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Morphological and molecular aspects of sclerotial development in the phytopathogenic fungus *Sclerotinia sclerotiorum*

Bruna Sousa Melo[a], Aline Raquel Voltan[a], Walquiria Arruda[a], Fabyano Alvares Cardoso Lopes[b], Raphaela Castro Georg[a], Cirano José Ulhoa[a,*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Goiás (ICB II), Campus Samambaia, Instituto de Ciências Biológicas, CEP 74001-970, Goiânia, GO, Brasil
[b] Laboratório de Microbiologia, Universidade Federal do Tocantins, 77500-000, Porto Nacional, TO, Brasil

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary produces a resistance structure called sclerotium, which guarantees its survival in soil for long periods. Morphological and melanization aspects during sclerotial development were evaluated by microscopy and qRT-PCR techniques. *S. sclerotiorum* produces sclerotia with different phases of maturation and melanization during growth in PDA medium. Using scanning electron microscopy we observed that there are no structural differences in the three stages of formation of melanized and non-melanized sclerotium. Through histochemical analysis we observed that the melanized sclerotium accumulates more glycogen and produces less protein than non-melanized sclerotia. Melanin was most commonly found in the rind of melanized sclerotia, and the highest concentration of lipofucsins was found in non-melanized sclerotia. These molecules are products of the lipid peroxidation pathway and are associated with oxidative stress during differentiation in fungi. The expression of histidine kinase (*shk*) and adenylate cyclase (*sac*) genes in melanized and non-melanized sclerotiawere also evaluated. The higher gene expression of *shk* and lesser expression of *sac* in non-melanized sclerotiais an indication of the participation of cell signaling in the development of these structures. The higher expression of polyketide synthase (*pks)*, tyrosinase (*tyr*) and laccase (*lac*) in non-melanized sclerotia suggested that *S. sclerotiorum* can use the DHN and L-dopa pathways to produce melanin. Expression studies of the enzymes chitin synthase and glucan synthase suggest that this process occurs along with the formation of melanin. This is interesting since polysaccharides, such as chitin and β-1,3-glucan, serve as a scaffold to which the melanin granules are cross-linked.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic and a plant pathogenic fungus that infects over 400 species of plants, including many important crops worldwide (Bolton et al., 2006; Willbur et al., 2018). In Brazil *S. sclerotiorum* was first reported in 1921 in São Paulo, in a potato crop, and since then has been described in soybean, beans, cotton, tomato, sunflower, and crotalaria, among other species of vegetables . White mold caused by this fungus interferes with the production of these crops, especially in Cerrado regions and in winter seasons in irrigated areas. This disease can be identified by the observation of mycelia accompanied by sclerotia (Smolinska and Kowalska, 2018). Sclerotium is a pigmented, multihyphal structure composed of three layers: a thick wall covered with melanin, a thin wall known as the cortex, and the white medulla. Three stages of sclerotial development have been characterized: (i) initiation - aggregation of hyphae to form a white mass called sclerotial initials, (ii) development - hyphal growth and further aggregation to increase size, and (iii) maturation - melanin deposition in peripheral rind cells, and internal matrix consolidation (Townsend and Willetts, 1953; Ordóñez-Valencia et al., 2014). The survival of sclerotia in the soil makes it difficult to handle white mold, since these resistance structures remain viable for several years, even in adverse conditions (Fernando et al., 2004). Sclerotia can germinate in two ways: carpogenically, to form apothecia from which ascospores are liberated, or myceliogenically to produce hyphae that can directly attack young plant tissues through the production of oxalic acid (Bolton et al., 2006).Thus, the understanding of the molecular mechanisms involved in the formation of sclerotia in *S. sclerotiorum* may identify new potential strategies for the control of disease caused by this fungus.

* Corresponding author.
E-mail address: ulhoa@ufg.br (C.J. Ulhoa).

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There are several factors that affect the formation and development of sclerotia, including many environmental changes, primary metabolism and secondary messengers such as cyclic AMP (Erental et al., 2008). The deletion of the adenylate cyclase gene (*sac1*) in *S. sclerotiorum* affected sclerotial development and pathogenicity, as reported by Jurick and Rollins (2007). Among other proteins involved in the development of sclerotia, one can cite ERK-like MAPK (Chen et al., 2004), Ser/Thr phosphatase (Kim et al., 2005) and the histidine kinase Shk1 (Duan et al., 2013). Although there is much information about sclerotial development, little is known about the mechanisms of melanogenesis in *S. sclerotiorum*. Melanin confers resistance to sclerotia when in the presence of unfavorable climate and soil conditions, and plays a very important role in sclerotium survival (Tourneau, 1979). Melanin is an amorphous polymer that is synthesized in fungi by two synthetic pathways, using 1,8-dihydroxynaphthalene (DHN) or L-3,4-dihydroxyphenylalanine (L-DOPA) as substrates (Eisenman and Casadevall, 2012). DHN is synthesized through the pentaketide pathway, which has been identified previously in *S. sclerotiorum* (Butler et al., 2009; Liang et al., 2017).

The production of sclerotia can be easily obtained after growth in culture media normally used for the maintenance of *S. sclerotiorum*. We observed that during the course of culture we can find sclerotia in different stages of development and melanization. In the present study, we investigated the morphological development of melanized and non-melanized sclerotia of *S. sclerotiorum* collected from potato dextrose agar (PDA) medium. Light and scanning electron microscopy were used to evaluate morphological aspects of sclerotium development. In addition, real-time PCR was used to evaluate the expression of genes involved in cell signaling, melanization and cell wall synthesis in melanized and non-melanized sclerotia of *S. sclerotiorum*.

2. Material and methods

2.1. Fungal culture and growth conditions

The strain of *S. sclerotiorum* used throughout this study was isolated from bean plants (*Phaseoulus vulgaris*), and obtained from the Brazilian Agricultural Research Corporation (EMBRAPA rice and beans) collection. This isolate was cultured routinely on PDA (Difco Laboratories, Detroit) at 28 °C and stored at 4 °C. To produce sclerotia, a single mycelium-agar plug was inoculated on the edge of 9 cm Petri dish containing PDA, and incubated at 28 °C. Sclerotia were normally observed as fluffy white hyphae after 3 days of growth. Non-melanized and mature melanized sclerotia were available after 5 and 7 days of growth, respectively. The sclerotia formed were harvested using a sterile forceps and stored at −20 °C for further study.

2.2. Scanning electron microscopy (SEM)

Melanized and non-melanized sclerotia were fixed overnight at 4 °C in a solution containing 2% (v/v) glutaraldehyde, and 2% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer at pH 7.2. After fixation, the samples were rinsed in the same buffer and dehydrated in a series of ascending ethanol concentrations (30–100%) (v/v), dried at the critical point in CO_2 (Autosamdri, 815, Series A) and coated with gold in a sputter-coater (Denton Vacuum, Desk V). The material was observed with a Jeol, JSM 6610 scanning electron microscope at the High Resolution Multiuser Laboratory of the Federal University of Goiás (LabMic-UFG).

2.3. Histological procedures

Melanized and non-melanized sclerotia were fixed overnight at 4 °C in 2% (v/v) glutaraldehyde, and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 7.2. Subsequently, the samples were rinsed in the same buffer, dehydrated in a series of 30–100% ethanol solutions, and embedded in historesin (HistoResin Embedding Kit®, Leica Germany). Sections of 3 µm thickness were cut on a microtome (RM224, Leica Biosystems Germany), and stained with Periodic Acid-Schiff reaction (PAS), Ponceau Xylidine and Nile Blue, for polysaccharides with 1:2 glycol groups (including glycogen), proteins, and melanin detection, respectively. The sections were observed with a Zeiss Axio Scope.A1 light microscope (Zeiss Germany) and photographed with AxioCam MRC (Zeiss Germany).

Table 1
Sequences of oligonucleotides used for analysis of gene expression in real-time PCR.

Name	Sequence (5′ → 3′)
Actin	F-CCCCAGCGTTCTACGTCT
	R-CATGTCAACACGAGCAATG
Polyketide synthase (*pks*)	F-TGCTGCCTTAGGTGACACAG
	R-AAACCCGGCTGGAATAGACT
Laccase (*lac*)	F-GGGGAATCAACTGATGCTGT
	R-CGATACACCTGTCGTTGTCG
Tyrosinase (*tyr*)	F-CTACATTCCCCACAGCCACT
	R-ATCAAGGGCTGTCCAACAAC
Chitin synthase 1 (*chs1*)	F-AGATGCGCCATATGGAAGAC
	R-CACAGATGGCTGAGGCTGTA
Chitin synthase 2 (*chs2*)	F-TGTTGCAGGTCAGAACTTCG
	R-TCCCATGATAGCTCGGAATC
Chitin synthase 3 (*chs3*)	F-ATGTATCTGGCGGAAGATCG
	R-ACTTACCCGGCACATCAGTC
Glucan synthase (*gsh*)	F-ATCCCGAAGTCGTTCAAATG
	R-TCGGTGTTTTCCATCTCCTC
Histidine kinase (*shk*)	F-CGACGGTACATGGAAGGAGT
	R-CTTGGTCGGTGAGGTTTTGT
Adenylate cyclase (*sac*)	F-CACCTCCCTGGACATCATCT
	R-CCACCCTTCGTTGTTTGACT

2.4. Real time PCR (RT-qPCR)

RNA was isolated from melanized and non-melanized sclerotia by grinding in a mortar and pestle under liquid nitrogen, followed by extraction using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was treated with DNase I (Invitrogen). RT-qPCR was used to evaluate the expression of genes potentially involved in sclerotial development and melanization (Table 1). The actin (HS574101) transcript was used as an internal reference to normalize the amount of total RNA present in each reaction. Primers used in real-time qPCR were designed using the Perl-Primer v1.1.20 software. The nucleotide sequences of the primers used in this work are presented in Table 1. RNA (5 µg) from each pooled sample was reverse transcribed into cDNA with the oligo(dT) primer in a volume of 20 µl, using the Maxima™ First Strand cDNA synthesis kit for RT-qPCR (Fermentas). The synthesized cDNA was diluted with 80 µl of water, and used as a template for real-time PCR. Reactions were performed in the iQ5 real-time PCR system (Bio-Rad). Each reaction (20 µl) contained 10 µl of MAXIMA® SYBR-green PCR Master mix (Fermentas), forward and reverse primers (500 nM each), cDNA template, and nuclease-free water. PCR cycling conditions were 10 min at 95 °C (1 cycle), 15 s at 95 °C followed by 1 min at 60 °C (40 cycles), and a melting curve of 1 min at 95 °C followed by 30 s at 55 °C, and a final ramp to 95 °C with continuous data collection (1 cycle) to test for primer dimers and non-specific amplification.

Quantification of gene expression was calculated according to the Livak method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). To compare the relative expression levels of the genes analyzed in the experimental conditions with the conditions used as control, a simple variance analysis (ANOVA) was performed, using the program GraphPad Prism version 7.00. The significance level considered was 5%.

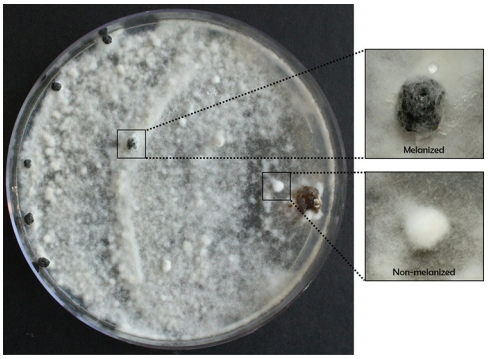

Fig. 1. Photograph showing melanized and non-melanized sclerotia after 9 days of growth of *S. sclerotiorum* on PDA medium.

3. Results and discussion

3.1. Morphological development of sclerotia from S. Sclerotiorum

S. sclerotiorum represents a good model system for studying the mechanisms involved in sclerotial development in many species of fungi. *S. sclerotiorum* produces multiple large sclerotia in laboratory and field conditions. Also, its full genome is available and genetic transformation methods are well established for this organism (Smith et al., 2014). Sclerotium is a hyphal aggregate, with an outer black rind containing melanin, a compound that plays an important role in many fungi. During maintenance of *S. sclerotiorum* in PDA medium, it was observed that in the course of culture, some mature sclerotia become melanized while others take more time to mature (Fig. 1). This is interesting because one can obtain sclerotia at different stages of maturation. We use this model as an alternative method to study the development and synthesis of melanin in sclerotia of *S. sclerotiorum*.

The colonies of *S. sclerotiorum* were fast-growing with white, floccose, aerial mycelia and completely occupied the plate after 6 days of growth. During growth, hyphal aggregation occurred at the middle and at the edge of Petri dishes (Fig. 1). Most sclerotia emerged from these regions, and after 9–10 days some of then turned grayish until they attained a hard black structure (Fig. 1). The fungus produced 15 to 20 sclerotia per PDA Petri plate. It is known that many environmental and nutritional factors can influence the formation of sclerotia, such as a decrease in nutrients, and alteration in the pH of the medium (Erental et al., 2008).

In the present study, we used scanning electron microscopy (SEM) to observe the sclerotial development in these three stages (Fig. 2). The initiation stage was visualized after 5 days of fungal growth when a cluster of white hyphae were observed on the surface of the medium (Fig. 2A–C). A previous study showed that the formation of hyphal clusters is preceded by dichotomous branching of hyphal tips (Ordóñez-Valencia et al., 2014). We also observed the secretion of a mucilage-like substance, which may function as a hyphal adhesive (Fig. 2C) as reported by Erental et al. (2008). However, the characterization of these substances has not yet been carried out. The developmental stage occurred after 6 days of growth when the hyphae coalesced and formed a slightly rounded and compacted structure containing the mucilage-like substance (Fig. 3D–F). Liquid droplets were also observed at the sclerotial surface after this phase, and this is described as a common feature during sclerotial development in *S. sclerotiorum* (Willetts and Wong, 1980). A proteome study of these liquid droplets identified proteins involved in amino acid, carbohydrate and lipid metabolism (Liang et al., 2010). At the maturation stage a boundary surface was observed with increased sclerotial size and deposits of a mucilage-like substance (Fig. 2G–I). At this stage the sclerotial structures were surrounded by hyphae and this is when formation and deposition of melanin in the rind walls begins. After 9 days of fungal growth, the presence of non-melanized (2J-L) and melanized (Fig. 2M–O) sclerotia was observed on the surface of the medium. The mature melanized sclerotia had dark pigmentation with a round shape, rough texture and swelling cells (Fig. 2M–O).

3.2. Histochemical studies of sclerotia from S. Sclerotiorum

The sclerotium is a pigmented, multihyphal structure composed of three layers: an outer rind, the cortex and the medulla (Townsend and Willetts, 1953). The rind is the outer-most layer, composed of thickened, pigmented, parenchyma-like cells. The outer rind pigmentation is due to deposition of melanin in the cell wall. The cortex layer is positioned to the interior of the rind, and this dense tissue layer separates the rind from the medulla. The medulla consists of an aggregation of hyphal tissue embedded within an extracellular matrix. Both the cortex and medulla accumulate nutrient reserves such as glycogen, protein, polyphosphate and lipids (Bullock et al., 1980; Bullock and Willetts, 1996).

With the aim of evaluating the distribution of polysaccharides, proteins and melanin in melanized and non-melanized sclerotia from *S. sclerotiorum*, thin sections of these structures were stained with Periodic Acid-Schiff (polysaccharides), Ponceau xylidine (proteins) and Nile blue (melanin and lipofucsin). In all samples we observed a brown pigmentation in the outer rind, only in the melanized sclerotium, suggesting that this represents melanin (Fig. 3).

The Periodic Acid-Schiff dye (PAS) is specific for polysaccharides, mainly glycogen, an important source of energy in many fungi. Through the histochemical reaction of PAS it was possible to observe the presence of glycogen inside the hyphae of melanized and non-melanized sclerotia (Fig. 3A–B). However, the amount of glycogen in mature melanized sclerotia was much higher than in non-melanized structures. In mature melanized sclerotia, the glycogen content in rind cells is lower than in cortex and medulla cells (Fig. 3A). Similar results have previously been described in the literature, in which the authors showed a reduction in glycogen content during rind differentiation (Bullock et al., 1980). The accumulation of energy in the form of glycogen, in cortex and medulla cells, will be important for the germination and formation of apothecia or mycelia (Bolton et al., 2006).

The histochemical reaction of Ponceau xylidine showed the presence of protein inside the hyphae of mature melanized and non-melanized sclerotia (Fig. 3C–D). We observed that the amount of protein in mature melanized sclerotia is much lower than in non-melanized. Bullock et al. (1980) previously showed that the number, size and distribution of protein-containing structures changed markedly during sclerotial maturation. This is due to the fact that the non-melanized sclerotium is a stage characterized by intense protein synthesis in order to reach the stage of maturation and formation of melanin in the rind.

The histochemical reaction with Nile blue showed the presence of a brown stain in the rind of melanized sclerotia, suggesting the presence of melanin (Fig. 3E). We also observed a more intense blue staining in the hyphae of the cortex and medulla of the non-melanized sclerotia (3 F). However, Nile blue is not specific for melanin and may also stain lipofuscins in histological sections of fungi. Lipofucsins are formed by lipid peroxidation products which react with amino groups of amines, amino acids, proteins, nucleic acids and phospholipids. These molecules are associated with the mechanism of sclerotial biogenesis in fungi such as *Sclerotium rolfsii*, *Rhizoctonia solani*, *Sclerotium minor S. sclerotiorum, S. trifoliorum and S.cepivorum* (Georgiou and Zees, 2001; Arseniuk and Macewicks, 1994; Backhouse and Stewart, 1987). These authors showed that a significant increase occurs in the production of lipofuscins during the formation of sclerotia in *S. sclerotiorum*. We observed that in mature melanized sclerotia, the blue coloration is less intense than in non-melanized structures (Fig. 3E), probably due to the degradation of the lipofucsins as shown previously (Georgiou and Zees, 2001). Our data are consistent with these hypotheses relating oxidative stress to sclerotial differentiation in filamentous fungi (Geourgiou,

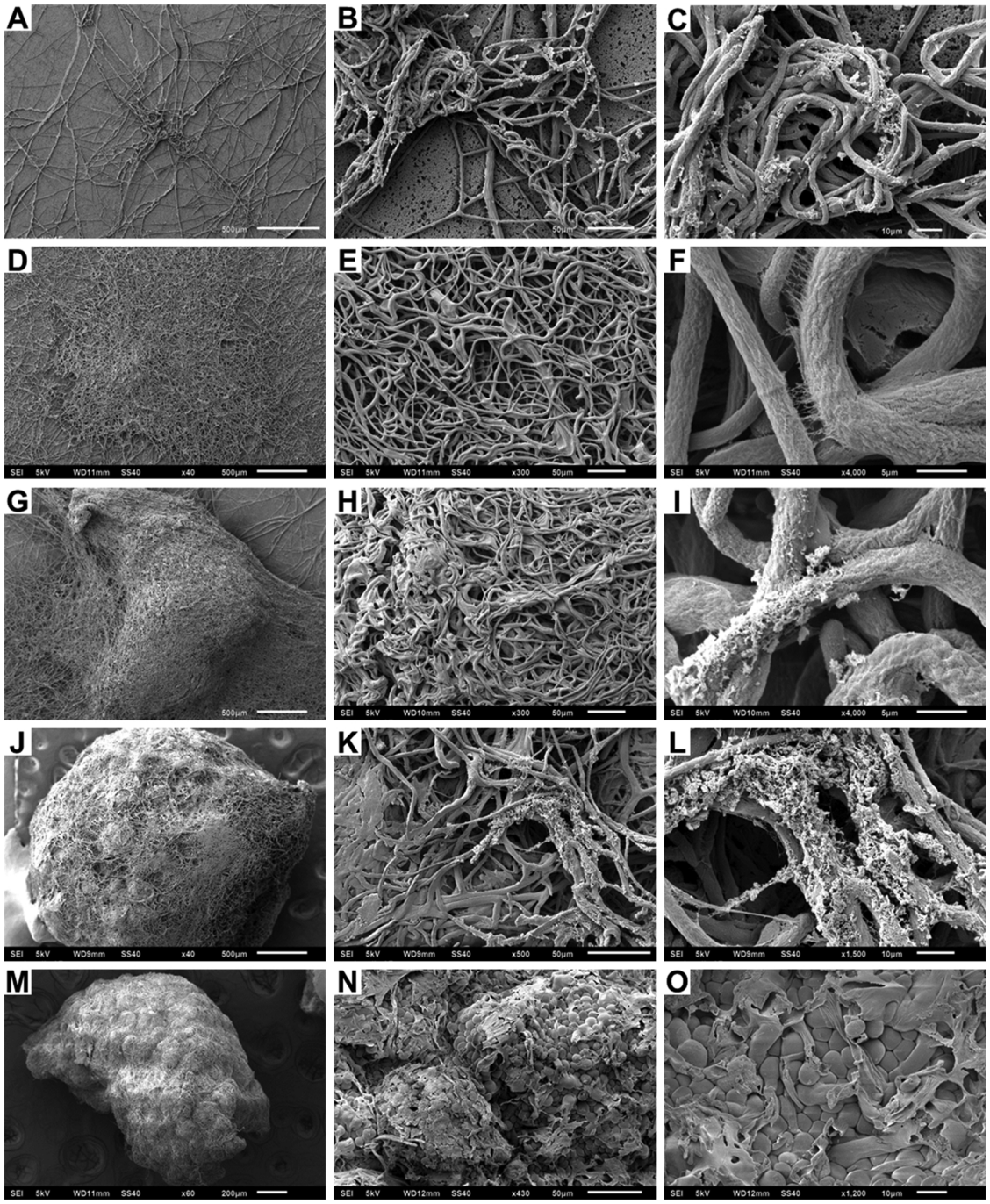

Fig. 2. Scanning electron microscopy (SEM) of the stages of development of sclerotia from *S. sclerotiorum*. Initiation stage, after 5 days of incubation (A–C). Development stage, after 6 days of incubation (D–F). Maturation stage, after 9 days of incubation (G, H and I). Non-melanized sclerotia (J–L) and mature melanized sclerotia (M–O).

1997).

3.3. Expression analysis of genes related to morphogenesis and melanogenesis in sclerotia

We are interested in the molecular events that regulate morphogenesis and melanogenesis during the development of sclerotia in *S. sclerotiorum*. In order to examine the expression of genes potentially involved in these mechanisms, RT-qPCR was performed using total RNA extracted from melanized and non-melanized sclerotia (Fig. 1). Two genes related to cell signaling (adenylate cyclase and serine protein kinase), three related to the melanization pathway (polyketide synthase, tyrosinase and laccase), and four related to cell wall synthesis (chitin synthase 1, 2 and 3 and glucan synthase), were selected for analysis (Table 1). Our study demonstrated that the levels of expression of these genes varied according to the stage of sclerotial maturation (Fig. 4).

We initially evaluated the expression of the histidine kinase (*shk*) and adenylate cyclase (*sac*) genes in melanized and non-melanized sclerotia. The gene expression of histidine kinase (*shk*) was 2.7-fold higher in non-melanized as compared to melanized sclerotia (Fig. 4A). These data show that the expression of *shk* is important for sclerotial maturation since its expression is greater in non-melanized sclerotia. A similar result was previously reported, in which the authors showed

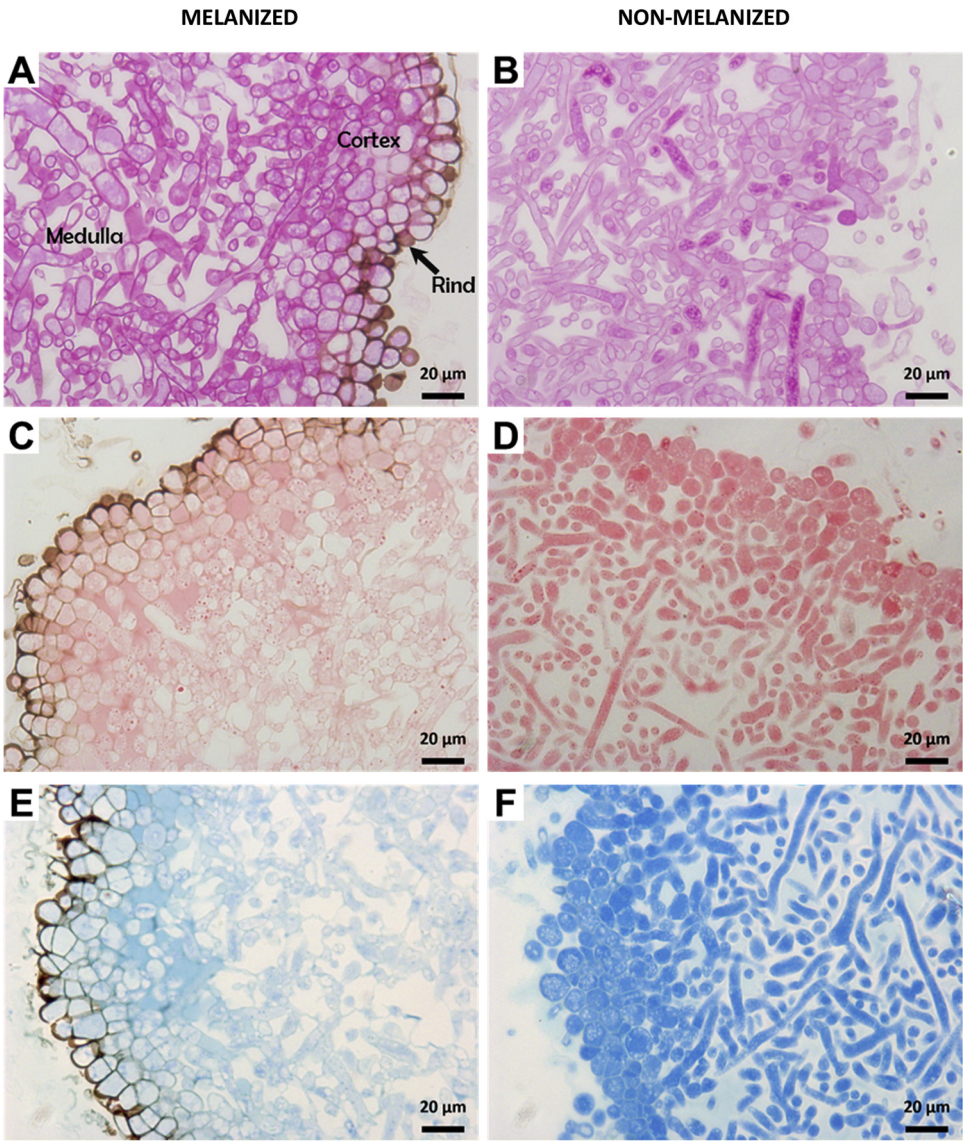

Fig. 3. Light micrographs of rind, cortex and medullar hyphae of the melanized (A, C and D) and non-melanized (B, D and F) sclerotia of *S. sclerotiorum*. Sections stained with Periodic Acid-Schiff showing a positive reaction for polysaccharides (A and B). Sections stained with Ponceau xylidine showing a positive reaction for proteins (C and D). Sections stained with Nile blue showing a positive reaction for melanin and lipofucsin (E and F).

that a *shk1* mutant of *S. sclerotiorum* had a significant reduction in vegetative hyphal growth, and was unable to produce sclerotia (Duan et al., 2013). The gene expression of adenylate cyclase (*sac*) was 1.7-fold higher in melanized as compared to non-melanized sclerotia (Fig. 4A). It is known that that compounds that inhibit phosphodiesterase (caffeine) or activate adenylate cyclase (NaF) can reduce sclerotial development in *S. sclerotiorum* (Rollins and Dickman, 1998; Harel et al., 2005). Our data suggest that a decrease in the activity of adenylate cyclase in non-melanized sclerotia maintains cyclic AMP at low levels until they reach maturity.

During the sclerotial development we observed a change in coloration due to the deposition of melanin in the rind layer (Fig. 1). Melanin is an amorphous polymer that in *S. sclerotiorum* reduces cell permeability, and protects the sclerotia from deleterious environmental agents and degradation by enzymes produced by antagonistic microorganisms (Willetts and Bullock, 1992). The biosynthesis pathways of fungal melanin polymers have been divided into two classes; the 1,8-tetrahydroxynaphthalene (DHN) or L-3,4-dihydroxyphenylalanine (L-dopa) pathways (Casadevall and Eisenman, 2012). The *S. sclerotiorum* genome contains all genes necessary for the synthesis of DHN or L-dopa melanin, and some of these have already been studied (Butler et al., 2009; Liang et al., 2017).

The gene expression of polyketide synthase (*pks)* was 3.7-fold higher in non-melanized as compared to melanized sclerotia (Fig. 4B). Polyketide synthase participates in the first step of DHN melanin synthesis pathways, forming 1,3,6,8-tetrahydroxynaphthalene (4THN) from acetyl CoA and malonyl CoA (Casadevall and Eisenman, 2012). Higher expression of *pks* in non-melanized sclerotia compared to melanized, shows that the DHN pathway is more active at this stage of maturation (Fig. 2 and 3). Other studies have already demonstrated the participation of the enzymes tetrahydroxynaphthalene reductase (THR) and scytalone dehydratase (SCD) in DHN melanin synthesis in *S. sclerotiorum*. SCD and THR participate in the pentaketide pathway, through which the fungal melanin DHN is produced (Casadevall and Eisenman, 2012). Butler et al. (2009), using tricyclazole, a specific inhibitor of the THR, showed that the melanin produced in sclerotia of *S. sclerotiorum* is a DHN melanin. The authors also showed the presence of SCD in melanized sclerotia of *S. sclerotiorum* by measuring the enzyme activity in PAGE gels. Liang et al. (2017), using the knockout gene technique, showed that the *scd1* and *thr1* genes play important roles in

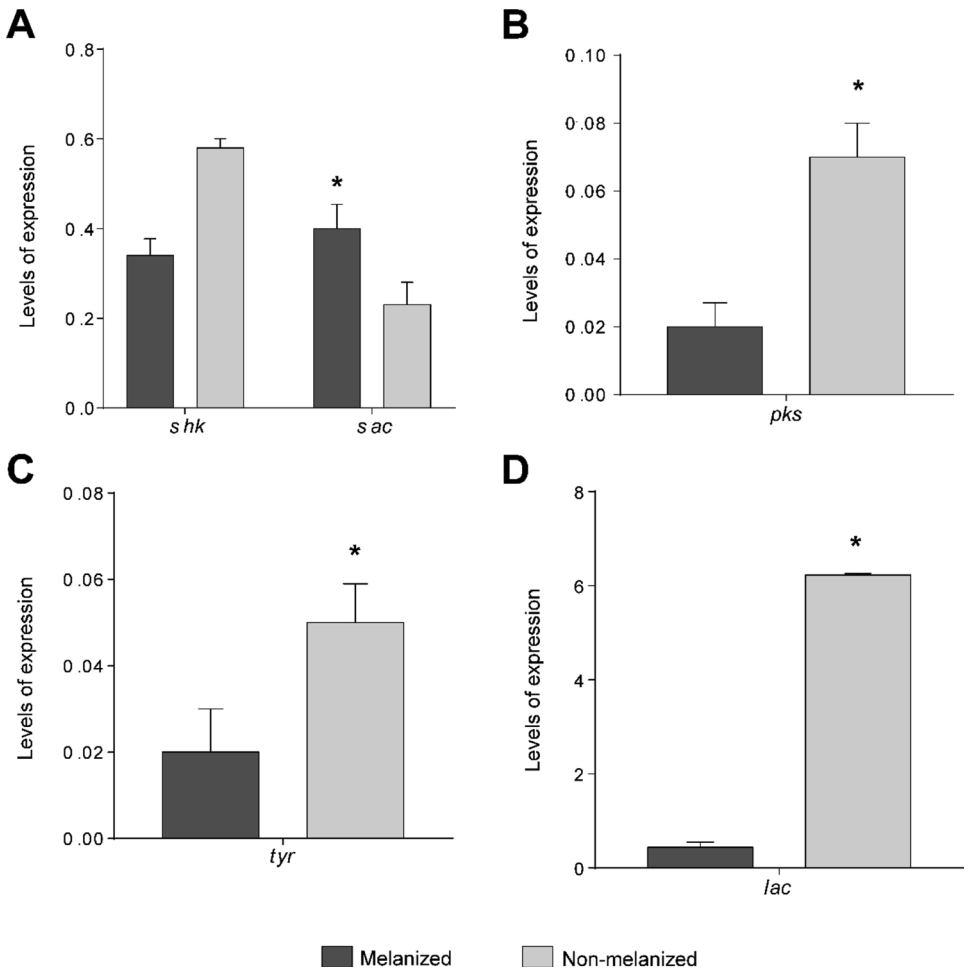

Fig. 4. Relative gene expression of the serine protein kinase (*shk*), adenylate cyclase (*sac*), polyketide synthase (*pks*), tyrosinase (*tyr*) and laccase (*lac*) genes of *S. sclerotiorum* in melanized and non-melanized sclerotia. Data are represented as means ± standard deviation from three biological replicates. Asterisks indicate significant differences.

mycelial growth and sclerotial formation in *S. sclerotiorum*. They observed that the disruption of the *scd1* and *thr1* genes did not affect the pathogenicity of this fungus. However, the sclerotial pigmentation was not fully inhibited by the disruption of either gene, suggesting the existence of an alternative pathway for melanization in sclerotia from *S. sclerotiorum*.

In order to examine if an alternative melanogenesis pathway exists in *S. sclerotiorum*, we evaluated the expression of the tyrosinase (*tyr*) and laccase (*lac*) genes in melanized and non-melanized sclerotia (Fig. 4C and D). These enzymes are involved in melanin biosynthesis through the L-dopa pathway (Casadevall and Eisenman, 2012). Tyrosinase is a mono-oxygenase enzyme that catalyses the *O*-hydroxylation of monophenols and subsequent oxidation of *O*-diphenols to quinones, which polymerize spontaneously to form melanin (Kanteev et al., 2015). Laccase is a copper-containing enzyme that catalyzes the oxidation of phenolic substrates by coupling them to the reduction of oxygen to water (Bolton, 2006). If tyrosine is the precursor, tyrosinase converts tyrosine into L-dopa and then into dopaquinone. However, if the precursor is L-dopa, it is oxidized to dopaquinone by laccase. The gene expression levels of *tyr* and *lac* were 2.6 and 15-fold higher in non-melanized than in melanized sclerotia, respectively (Fig. 4C and D). The involvement of laccases in both melanin synthesis pathways is well documented (Nosanchuk et al., 2015). However, the high expression of *lac* associated with significant expression of *tyr* suggest that *S. sclerotiorum* also uses the L-dopa pathway during the melanization of sclerotia. Previous studies have shown that there are many fungi that can use both pathways, while others such as *Cryptococcus neoformans* use only the L-dopa pathway (Casadevall and Eisenman, 2012).

Melanin can be associated with the cell wall or secreted into the culture medium (Nosanchuk et al., 2015). In order to evaluate whether there is a relationship between cell wall synthesis and melanin synthesis, we examined the expression of three chitin synthase genes (*chs1, chs2 and chs3*) and one glucan synthase (*gsh*) in non-melanized and melanized sclerotia of *S. sclerotiorum* (Fig. 5). The gene expression of chitin synthase 1 (*chs1*) was 4.8-fold higher in non-melanized as compared to melanized sclerotia. However, the expression of chitin synthase 2 and 3 (*chs2* and *chs3*) was not statistically different (Fig. 5). The gene expression of glucan synthase (*gsh*) was 1.2-fold higher in non-melanized as compared to melanized sclerotia. These data showed that cell wall synthesis in non-melanized sclerotia is more intense than in melanized sclerotia. Cell wall synthesis associated with melanin synthesis is an interesting finding since in fungi, polysaccharides such as chitin and β-1,3-glucan, serve as a scaffold to which the melanin granules are cross-linked (Casadevall and Eisenman, 2012; Latgé, 2007.

4. Conclusions

The results of the present study showed that sclerotia produced in PDA medium can be used as a model to study sclerotial development and melanization in *S. sclerotiorum*. The sclerotial initiation, development and maturation phases were well defined through scanning electron microscopy. The histochemical studies showed that there is a difference in the concentration of polysaccharides, proteins, melanin and lipofucsin in the melanized compared to non-melanized sclerotia.

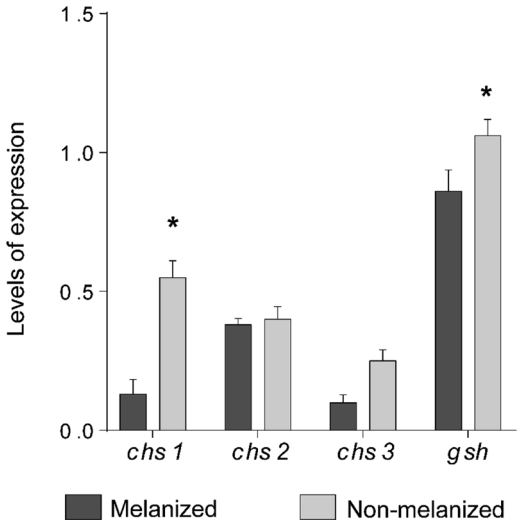

Fig. 5. Relative gene expression of the chitin synthase (*chs1, chs2 and chs3*) and glucan synthase (*gsh*) genes of *S. sclerotiorum* in melanized and non-melanized sclerotia. Data are represented as means ± standard deviation from three biological replicates. Asterisks indicate significant differences.

Molecular analysis through real-time PCR allowed evaluation of the expression levels of genes involved in cell signaling, melanin and cell wall synthesis. High laccase expression suggested for the first time that *S. sclerotiorum* may use the DHN and L-dopa pathways to produce melanin. This fact makes this enzyme an interesting target for the development of melanin synthesis inhibitors in this fungus.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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