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Regular paper

Detection of a pheomelanin-like pigment by EPR spectroscopy in the mycelium of *Plenodomus biglobosus**

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Melanin occurrence in *Plenodomus biglobosus* was investigated using the electron paramagnetic (spin) resonance (EPR, ESR) spectroscopy. The fungus was isolated from living and dead leaves of European ash (*Fraxinus excelsior* L.). Dark pigmentation of *P. biglobosus* mycelium *in vitro*, especially on the reverse side, was observed. The black coloration intensified with the age of the culture and inspired us to check if the analyzed fungus species synthesizes melanin. Melanin contains unpaired electrons, thus, EPR spectroscopy was applied as a specific technique to verify its presence in *P. biglobosus*. The EPR spectrum of the mycelium showed a very strong melanin signal, revealing pheomelanin-like features. Thus, the black pigment of *P. biglobosus* was clearly identified as melanin. However, no melanin was detected in the apparently dark culture medium even when zinc (II) acetate was added to increase the sensitivity of detection. Pheomelanin has many unusual biological functions but it is not commonly found in fungi. Detection of this type of melanin in *P. biglobosus*, which can be both endophytic or pathogenic, suggests a closer examination of the potential role of this melanin in the host-parasite interaction.

Key words: endophytes, ESR, *Fraxinus*, melanin, *Leptosphaeria biglobosa*, *Phoma*

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Abbreviations: DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHN, dihydroxynaphthalene; DOPA, 3,4-dihydroxyphenylalanine; EPR, electron paramagnetic resonance (called also ESR, electron spin resonance); ME, malt extract

INTRODUCTION

Fungi constitute a separate Kingdom in Eukaryotes, and are a very diverse group of organisms occurring all over the world. Despite their ubiquity, fungi are still relatively poorly known regarding the production of biologically-active substances. On the other hand, they are potentially considered as the richest source of such substances (Pusztahelyi *et al.*, 2015). In this paper we examine if the black color of a fungus isolated from leaves of European ash (*Fraxinus excelsior* L.) is determined by melanins. Ash leaf petioles play an important role in the development of the ascomycete fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral *et al.* (anam. *Chalara fraxinea* T. Kowalski), which is a dangerous pathogen of ash, presumably introduced from East Asia (Kowalski, 2006; Baral *et al.*, 2014; Gross *et al.*, 2014). *H. fraxineus* causes an epidemic disease commonly known as ash dieback, spreading across Europe for about 20 years. It is a serious threat for European ash (Gross *et al.*, 2014). After leaf infection, mycelium of *H. fraxineus* overgrows through the petioles to the ash branches and initiates dieback of the tree crown. In the following year, *H. fraxineus* can produce fruitbodies on infected leaves which fell onto the ground. These fruitbodies eject ascospores, which can cause new infections on other, so far symptomless ash trees.

In Poland, the fungal community living on both, the alive and dead petioles, has been studied for several years. In this context, competitive ability of various fungi towards the ash pathogen *H. fraxineus* is being investigated aiming at identification of potential antagonists against the ash dieback pathogen (Kowalski, unpublished). Among the fungal community obtained in these studies, several strongly pigmented slow growing isolates were found. The dark pigmentation of the colonies was observed both in monocultures and, even more pronounced, in dual cultures with *H. fraxineus* (unpublished data). Thus, we hypothesized that this pigmentation is caused by a melanin, which is possibly biologically active.

Melanins are ubiquitous biopolymers found in each group of organisms: Prokaryotes (Drewnowska *et al.*, 2015; Bolognese *et al.*, 2019), plants (Varga, 2016; Shoeva *et al.*, 2020), animals and humans (Barek *et al.*, 2018; Ali & Naaz, 2018; Slominski *et al.*, 2005b), slime molds (Płonka & Rakoczy, 1997) and fungi (Dadachova & Casadevall, 2008; Nosanchuk *et al.*, 2015; Suwannarach *et al.*, 2019). They may be synthesized *via* enzymatic reaction pathways or by spontaneous oxidative processes (Kaney & Knox, 1980; Martin Gonzalez *et al.*, 1997; Płonka & Garbacka, 2006). Melanins may act as antioxidants (ROS scavengers) (de Cassia R. Goncalves & Bonperio-Sponchiado, 2005; Wang *et al.*, 2006), protective pigments against intense irradiation (Kollias *et al.*, 1991; El-Bialy *et al*, 2019; Zadlo 2019), chelators for metals (Thaira *et al.*, 2018; Zadlo & Sarna, 2019) and toxins (Karlsson *et al.*, 2009), ecologically and evolu-

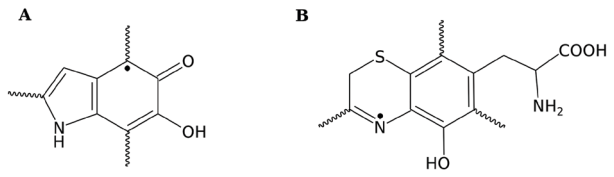

Figure 1. Mers characteristics for:
A – eumelanin (semiquinone radical), **B** – pheomelanin (semiquinonimine radical). '•' – free electron of radical molecule (**A** – modified after Godechal & Gallez, 2011; **B** – modified after Sealy *et al.*, 1982b; Płonka & Garbacka, 2006)

tionary important pigments supporting cryptic pigmentation (Kelley *et al.*, 2016; Polo-Cavia & Gomez-Mestre, 2017) and mimicry (Hines *et al.*, 2017), as a virulence factor in various fungal species (Polak, 1990; Płonka & Grabacka, 2006) or even as metabolites necessary for proper spore formation (Yu *et al.*, 2015; Zhang *et al.*, 2017). Physicochemical and biological properties of melanin, such as its color, are determined by the level of polymerization, and the structure of its mers (Fig. 1) that build a given melanin polymer (Micillo *et al.*, 2016).

Melanins are divided into three main groups: eumelanins, pheomelanins and allomelanins. Eumelanins are dark (black, brown) colored pigments synthesized from tyrosine and/or phenylalanine during oxidation processes. An important stage of eumelanin synthesis is cyclization of DOPAquinone (derived from o-dihydroxyphenylalanine; DOPA) to leucodopachrome (Fig. 2A) and further to 5,6-dihydroxyindole (DHI) and/or 5,6-dihy-droxyindole-2-carboxylic acid (DHICA) (Land *et al.*, 2004).

Pheomelanins are yellow-red pigments synthesized similarly to eumelanins at the early stage, but DOPAquinone is cysteinylated (Fig. 2B) before cyclization. Thus, pheomelanins are pigments containing sulphur in their molecular structure (Land *et al.*, 2004).

Allomelanins are the most diverse group among melanins, and generally do not contain nitrogen (if they are not synthesized from tyrosine). Characteristic subgroups of allomelanins are DHN-(1,8-dihydroxynaphthalene)-derived melanins and pyomelanins. Molecules specific for synthesis pathways of these subgroups are 1,8 DHN (Fig. 2C) and the homogentisate anion (Fig. 2D), respectively (Płonka & Garbacka, 2006; Wang *et al.*, 2015).

Melanin biosynthesis in fungal cells takes place in subcellular organelles called melanosomes (Eisenman & Casadevall, 2012; Nosanchuk *et al.*, 2015), analogous to the mammalian melanosomes where mammalian melanogenesis occurs (Slominski *et al.*, 2004; Eisenman & Casadevall, 2012; Ali & Naaz, 2018). The synthesized fungal melanins can be released to the extracellular space or they associate with cell walls, thus also affecting their structure and porosity (Fogarty & Tobin, 1996; Eisenman & Casadevall, 2012).

A characteristic property of melanins is paramagnetism caused by the presence of an unpaired electron in the semiquinone (Fig. 1A) or semiquinonimine (Fig. 1B) group (Sealy *et al.*, 1982b, Godechal & Gallez, 2011; Chikvaidze *et al.,* 2014). Thus, melanins are unique pigments, which are stable radicals. As a stable free radical systems, melanins can be analyzed with electron paramagnetic (spin) resonance (EPR, ESR) spectroscopy, which is considered to be one of the most selective and sensitive analytical methods of spectroscopy for these pigments studies. EPR spectroscopy is a method for analysis of materials containing unpaired electron systems. The presence of stable semiquinone or semiquinonimine radicals makes melanin measurements independent on the use of additional, exogenous spin labels. Furthermore, EPR spectroscopy excels over UV-Vis and other methods due to much greater specificity.

Spectroscopy not only allows for detection of melanin in a biological system but also for its qualitative analysis – melanin systems made of benzothiazine are characterized by the spectrum containing hyperfine structure, whereas melanins produced only of tyrosine (without participation of the sulfur-containing components) are characterized by a simple spectrum (Sealy *et al.*, 1982b). Additional advantages of EPR spectroscopy are non-invasiveness – the sample does not need to be processed before testing, and non-destructiveness – the sample is not damaged under measurement conditions (Chikvaidze *et al.*, 2014). Therefore, EPR spectroscopy was applied as a sensitive and highly specific method to test the hypothesis that the black color of the fungus from ash leaves is determined by melanins, and if so, to determine which kind of melanin is produced by the mycelium.

MATERIALS AND METHODS

Fungal culture. *Plenodomus biglobosus* was isolated both from living symptomless leaf's petioles and from dead previous year petioles of the European ash collected from litter. They were not infected by *H. fraxineus*. The petioles were surface-sterilized by first soaking for 1 min in 96% ethanol, then for 5 min in a solution of sodium hypochlorite (approx. 4% available chlorine) and finally for 30 s in 96% ethanol. After drying in layers of blotting paper, twelve pieces from each petiole were cut out

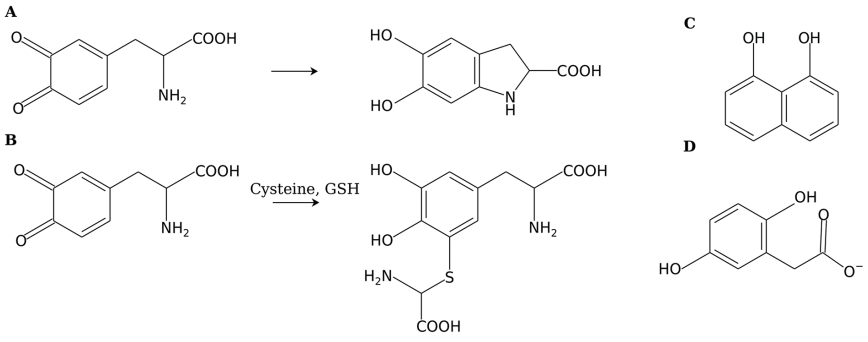

Figure 2. Characteristic stages of synthesis of:
A – eumelanin (cyclization of dopaquinone to leucodopachrome), **B** – pheomelanin (cysteinylation of dopaquinone to cysteinylDOPA directly or *via* glutathione (GSH)); characteristic molecules in syntheses of: **C** – DHN-melanins (1,8 DHN, 1,8- dihydroxynaphthalene), **D** – pyomelanins (homogentisate anion). Modified after Płonka & Garbacka, 2006

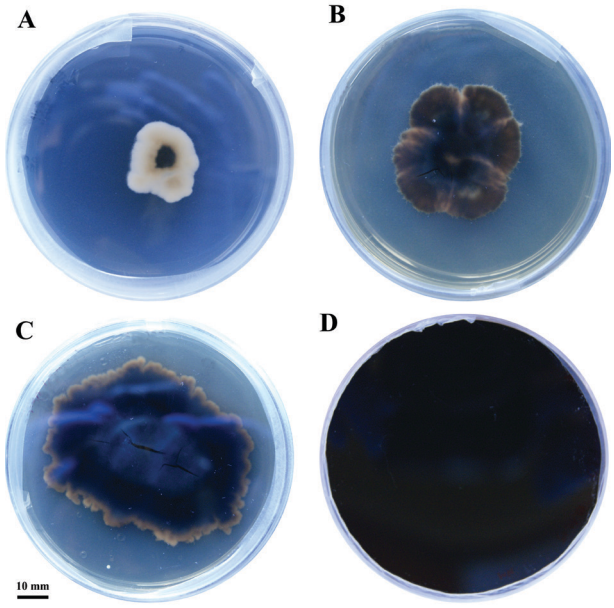

Figure 3. Images of fungal colonies documented after:
A – 2 weeks, **B** – 3 weeks, **C** – 6 weeks, **D** – 3 months

and placed on the surface of malt extract agar (MEA; 20 g l^{-1} malt extract (Difco; Sparks, MD, USA), 15 g l^{-1} agar Difco supplemented with 100 mg l^{-1} streptomycin sulphate) in Petri dishes (diameter 9 cm). Among the many isolated fungal taxa, one, getting black during *in vitro* culture, was selected to further studies. It was isolated on 29 August 2018 from a living symptomless petiole sampled from young European ash growing in the Myślenice Forest District (Malopolska, Poland). In dual cultures, both species, *P. biglobosus* and *H. fraxineus*, showed growth inhibition towards their counterpartner, and between them an inhibition zone was formed wider than 5 mm. The examined strain is deposited at the Department of Forest Ecosystem Protection at Agriculture University in Kraków (HMC 21532).

The selected strain was cultivated at 20°C in the dark on the malt extract medium (ME; 20 g/l (BIO-CORP)) as liquid, and on 1.5% agar as solid cultures. The change of mycelium color during growth was photodocumented after 2, 3, 6 weeks and 3 months (Fig. 3). DNA from the mycelium was isolated using DNA Mini Kit (Syngen Biotech, Wroclaw, Poland) and amplified using the following primers: ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes & Bruns, 1993) and LR6 (5'-CGC CAG TTC TGC TTA CC-3') (Vilgalys & Hester, 1990).

Identification of the fungus was based on internal transcribed spacer (ITS) sequence which was verified using both, GenBank (https://www.ncbi.nlm.nih.gov/, accessed 24.04.2020, Altschul *et al.*, 1990) and UNITE (https://unite.ut.ee/, accessed 24.04.2020, Nilsson *et al.*, 2019) databases.

EPR studies

Analysis of mycelium and synthetic melanins. The mycelium with agar from a solid culture was resected with a lancet and packed densely into an EPR glass tube (inner diameter 4 mm, length 2.5 cm) whereupon the glass tube was frozen in liquid nitrogen to form an icicle. The sample was subsequently transferred to a quartz finger Dewar filled with liquid nitrogen.

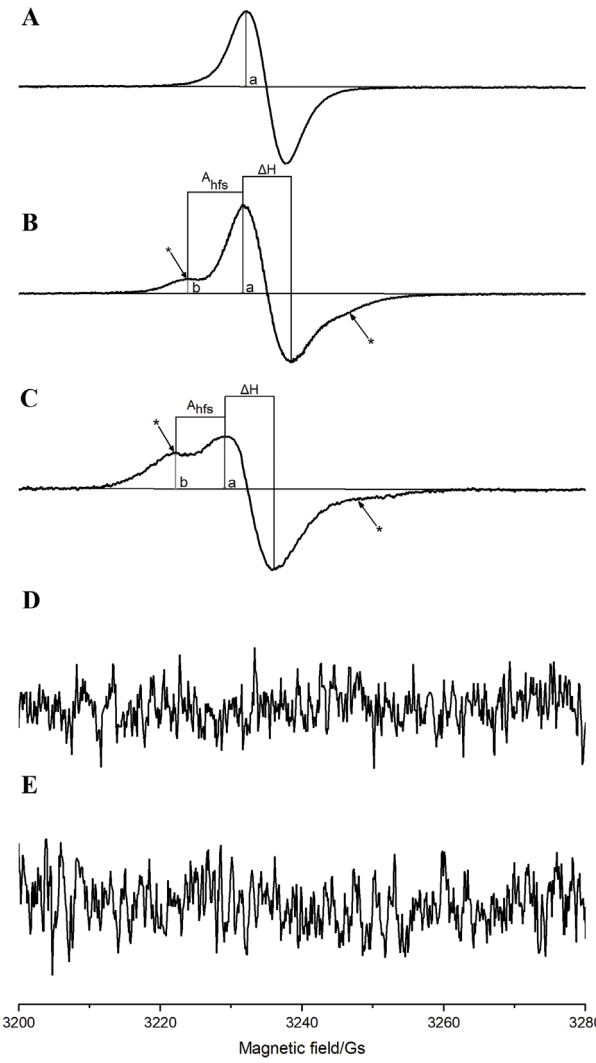

Figure 4. Representative EPR spectra of analyzed materials:
A – synthesized eumelanin, **B** – studied fungus mycelium, **C** – synthesized melanin copolymer, **D** – supernatant without addition of zinc (II) acetate, **E** – supernatant with addition of zinc (II) acetate. 'a' – intensity of superpositioned bands characteristic for eu- and pheomelanin, 'b'– intensity of the low-field pheomelanin band, '*' – high- and low-field bands characteristic for pheomelanin (the high-field line is poorly resolved), Ahfs – hyperfine splitting constant. ΔH – linewidth of the EPR signal

The EPR measurements were performed with a Varian E-3 X-band spectrometer (Sunnyvale, LA, USA) at 77K (–196°C). Initially, technical parameters were tested to obtain the spectrum of the best quality, with the lowest values of the gain and power, which may cause signal distortion. The final, established parameters for fungal mycelium analysis were as follows: modulation amplitude 1.0 Gs, receiver gain 50 000, microwave power 1 mW, time of one scan 200 s, time constant 0.1 s, number of scans 3 and were then averaged.

Spectra were analyzed by calculating the 'a' and 'b' parameters, and the hyperfine splitting constant A_{hfs} (which is the distance between the centers of the bands a and b). The 'a' parameter is the intensity of the band which is a linear combination (superposition) of bands of eu- and pheomelanin; the 'b' parameter is characteristic for pheomelanin (Sealy *et al.*, 1982a, b). To analyze the relative proportions between eu- and pheomelanin, the a/b

ratio was calculated. Additionally, the ΔH parameter was calculated as a linewidth of the EPR signal connected to the 'a' band. The final values of A_{hfs}, a/b ratio and ΔH were the means of values calculated from 5 spectra (values of standard deviation and standard error were calculated and the second one was presented as the measurement of uncertainty in the text). Spectra were obtained from separate colonies, cultured for 21 days under the same conditions, and of comparable diameter (5 cm).

The calculated parameters were compared to the values obtained from a synthetic eumelanin/pheomelanin copolymer (predicted composition: 90% of pheomelanin, 10% of eumelanin) (Fig. 4C). The spectrum of synthetic eumelanin (i.e. DOPA melanin) was also shown (Fig. 4A). Synthetic melanins were prepared based on the protocols by d'Ischia *et al.* (2013).

Analysis of pigment from the liquid medium. 30 days old mycelium from liquid culture was separated from the medium by centrifugation (1 h, 9600 rpm) and the supernatant was transferred into a Falcon tube. A portion of the supernatant was divided into two parts, and two specimens were prepared: 1 ml of supernatant without any additions and 1 ml of supernatant with an addition of 1 ml of 100 mM $Zn(CH_3COO)_2{\times}2H_2O$ solution (to increase melanin bands resolution: Sarna & Lukiewicz, 1972; Felix *et al.*, 1978); glass tubes, such as the one described earlier were used to prepare the samples. Frozen samples were then measured as presented earlier, except of the receiver gain – 620 000.

RESULTS AND DISCUSSION

Fungus identification

Identification by ITS sequencing of the selected isolate (GenBank accession no. MT651609) resulted in *Plenodomus biglobosus* (*Leptosphaeria biglobosa*) (Shoemaker & Brun, 2001; de Gruyter *et al.*, 2013) with 100% matches with sequences in the employed databases. However, the current species concept of *L. biglobosa* is broadly defined with six distinct subclades, thus its systematic allocation and characterization may be re-described in the future (de Gruyter *et al.*, 2013). In this paper, *P. biglobosus* was for the first time identified as an endophyte and saprotroph of the European ash. Ibrahim *et al.* (2017) previously characterized this species as a *Fraxinus ornus* endophyte. On the other hand, *P. biglobosus* was also described as one of etiological factors of phoma stem canker of oilseed rape (*Brassica napus* L. var. *napus)* (Stonard *et al.*, 2010). Oilseed rape is an important oleaginous plant, and the mentioned disease is one of the major causes of loss of winter oilseed rape crops in Europe, Australia and North America (Fitt *et al.*, 2006). It is worth noting that Polish strains of *P. biglobosus* are considered to be more pathogenic than strains from France, Canada or Germany, due to their production of polanrazines and phomapyrones (Stonard *et al.*, 2010).

The cultures of *P. biglobosus* were blackening with time (Fig. 3), suggesting that they were generating and accumulating a dark pigment, most probably melanin. It is known that on oilseed rape, *P. biglobosus* often co-occurs with *Leptosphaeria maculans* (Stonard *et al.*, 2010), which does not produce blackish pigments (Lob *et al.*, 2013). However, there are articles in which *L. maculans* was described as a melanized fungus, therefore *P. biglobosus* could have been incorrectly described as *L. maculans* (Shoemaker & Brun, 2001). The authors also used term 'melanin', but there were no citations or evidences that the pigment was melanin indeed, and not another pigment (Jędryczka *et al.*, 1998; Jedryczka *et al.*, 2002). Other authors determined *P. biglobosus* as *Leptosphaeria maculans* or *Leptosphaeria maculans/Phoma lingam*, and described sulphur-containing chemical species which were considered only as shunt metabolites connected to DHN-melanins synthesis pathways (Pedras & Yu, 2009a, b).

Table 1. Mean values of EPR spectra parameters calculated for fungal mycelium (n = 5).
'a/b' – ratio of intensity of the band 'a' (superposition of eu- and pheomelanin bands) and 'b' (characteristic for pheomelanin), Ahfs – hyperfine splitting constant – distance between 'a' and 'b' bands, ΔH – linewidth of the EPR signal connected to 'a' band, S.D. – standard deviation, S.E. – standard error

Parameter	Calculated mean value	± S.D.	± S.E.
a/b ratio	6.0	0.3	0.1
Ahfs/Gs	7.9	0.2	0.1
ΔH/Gs	6.6	0.3	0.1

The authors claimed in these papers that they described for the first time sulphur-containing variations of DHN-related allomelanins. However, they did not support their discoveries with EPR measurements and little is known on the pheomelanin-like DHN melanins. Nevertheless, the work of these authors opened up a new field of melanin research.

In the work presented here we finally confirm by using EPR that the pigment produced by *P. biglobosus is* melanin, and in particular – pheomelanin.

EPR analysis of *P. biglobosus*

EPR analysis of the fungal mycelium revealed a strong EPR signal around g = 2.004, typical of melanin (Commoner *et al.*, 1954). Analysis of the spectrum (Fig. 4B) revealed a hyperfine splitting of the signal with A_{hfs} ca 7.5 (Table 1) suggesting that *P. biglobosus* produced pheomelanin (Sealy *et al.*, 1982a, b), which is a rare type of melanins found in fungi. The splitting is a result of magnetic interaction of the unpaired electron with the magnetic nucleus of nearby ^{14}N. The third, high-field component of the splitting is usually poorly resolved (Sealy *et al.*, 1982a; Sealy *et al.*, 1982b). Some authors mentioned a possibility of pheomelanin being produced by fungi (Eisenman & Casadevall, 2012; Nosanchuk *et al.*, 2015), but few papers showing EPR analysis of fungi containing pheomelanin pigment can be found. Well-defined EPR spectra of fungal pheomelanin were published e.g. for *Cladosporium cladosporioides* (Buszman *et al.*, 2006), *Cladosporium sphaerospermum* (Dadachova *et al.*, 2007) and *Cladosporium herbarum* (Zdybel *et al.*, 2009). Pheomelanin is also known to have a stronger prooxidant activity than other melanins (Tanaka *et al.*, 2018). As *P. biglobosus* maintains this type of pigment, it must be suggested that its production is somehow evolutionarily advantageous for this species.

In the studied fungus the calculated a/b ratio was estimated to be 6.0±0.1 (Table 1). For a more detailed analysis of the fungal melanin spectrum, it was compared to a synthetic melanin spectrum (Fig. 4C) which was obtained from analysis of a copolymer predicted to contain 90% of pheomelanin and 10% of eumelanin. The calculated ratio of a/b parameters was 1.4, which stays in a good agreement with the predicted value for pure synthetic pheomelanin (1.2±0.1, Sealy *et al.*, 1982a) and suggests that the spectrum of *P. biglobosus* also contains a copolymer of melanins with quite a substantial par-

ticipation of pheomelanin. For comparison, for common animal pheomelanotic materials, the a/b value equals e.g. 3.5–4 for yellow A^y/a mice, up to approx. 9 for an agouti phenotype (Slominski *et al.*, 2005b; Wolnicka-Głubisz *et al.*, 2013). Another typical EPR parameter – the linewidth (ΔH) 6.6±0.1 Gs (Table 1) was also comparable to pheomelanin, as revealing a wider signal than eumelanin (ca. 3.5 – 4 Gs, e.g. Slominski *et al.*, 2005a; Wolnicka-Głubisz *et al.*, 2013).

The A_{hfs} (Fig. 4B) calculated for fungal mycelium was 7.9±0.1 Gs (Table 1), which was slightly higher than the parameter calculated for synthetic pheomelanin (A_{hfs} = 7.1 Gs, Fig. 4C). According to the literature, the value for synthetic pheomelanin should vary in the range of 7–7.4 Gs, but it is strongly dependent on the microenvironment of the sample, especially pH (Sealy *et al.*, 1982b). This may be the reason of the discrepancy between our result and values found in the literature. This discrepancy remains within the frame of experimental errors but at the same time it suggests taking a closer look into the structure of the fungal pheomelanin in the future. In particular, the abovementioned suggestion on the potential structure of mers of sulphur-containing DHN-derived melanin (Pedras & Yu, 2009a, b) should be seriously taken into consideration.

The deep-dark, almost black color of the culture (Fig. 3) may seem incongruent with the popular view that pheomelanins give 'yellow-red' coloration of biological materials, but this may be due to a purely optical phenomenon, as some samples of dried pheomelanin seem to be apparently black, and only after dissolving or spreading on a white background as a thin layer, they reveal a reddish tint (Wolnicka-Głubisz *et al.*, 2012). Due to the high specificity of EPR parameters, our analysis revealed beyond any doubt that pheomelanin or a similar pigment with analogical structure of paramagnetic centers is produced by the mycelium of *P. biglobosus*.

EPR analysis of the supernatant from the liquid culture showed that melanin was not secreted outside the fungal cell. The dark color of liquid was probably caused by secretion of melanin precursors or might also result from secretion of other colored metabolites. It is possible that monomers with conjugated systems of double bonds were colored, but before polymerization they were not paramagnetic, thus, an EPR signal was not detected (Fig. 4D) even with zinc (II) acetate addition (Fig. 4E).

Role of the pigment – suggestions

Increasing intensity of the dark color of the mycelium indicates that the fungus is accumulating melanin pigments during growth and aging. Melanins, especially eumelanin, play a role as photoprotectants able to absorb and dissipate electromagnetic radiation (Kollias *et al.*, 1991). Analyses conducted by Drewnowska and others (Drewnowska *et al.*, 2015) showed that production of eumelanin-like pigments among bacterial strains of *Bacillus weihenstephanensis* differed, and the main differentiating factor was exposure to light. However, according to our observations, the pheomelanin-like pigment accumulation was present when *P. biglobosus* cultures were not exposed to light. This suggests that the role of pheomelanin in the fungus is different than the role of eumelanin in the mentioned bacteria. As pheomelanin can be phototoxic (Ito *et al.*, 2018), this observation seems logical, but it does not explain what is the reason for generating pheomelanin in the dark. It would be also interesting to check if allo-, or eumelanin content is growing during exposure to light. Riley (Riley, 1992) suggested that initially melanin might have been a 'dustbin' for the excess of toxic orthoquinones, free radicals and metals (and maybe thiols, too), while Ito (Ito, 2003) emphasized a possibility that during biological melanogenesis initially pheomelanin is always produced, up to the exhaustion of the cellular thiol donors, and the eu/pheomelanin composition would depend on the availability of intracellular cysteine. To examine the role and production kinetics of melanin pigments in *P. biglobosus* further experiments are planned.

Pigmentation of *P. biglobosus* was already noticed by Lob and others (Lob *et al.,* 2013), but no information on the pigment type was available. We provide the first data showing that not only eu-, or allomelanins but also pheomelanins occur in the *P. biglobosus* mycelium (Fig. 4). Besides photoprotection, another probable role of melanin in this species is its contribution to virulence. In numerous pathogens of plants, fungal melanin has been documented as a factor facilitating penetration of the host tissues and protecting the fungus against the host defense mechanisms (Płonka & Grabacka, 2006; Taborda *et al.*, 2008).

Nevertheless, a possible involvement of melanin in the phoma stem canker etiology (see above) remains to be shown. It is also possible that melanin is just a side product of enzymes (such as polyketide synthases) whose primary role is different (e.g. detoxication) or still unknown (Yu *et al.*, 2015). Such studies demand a thorough and painstaking analysis at the level of molecular genetics and engineering (Takano *et al.*, 1997, Yu *et al.*, 2015).

The melanin pigment is probably not being secreted into the plant tissues, but nevertheless comes into their close contact. On the other hand, no pathogenic effect of *P. biglobosus* was observed in *F. ornus* (Ibrahim *et al.,* 2017). Similarly, in our study, living leaves of *F. excelsior* from which the fungus was isolated, were free of visible symptoms of disease. Nevertheless, some apathogenic endophytes may become virulent under certain environmental conditions or due to accumulation of mutations (Sieber, 2007). For this reason, further studies of the significance of *P. biglobosus* melanins for the host-parasite interaction involving *Fraxinus* spp., as well as oilseed rape, are necessary.

SUMMARY

We isolated and identified a fungal endophyte of the European ash as *Plenodomus biglobosus* (*Leptosphaeria biglobosa).* This ascomycete, which is a pathogen of Brassicaceae and an endophyte of *F. ornus*, is shown to colonize the living and dead leaves of *F. excelsior.* Pronounced darkening during colony development encouraged us to analyze the pigments of *P. biglobosus*. Our study provides the first EPR analysis of melanins produced by *P. biglobosus.* This fungus synthesizes significant amounts of pheomelanin, which is rarely found in fungi. However, the actual role of this metabolite in pathogenicity, stress resistance or interactions with other microorganisms still needs to be investigated.

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