CAS number 22358–80-1), purchased from BLD Pharmatech (Kaiserslautern, Germany) for pheomelanin pigment identification and 1,8-dihydroxynaphthalene (1,8-DHN) (CAS number 569–42-6), purchased from Sigma-Aldrich (Darmstadt, Germany) for DHN-melanin pigment identification.

For the identification of eumelanin and pheomelanin pigments (L-DOPA pathway), a solution of the mixture of melanin standards was prepared using water/acetonitrile (98:2) 0.1% formic acid (Fisher Scientific, Madrid, Spain). A seven-point calibration curve was obtained to cover 1.0–175 ng mL^{-1} range with 18 µg mL^{-1} for both standards (PDCA and TDCA). For the identification of 1,8-DHN, a solution of the standard was prepared using water/acetonitrile (98:2) (Fisher Scientific, Madrid, Spain). A five-point calibration curve was obtained to cover the range of 4.5–18 ng mL^{-1} with 10 µg mL^{-1} for the 1,8-DHN standard.

UHPLC-MS/MS analysis was performed on Waters Acquity UPLC binary system coupled to a Xevo TQ-S triple-quadrupole mass spectrometer equipped with an ESI source (Waters, Milford, MA, USA). A Cortecs™ Phenyl, UPLC® 1.6 µm 2.1×150 mm column (Milford, MA, USA) was used for chromatographic separation. For the detection of PDCA (eumelanin) and TCDA (pheomelanin), a water/acetonitrile (98:2) 0.1% formic acid solution was used as the mobile phase A, the acetonitrile was used as the mobile phase B with a gradient elution and a flow rate between 0.25–0.30 mL min^{-1} (Table S1). The sample injection volume (5 µL) was the same for all samples, the column oven temperature was kept at 30 °C and the sample temperature was at 10 °C. The tandem mass spectrometer (MS/MS) was operated in ESI positive mode and data were acquired in the multiple reaction monitoring (MRM) mode (Table S2). The MS/MS parameters were set as follows: capillary voltage 3200 V, source temperature 150 °C, desolvation temperature 400 °C, desolvation gas (nitrogen) flow 800 L h^{-1}, cone gas (nitrogen) flow 150 L h^{-1} and collision gas (argon) flow 0.15 mL min^{-1}.

For the detection of DHN-melanin (1,8-DHN), a methanol solution with 0.1% formic acid was used as mobile phase A, water/methanol (98:2) and 0.1% formic acid was used as mobile phase B with a gradient elution and a flow rate of 0.30 ml min^{-1} (Table S3). The sample injection volume (2.5 µL) was the same for all samples, the column oven temperature was kept at 35 °C and the sample temperature at 10 °C. The tandem mass spectrometer (MS/MS) was operated in ESI negative mode and data were acquired in MRM mode (Table S4). The MS/MS parameters were set as follows: capillary voltage 2500 V, source temperature 150 °C, desolvation temperature 400 °C and desolvation gas (nitrogen) flow 800 L h^{-1}.

Acquired data were processed by MassLynx™ MS Software with TargetLynx™ program version 4.1 (Waters, Milford, MA, USA).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using JMP® 16 (v.16.0.0; SAS Institute Inc., Cary, NC, USA). When the analysis was statistically significant, Tukey's HSD test was performed at the $p \leq 0.05$ level for the comparison of means among species. In the case of the comparison between two conditions and/or two *Monilinia* species, the student's t-test was conducted at the level of $p \leq 0.05$.

Results

Inhibition of the DHN pathway in *Monilinia* spp. using specific inhibitors

Effect of tricyclazole and kojic acid on the phenotype of Monilinia spp.

The colonies of the three *Monilinia* spp. treated with tricyclazole at 50 µg mL^{-1} showed changes in pigmentation while at 150 µg mL^{-1} of kojic acid no phenotypic changes were observed (Fig. 1). In particular, the pigmentation of *M. laxa* changed from white coloration (control) to cream coloration (tricyclazole condition) with the presence of a white growth halo in both conditions. In the case of *M. fructicola*, an olivaceous pigmentation with a white peripheral halo was observed in the control condition, while in the colony grown in tricyclazole, a brownish colour in the central part and a grey coloration in the peripheral halo was observed. Finally, in *M. fructigena* predominated a light olivaceous pigmentation in the control condition, while in the presence of tricyclazole showed a brownish coloration.

Overall, the growth rate data (Table 1) for the conditions tested indicated that there are differences in *Monilinia* spp. Particularly, both *M. laxa* and *M. fructicola* reduced their growth rate in the presence of both tricyclazole and kojic acid compared to the control. However, in *M. fructigena* no significant differences were observed between the tricyclazole and kojic acid conditions compared to the control. Among species, in the tricyclazole condition, a higher growth rate was observed in *M. fructigena* compared to the rest of the species. In the kojic acid condition, *M. laxa* significantly reduced its growth rate compared to the other two *Monilinia* species.

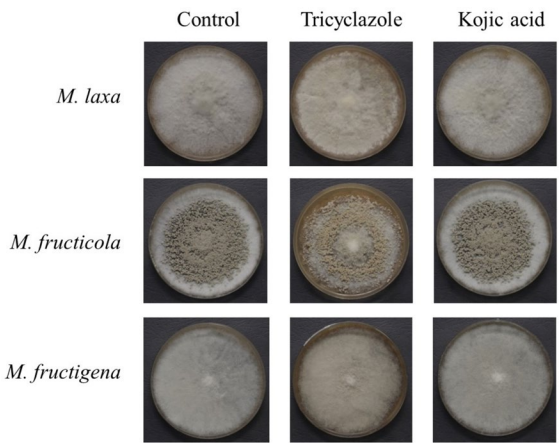

Fig. 1 Colony phenotype of *M. laxa*, *M. fructicola* and *M. fructigena* grown in PDA-T medium without melanin inhibitors (control), with tricyclazole (50 µg mL^{-1}) and with kojic acid (150 µg mL^{-1}) and incubated under darkness for 7 days at 20 °C

Table 1 Growth rate of *M. laxa*, *M. fructicola* and *M. fructigena* grown without the presence of inhibitors (control), with tricyclazole (50 µg mL^{-1}) and with kojic acid (150 µg mL^{-1}) during 7 days at 20 °C under darkness. Data represent the mean of replicates (n = at least 3) ± standard deviation of the means. Asterisks indicate significant differences among species for each condition with a significance level of $p \leq 0.05$. Letters indicate significant differences among conditions for each species

Growth rate	Control	Tricyclazole	Kojic acid
M. laxa	1.59 ± 0.00 A	1.07 ± 0.01 B	0.75 ± 0.02 C *
M. fructicola	1.52 ± 0.01 A	1.12 ± 0.02 B	1.16 ± 0.01 B
M. fructigena	1.18 ± 0.37 A	1.28 ± 0.03 A *	1.22 ± 0.16 A

Effect of tricyclazole on the transcriptional regulation of the DHN pathway in Monilinia spp.

A more detailed study was carried out to verify the inhibition of the DHN pathway using the specific inhibitor, tricyclazole.

On the one hand, the final compound 1,8-DHN was quantified by UHPLC MS/MS, observing a decrease or even a no detection of this compound in the presence of tricyclazole in *Monilinia* spp. (Table 2). In both *M. laxa* and *M. fructicola*, the 1,8-DHN final compound was not detected in the

Table 2 Quantification (ng g^{-1}) of the final compound 1,8-DHN by UHPLC MS/MS analysis grown without the presence of tricyclazole (control) and in the presence of tricyclazole. Data represent the mean of replicates (n = 4) ± standard deviation of the means. Capital letters indicate significant differences between treatment conditions for each species based on Student's t test (p <0.05)

1,8-DHN	Control	Tricyclazole
M. laxa	77.13 ± 2.65 A	ND B
M. fructicola	39.63 ± 5.51 A	ND B
M. fructigena	35.52 ± 5.26 A	10.06 ± 0.36 B

ND (Not Detected): < 3 ng g^{-1} sample

presence of tricyclazole. In the case of *M. fructigena*, the 1,8-DHN was detected in both control and tricyclazole conditions. However, a significant reduction (4.17-fold) of 1,8-DHN was observed in *M. fructigena* in tricyclazole condition compared to the control condition.

On the other hand, the expression of 5 genes (*PKS12*, *YGH1*, *BRN1*, *BRN2* and *SCD1*) located upstream and downstream the inhibition site of tricyclazole in the DHN pathway of *Monilinia* spp. were analysed**.** Upstream the inhibition site of tricyclazole, are located both *PKS12* and *YGH1* genes (Fig. 2). In particular, *PKS12* gene displayed a significant upregulation in the presence of tricyclazole compared to the control, being 3.64-fold, 3.95-fold, and 4.23-fold higher in *M. laxa*, *M. fructicola*, and *M. fructigena*, respectively. However, for *YGH1* gene, significant differences were only observed in *M. laxa*, inducing the inhibitor a higher expression (2.24-fold) compared to the control. Downstream the inhibition site of tricyclazole, are located *BRN1*, *BRN2* and *SCD1* genes (Fig. 2). A higher expression of *BRN1* gene was observed in tricyclazole condition for *M. fructicola* (2.97-fold) and *M. fructigena* (3.01-fold) compared to the control. However, in the case of *M. laxa*, no significant differences were observed between treatments. For the *BRN2* gene, *M. laxa* and *M. fructigena* displayed a significantly higher expression (2.18-fold

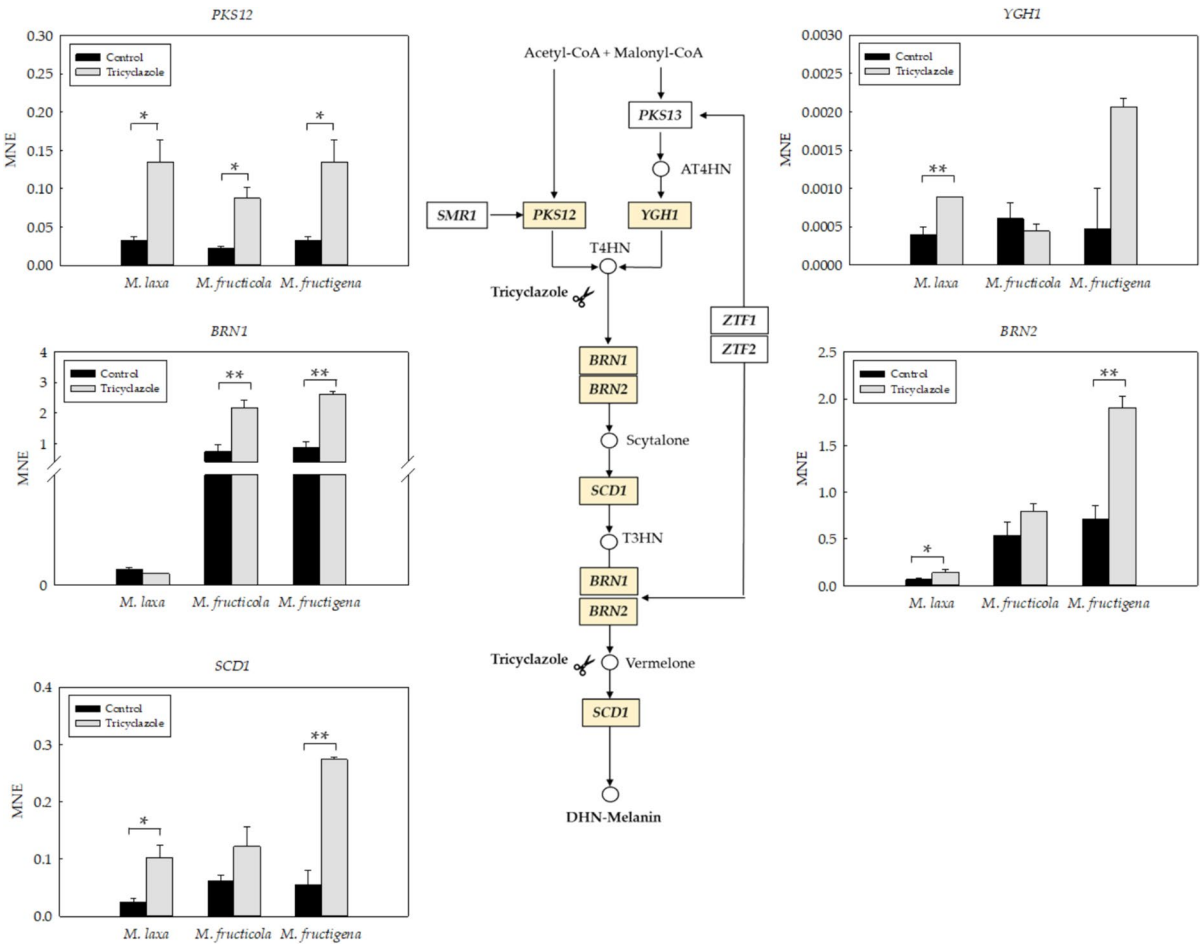

Fig. 2 Mean normalized expression (MNE) of DHN-melanin biosynthetic genes (*PKS12*, *YGH1*, *BRN1*, *BRN2* and *SCD1*) of *M. laxa*, *M. fructicola*, and *M. fructigena* grown for 7 days on PDA-T under darkness without the presence of tricyclazole (■) or with tricyclazole (50 µg mL^{-1}) (□). Error bars represent the standard deviation of the means (n = 3). For each species, asterisks indicate significant differences between treatment conditions based on Student's t test (*$p < 0.05$; **$p < 0.01$). The DHN-melanin pathway is represented, and the candidate genes are indicated in a yellow box

and 2.69-fold, respectively) when grown with tricyclazole. In contrast, in *M. fructicola* no significant differences were observed between treatment conditions for this gene. Finally, similar results to *BRN2* gene were obtained for *SCD1* gene. In both *M. laxa* and *M. fructigena*, the *SCD1* gene expression was 4.11-fold and 5.00-fold higher, respectively, in the presence of tricyclazole than in the control condition. Therefore, these results indicate that the gene expression of the genes located upstream and downstream of the inhibition site were induced in the tricyclazole condition (Fig. 2).

Identification and detection of eumelanin and pheomelanin in the three *Monilinia* spp.

After confirming the inhibitory effect of tricyclazole on the DHN pathway in *Monilinia* spp., the ability of *Monilinia* spp. to produce melanin using the L-DOPA pathway was evaluated.

The BLAST analysis on the genome of *Monilinia* spp. confirmed the existence of the L-DOPA biosynthetic and regulatory gene in the three main *Monilinia* species (Table 3). Overall, a low sequence coverage in the identified candidate gene (two isoforms) was obtained for all three *Monilinia* spp., and greater than 80% of sequence identity, revealing low gene conservation among species (Supplementary Figure S1).

The results obtained from the UHPLC-MS/MS analysis also confirmed the existence of the L-DOPA derived pigments and, in particular, eumelanin and pheomelanin in the three *Monilinia* species grown under the conditions tested in this study. The eumelanin pigment was quantified (Fig. 3a) by detecting DHI (PDCA) units. Among the conditions evaluated, significant differences were observed in both *M. laxa* and *M. fructicola*, highlighting the kojic acid condition as the one that induced the greater eumelanin production. Particularly, *M. laxa* synthesized a higher content of the eumelanin pigment under the kojic acid condition compared to the control (1.87-fold) and tricyclazole (1.93-fold). In the case of *M. fructicola*, the kojic acid condition was higher compared to the control (2.85-fold). However, in *M. fructigena* no differences were observed between the kojic acid condition and the control. In addition, in both *M. fructicola* and *M. fructigena*, the eumelanin pigment was not detected in the presence of the tricyclazole inhibitor. When comparing among species, it was observed that eumelanin production was higher in *M. laxa* compared to the rest of *Monilinia* spp. in all the conditions evaluated.

In the case of the pheomelanin pigment (Fig. 3b), detected through the benzothiazole units (TDCA), a greater production was observed in the kojic acid condition compared to the control in both *M. fructicola* (1.89-fold) and *M. fructigena* (1.69-fold), while not detecting the pheomelanin pigment in the tricyclazole condition. On the other hand, in *M. laxa* a greater production of the pheomelanin pigment was observed in both kojic acid condition (1.27-fold) and tricyclazole (1.25-fold) compared to the control. Therefore, *Monilinia* spp. is able to produce both eumelanin and pheomelanin pigments, both under control conditions and in the presence of the kojic acid. However, in the tricyclazole condition, only *M. laxa* synthetized these pigments.

Role of DHN pathway in the development and survival of *Monilinia* spp.

An analysis of the *in vitro* development and growth of the three *Monilinia* species grown in the presence of

Table 3 Identification of L-DOPA biosynthetic and regulatory genes from *M. laxa*, *M. fructicola*, and *M. fructigena* obtained by BLAST analysis using previously described genes from *B. cinerea* as query sequences. The results for *Monilinia spp.* indicate the ID of the corresponding gene, and the identity and coverage result from the BLAST analysis

Gene	*M. laxa*			*M. fructicola*			*M. fructigena*		
	Gene ID	Identity (%)	Coverage (%)	Gene ID	Identity (%)	Coverage (%)	Gene ID	Identity (%)	Coverage (%)
K00505 tyrosinase BCIN_15g05090	Monilinia__010680	85.71	31.94	MFRU_065g00120.1	87.01	33.23	g2211.t1	84.55	33.23
K00505 tyrosinase BCIN_16g01460	Monilinia__077880	84.03	31.99	MFRU_025g00050.1	85.39	33.25	g5812.t1	85.14	33.25

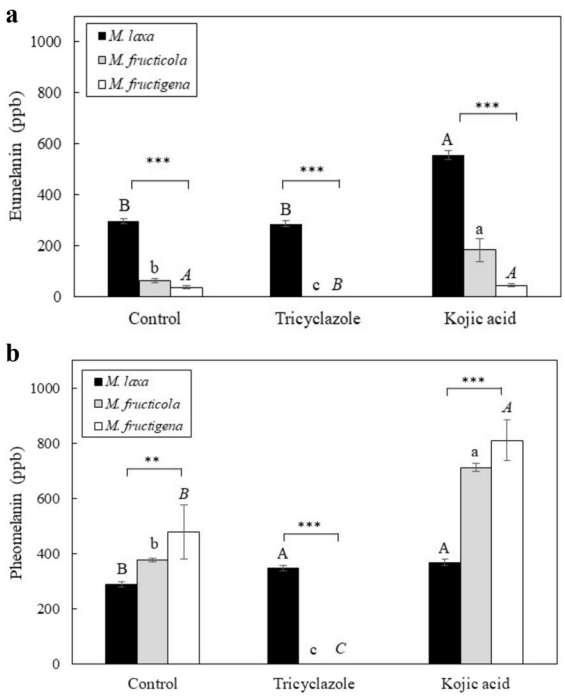

Fig. 3 Identification and quantification of the oxidation products derived from (**a**) the eumelanin pigment (PDCA) and derived from (**b**) pheomelanin pigment (TDCA) by UHPLC-MS/MS analysis of *Monilinia* spp. grown under control conditions, in the presence of tricyclazole (50 µg mL^{-1}) and kojic acid (150 µg $mL.^{-1}$). Error bars represent the standard deviation of the means. Asterisks indicate significant differences among species for each condition based on Tukey HSD test. Different uppercase, lowercase, and italic letters indicate significant differences ($p \leq 0.05$) for *M. laxa*, *M. fructicola*, and *M. fructigena* species, respectively, based on Tukey HSD test. Error bars represent the standard deviation of the means (n = at least 3)

tricyclazole was performed to determine the importance of this melanin biosynthetic pathway on the development of *Monilinia* spp.

Conidiation was only examined quantitatively for *M. laxa* and *M. fructicola* (Fig. 4A) since *M. fructigena* has almost no capacity to produce conidia in the tested conditions. The results between conditions did not show significant differences in any of the species, although it was observed that the production of conidia was higher in *M. fructicola* than in *M. laxa* both in the control (1.40-fold) and in the tricyclazole condition (1.39-fold). Therefore, tricyclazole at a concentration of 50 µg mL^{-1} did not affect conidiation in neither *M. laxa* nor *M. fructicola*.

Regarding cell viability (Fig. 4B), results revealed that *M. laxa* and *M. fructicola*, but not *M. fructigena*, showed significant differences between conditions. Indeed, the viability was higher in *M. laxa* (1.11-fold) and *M. fructicola* (1.06-fold) grown in the medium supplemented with tricyclazole than in the control condition. The comparison among species demonstrated that cell viability was significantly higher in *M. fructicola* (1.13-fold) and *M. fructigena* (1.12-fold) compared to *M. laxa* in the control condition, whereas in the tricyclazole condition it was observed only a significant higher viability in *M. fructicola* (1.07-fold) compared to *M. laxa*.

Concerning germination (Fig. 4C), a significant effect of tricyclazole was only observed in *M. laxa*. Specifically, the tricyclazole inhibitor reduced significantly (1.05-fold) the germination capacity of *M. laxa* compared to the control condition. In this case, the germination percentage was significantly higher in *M. fructicola* than in *M. laxa* both in the control (1.04-fold) and with the tricyclazole inhibitor (1.09-fold).

Discussion

Currently, several investigations have demonstrated that melanin is involved in the development and survival of *Monilinia* spp. (De Cal & Melgarejo, 1994; Verde-Yáñez et al., 2022). In addition, the transcriptional regulation of the DHN pathway has been described in the three main *Monilinia* species under different conditions (Verde-Yáñez et al., 2023a, b). The information regarding the types of pigment synthesized by fungi is limited. So far, only the most predominant pigments, eumelanin and pheomelanin (L-DOPA) have been identified in fungi such as *Cryptococcus neoformans* and *A. niger*, *A. terreus* and *A. fumigatus* (Bayram, 2022; Liu et al., 2022). However, in *Monilinia* species the information is still scarce since to date, any type of melanin pigments have been identified.

Previous studies in other fungi have described the use of different melanin biosynthetic pathways, leading to the presence of distinct melanin types, with ascomycetes mainly relying on the DHN pathway and basidiomycetes typically using the L-DOPA pathway (Butler et al., 2009; Schumacher, 2016; Bayram, 2022; Jia et al., 2025). However, it has been demonstrated that there are ascomycetes that can use both

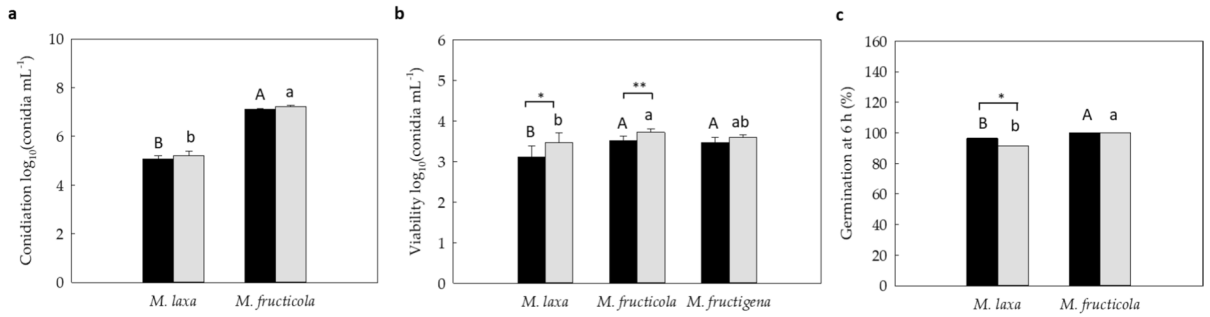

Fig. 4 Conidiation data (**A**) of *M. laxa* and M. *fructicola*, cell viability (**B**) of *M. laxa*, *M. fructicola* and *M. fructigena*, and germination (**C**) of *M. laxa* and *M. fructicola* grown in PDA-T without the presence of tricyclazole (■) or with tricyclazole (50 µg mL^{-1}) (□) and incubated for 7 days under darkness. Error bars represent the standard deviation of the means. Asterisks denote significant differences between treatment conditions for each species ($*p<0.05$; $**p<0.01$) based on Student's t test. Different uppercase and lowercase letters indicate significant differences ($p \leq 0.05$) for the control and tricyclazole condition, respectively, among species, based on Tukey HSD test for viability data, and on Student's t test for conidiation and germination

DHN and L-DOPA pathways to synthesize melanin (Pacelli et al., 2020; Pal et al., 2014). The presence of specific melanin pigments has been determined, for years, by using specific inhibitors of both pathways. Therefore, considering the existence of the DHN pathway in *Monilinia* spp. (De Miccolis Angelini et al., 2022; Verde-Yáñez et al., 2023a), and the possible existence of other biosynthetic pathways (Eisenman & Casadevall, 2012) such as the L-DOPA pathway (Pal et al., 2014), in this study it was confirmed the presence of the L-DOPA pathway by identifying the presence of the biosynthetic genes, as well as through the identification of its derived pigments (eumelanin and pheomelanin). In addition, the detection of this pathway in *Monilinia* spp. was determined using specific inhibitors of the DHN and L-DOPA pathways (tricyclazole and kojic acid, respectively). Finally, the possible role of L-DOPA in the ecophysiology of *Monilinia* was determined, demonstrating its importance in parameters such as conidiation and germination of conidia, independently of the availability of the DHN pathway.

According to previous studies performed in *B. cinerea* by Schumacher (2016), it was demonstrated that tricyclazole inhibits two reduction steps (T4HN to scytalone, T3HN to vermelone) of the DHN pathway, and thus induces the accumulation of T4HN and T3HN and their autooxidation products, flaviolin and 2-hydroxyjuglone (2-HJ), giving an orange coloration. In this line, we also observed that the pigmentation of *Monilinia* spp. colonies (Fig. 1), in the presence of 50 µg mL^{-1} tricyclazole changed, being, in general, slightly brownish-orange compared to the control (Supplementary Figure S2). Similar results were obtained in a study carried out with *M. laxa*, in which an orange coloration was observed in the colony grown with tricyclazole (1 µg mL^{-1}) (De Cal & Melgarejo, 1994). In the same line, the ascomycete fungus *E. mesophila* showed colony changes ranging from black to reddish-brown when using the inhibitor tricyclazole at a concentration of 50 µg mL^{-1} (Medina-Armijo et al., 2024). However, in *B. cinerea* were required concentrations of 10 µg mL^{-1} and 50 µg mL^{-1} of tricyclazole to observe a change on the pigmentation of the conidia and sclerotia, respectively (Schumacher, 2016). These differences between studies could be due to the media used, the growth conditions, the compounds in which the inhibitor was diluted and even the strains of *Monilinia* spp. used for each study. According to other species of fungi, our results are in line with what occurred in *B. cinerea*, since a high concentration of tricyclazole (50 µg mL^{-1}) was necessary to observe changes in the pigmentation of the *Monilinia* spp. colonies. On the other hand, the use of one of the specific inhibitors of the L-DOPA pathway, kojic acid, did not produce any coloration changes in the colonies of *Monilinia* spp. at a concentration of 150 µg mL^{-1}. Similar results were observed in *Aspergillus fumigatus*, *A. nidulans*, *A. niger* and *A. terreus* grown in a medium containing

kojic acid at a concentration of 100 µg mL^{-1} (Suwannarach et al., 2019). However, the species *A. tamarii* and *A. flavus* showed phenotypic changes at a concentration of 100 µg mL^{-1} of kojic acid, demonstrating the genus and species dependent effect. Only in the species *A. niger* a higher concentration of kojic acid (5 mg mL^{-1}) was required to observe phenotypic changes (Pal et al., 2014). Therefore, it is possible that for *M. laxa*, *M. fructicola* and *M. fructigena* a higher concentration should be considered to observe a coloration change as occurred with *A. niger*. Probably, this species could not be able to synthesize kojic acid by itself like *Monilinia* spp., which could explain the need for a higher kojic acid concentration to observe the pigmentation changes.

In previous studies, it has been observed that, in general, the melanin pigment is not essential for growth and development in fungi (Medina-Armijo et al., 2024). However, in more recent studies, it has been demonstrated that fungal melanin does not appear to be essential for growth, but is required for certain development processes of ascomycetes and basidiomycetes fungi (Medina-Armijo et al., 2024; Jia et al., 2025). In this study we analysed the growth of *Monilinia* spp. to evaluate if the presence of tricyclazole and kojic acid in the media had an effect on the growth rate of the three species evaluated. The data obtained from the growth rate (Table 1) indicated that the growth was reduced in the presence of tricyclazole and kojic acid compared to the control only in *M. laxa* and *M. fructicola*. In the same line, De Cal and Melgarejo (1994) observed a significant reduction in the growth of *M. laxa* after 7 days when this species was grown in PDA containing tricyclazole at a concentration of 10 µg mL^{-1}, a lower concentration than that used in our study. However, Villarino et al. (2011) observed that the growth of *M. laxa* in PDA with pyroquilon (another melanin inhibitor of DHN pathway) at a concentration of 10 µg mL^{-1} was not affected. Therefore, *Monilinia* species could be dependent on different specific inhibitors and their concentrations, both for the DHN and the L-DOPA pathway, thus observing different effects on the synthesis of melanin pigment. In fact, it has been demonstrated that the use of tricyclazole at low doses can elevate ROS levels within cells, potentially affecting the integrity of several metabolic pathways (Medina-Armijo et al., 2024). In a recent study, we evaluated the transcriptional regulation of the DHN pathway in *Monilinia* spp. (Verde-Yáñez et al., 2023a). In this sense, it is important to note that herein, we observed that the different *Monilinia* species displayed distinct transcriptional patterns in the presence or absence of the inhibitor tricyclazole. These differences could, in part, be attributed to the findings of a previous study, in which we observed that both the biosynthetic and regulatory genes of *M. fructigena* were differentially clustered in the genome compared to those of *M. laxa* and *M. fructicola* (Verde-Yáñez et al., 2023a). Hence, this phenomenon raises the possibility that *M. fructigena* may employ different regulatory mechanisms or compensatory pathways compared to the other species. However, to date there are few studies that quantify the DHN-melanin pigment in fungi, which would help to understand the functionality and importance of this pigment in the secondary metabolism of the fungus. Herein, the results obtained from the quantification by UHPLC MS/MS of the final compound 1,8-DHN, allowed us to confirm that the production of DHN-melanin was reduced or even not detected when the three species of *Monilinia* were grown in a medium containing tricyclazole (Table 2). Therefore, and in our tested conditions, these results confirm the effect of tricyclazole as an inhibitor of the DHN-melanin on the three *Monilinia* species. The differences observed between *M. fructicola* and *M. laxa* (in which 1,8-DHN was not detected) and *M. fructigena* (in which the compound was still detectable, although with a 4.17-fold reduction) could be explained by several factors: (i) species-specific differences in inhibitor sensitivity; (ii) limited compound permeability under the conditions used (since *M. fructigena* is unable to sporulate as *M. laxa* and *M. fructicola* do); or (iii) putatively distinct regulatory mechanisms of the DHN-melanin biosynthetic pathway among *Monilinia* spp. (Verde-Yáñez et al., 2023a). Such differences in inhibitor response have already been demonstrated for other inhibitors in *Aspergillus* spp. (Pal et al., 2014).

In the same line as the results presented herein, Schumacher (2016) also confirmed the effect of tricyclazole and the importance of the DHN-melanin compound during the different stages of the cycle in *B. cinerea*. On the other hand, the enzymatic regulation of this pathway has also been demonstrated in other organisms such as *B. cinerea*, *A. fumigatus* and *Penicillium marneffei* (Bell & Wheeler, 1986; Tsai et al., 1999; Woo et al., 2010). Herein, we demonstrated how the presence of tricyclazole changed the

expression pattern of DHN-related genes (Fig. 2). Tricyclazole blocks the substrate binding sites of THNR enzymes, and thus, *Monilinia* spp. induced the expression of all the genes analysed, probably aiming to compensate the lack of enzyme activity.

Once the inhibition of the DHN pathway was confirmed, we studied the use of the L-DOPA pathway in *Monilinia* spp., through gene identification and detection of eumelanin and pheomelanin pigments, both products of the L-DOPA pathway.

The results of BLAST and UHPLC-MS/MS analysis demonstrated, for the first time, that *Monilinia* spp. are able to synthesize melanin through the L-DOPA pathway and producing the eumelanin and pheomelanin pigments. The BLAST analysis (Table 3) allowed us to identify two isoforms of the K00505 tyrosinase gene, which is related to the conversion of tyrosine into L-DOPA that occurs at the beginning of the pathway as demonstrated in *B. cinerea* (Smith and Casadevall, 2019) and *Scleroderma yunnanense* (Zhang et al., 2023).

Regarding the study on the quantification of pigments derived from the L-DOPA pathway, significant differences were observed among species and conditions (Fig. 3), which demonstrates that both pathways, DHN and L-DOPA, could be active at the same time. In detail, it was observed that in the three species of *Monilinia*, the production of eumelanin and pheomelanin in the presence of the specific inhibitor of the L-DOPA pathway (kojic acid) was the same or greater compared to the control, which would indicate that this compound is not working as an inhibitor in the three *Monilinia* species (at least at the concentrations tested in this study) and, therefore, does not block the production of both pigments. In addition, it was also observed that the production of L-DOPA derived pigments (eumelanin and pheomelanin) was reduced or not detected once the DHN pathway was blocked by tricyclazole in *M. fructicola* and *M. fructigena*. This could probably be due to the inhibition of the L-DOPA pathway caused by the inhibitor tricyclazole, affecting the synthesis of these pigments in *Monilinia*. In this line, it has demonstrated that the inhibitor tricyclazole can affect other metabolic pathways due to the levels of ROS it generates (Medina-Armijo et al., 2024).

In a study carried out by Chen et al. (2014), it was observed that the fungus *Wangiella dermatitidis* was able to use the two biosynthetic pathways, DHN and L-DOPA, when exposed to different pH and radiation conditions. This ability to produce melanin using both pathways has also been demonstrated in the fungus *Cryomyces antarticus*, which synthesizes melanin both through the DHN pathway and the L-DOPA pathway (Pacelli et al., 2020). In turn, in the study carried out by Pal et al. (2014), it was determined that the pathway (DHN or L-DOPA) used by *Aspergillus* was dependent on the species. Hence, *A. niger*, *A. tamarii* and *A. flavus* synthesize melanin through the L-DOPA pathway, while *A. terreus* and *A. tubingensis* produce melanin through the DHN pathway. Taken together, this preliminary study suggests that the L-DOPA pathway is also activated in *Monilinia*. However, further studies, including enzymatic analyses, are required to confirm this hypothesis, to determine whether the regulation of both melanin biosynthetic pathways is interdependent, and to establish whether the L-DOPA pathway is constitutively activated or only under specific conditions (Chhoker et al., 2025).

The developmental parameters of *Monilinia* spp. analysed, allowed us to demonstrate, through the inhibition of the DHN pathway caused by tricyclazole, that the DHN pathway was not essential for certain development parameters such as conidiation (Fig. 4A) in *M. laxa* and *M. fructicola*. Hence, the lack of DHN-melanin synthesis could be compensated by the activation of the L-DOPA pathway. However, it is still unknown if these parameters could be dependent on other biosynthetic pathways, such as L-DOPA. In contrast with our results, in *Magnaporthe grisea*, it was observed an inhibition of conidiation greater than 95% when tricyclazole was applied (5 μg mL^{-1}) (Kunova et al., 2013). Hence, it could point out that either melanin is not required for such process in *Monilinia* spp. or that L-DOPA-derived melanin (i.e., eumelanin or pheomelanin) is enough to maintain the conidiation capacity of both *M. laxa* and *M. fructicola*, although further studies are needed to support these hypotheses. On the other hand, viability data indicated significant differences in both *M. laxa* and *M. fructicola* while no significant differences were observed in *M. fructigena* in the presence of tricyclazole (Fig. 4B). In the case of *M. fructigena*, the absence of effect of tricyclazole could be due, in part, due to its greater proportion of mycelial structures and a low percentage of conidia, which are the structures where the melanin pigment could predominate.

Finally, the germination results (Fig. 4C) only showed significant differences in *M. laxa*, with fewer germinated conidia in the presence of tricyclazole compared to the control. In fact, in a study carried out with *M. oryzae*, it was observed that there is no correlation between the increase in tricyclazole concentration and germination (Kunova et al., 2013). On the other hand, the inhibition of melanin biosynthesis in the fungus *Pestalotiopsis microspore* by tricyclazole, showed little effect on growth but caused great alterations in the production, morphogenesis, and germination of conidia (Yu et al., 2015), probably because this fungus has not the ability to synthetize melanin by other pathways different to the DHN pathway, as does occur in *Monilinia* spp.

The contribution of L-DOPA–derived pigments to fungal development was small but significant. These findings suggest a supportive rather than essential role, and further studies are needed to determine whether this association reflects a direct causal effect.

All these results contribute to improve the knowledge about, for the time being, unknown role of melanin in *Monilinia* spp. and demonstrate the effect caused by tricyclazole and the presence of the L-DOPA pathway (and thus the production of eumelanin and pheomelanin pigments) on the development and survival of *Monilinia* spp. The evidence obtained so far suggests a potential activation of the L-DOPA pathway; however, further molecular and enzymatic analyses are required to confirm its functional activity and to elucidate the full role of melanin in the adaptation of this fungus to diverse environmental conditions.

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Data Availability Data will be made available upon request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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