Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 63 (2003) 687–691
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Melanin in the extracellular matrix of germlings of *Botrytis cinerea*

Robert P. Doss[a,b,*], Joachim Deisenhofer[c], Hans-Albrecht Krug von Nidda[c], Alfred H. Soeldner[d], Ruth P. McGuire[a,b]

[a]*USDA-ARS, Horticultural Crops Research Unit, 3420 NW Orchard Avenue, Corvallis, OR 97330, USA*
[b]*Department of Horticulture, Oregon State University, Corvallis, OR 97331, USA*
[c]*Experimentalphysik V, EKM, Institut fuer Physik, Universitaet Augsburg, D-86159 Augsburg, Germany*
[d]*Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA*

Received 11 February 2003; received in revised form 28 April 2003

Abstract

Previous work on the composition of the extracellular matrix of germlings of the plant pathogenic fungus *Botrytis cinerea* demonstrated the presence of carbohydrate, protein, and simple lipids; which, together, comprised 50–60% of the dry weight. Here we show that most of the remaining mass of the extracellular matrix consists of a chemically inert dark pigment with the electron paramagnetic resonance characteristics of a melanin. Scanning electron micrographs of the purified pigment, and transmission electron micrographs of thin sections made using the pigment indicate that it has a filamentous structure. We conclude that melanin is an important component of the extracellular matrix of germlings of *B. cinerea*. This is the first report of a melanin present in the extracellular matrix of a plant pathogenic fungus.

Keywords: *Botrytis cinerea*; Sclerotiniaceae; Grey mold; Extracellular matrix; Composition; Melanin

1. Introduction

Botrytis cinerea Pers:Fr is an important fungal pathogen of a number of food and ornamental crops (Jarvis, 1977). Germlings of *B. cinerea* attach to their host substrate through secretion of an extracellular matrix (ECM) (Doss et al., 1995). The ECM can be separated from fungal hyphae by vigorous washing of surfaces, such as glass, upon which conidia have been allowed to germinate (Doss, 1999; Doss et al., 1995). Such washing removes the fungal tissue, leaving the matrix attached to the substrate from which it can be removed for analyses. ECM isolated in this way contains carbohydrates, simple lipids, and proteins. The latter includes enzymes thought to be involved in the infection process.

To study the composition of the carbohydrate portion of the ECM, hydrolysis in a strong mineral acid was used to obtain monosaccharides for analysis by gas–liquid chromatography (Albersheim et al., 1967; Doss, 1999). It was noticed that even after prolonged periods of hydrolysis under vigorous conditions an insoluble dark substance remained. As melanins are associated with several fungal structures, and secretion of melanins into fungal culture has been reported (Bell and Wheeler, 1986; Wheeler and Bell, 1988), we hypothesized that the dark substance present in the ECM was such a pigment. The objectives of the study discussed here were to determine whether or not the dark pigment present as part of the ECM of *B. cinerea* was a melanin, and to learn something of the form of the pigment.

2. Results and discussion

It has already been established that the ECM secreted by germlings of *B. cinerea* is made up of a mixture of simple lipids, polysaccharides, and polypeptides that make up 50–60% of the total mass (Cooper et al., 2000; Doss, 1999). We now show that most of the remainder of the ECM consists of a dark pigment with the physicochemical characteristics of a fungal melanin.

* Corresponding author. Tel.: +1-541-738-4056; fax: +1-541-738-4025.
E-mail address: dossr@science.oregonstate.edu (R.P. Doss).

0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00323-6

This pigment, obtained after prolonged refluxing in 6 M HCl followed by repeated washing with organic solvents, comprised 43±4% of the dry weight of the matrix with *B. cinerea* isolate ATCC No. 204446, and 27±3% with isolate ATCC No. 11542 (Table 1). Table 1 also shows estimates of the percentages of the other major components of the ECM of these two isolates. In both cases the dark pigment represents a sizable fraction of the total mass.

Melanization of sclerotia of *B. cinerea* is inhibited in the presence of tricyclazole (Zeun and Buchenauer, 1985), a specific inhibitor of the reductase in the pentaketide biosynthetic pathway that gives rise to 1,8 dihydroxynapthalene (DHN), the precursor of the melanin polymer formed by this compound (i.e., DHN melanin) (Bell and Wheeler, 1986; Wheeler and Bell, 1988). In two cases, with isolate ATTC No. 204446, where germination and hyphal growth occurred in the presence of 12.5 μg/ml tricyclazole, the ECM, when collected and used for melanin preparation, yielded a material much lighter in color than that formed in the absence of tricyclazole. This material comprised 9% of the dry weight of the ECM in one case, and 11%, in the other. The effect of tricyclazole on the amount and appearance of the dark pigment present in the ECM, and its refractory nature are consistent with the idea that it is a fungal melanin of the DHN type.

Melanins are dark-colored pigments, formed by oxidative polymerization of various phenolic compounds (Bell and Wheeler, 1986; Wheeler and Bell, 1988). Generally these pigments are insoluble in water, are intractable to the methods usually used for chemical characterization, and can act as both oxidants and reductants (Bell and Wheeler, 1986). One defining feature of all melanins is the presence of "...a stable population of organic free radicals...," which results in characteristic electron paramagnetic resonance behavior (Enochs et al., 1993, Sealy et al., 1980). In particular, Enochs et al. (1993) describe, "[a] standardized test for the identification...of melanins using electron paramagnetic resonance (EPR) spectroscopy." Application of this test involves examination of the effects of illumination, of basic pH, and of Zn^{2+} ion on the ESR spectrum of pigment suspended in water. A pigment is identified as a melanin if each of these treatments causes a significant increase in signal intensity.

In Fig. 1 we show the electron paramagnetic resonance spectra of the dark pigment from *B. cinerea* before and after exposure to a red laser and a white light source (Fig. 1a), after suspension in $ZnCl_2$ (Fig. 1b) and at high pH (Fig. 1c). The spectra could be fitted by a Lorentzian lineshape derivative showing slight deviations due to inhomogeneous broadening. The half width at half maximum linewidth (ΔH) of a Lorentzian line is related to the peak-to-peak linewidth (ΔH_{pp}) by the equation $\Delta H_{pp} = 1.16^{*}\Delta H$ and the intensity (I) is calculated using $I = A^{*}\Delta H^2$ with the amplitude (A) of the measured signal dP/dH. The unprocessed pigment yields an EPR spectrum (Fig. 1) that is very similar to the EPR spectra reported for synthetic DOPA melanin or other fungal melanins (Enochs et al., 1993; Loganathan and Kalyanasundaram, 1999). The corresponding lineshape parameters of the unprocessed pigment, the g-factor (g) = 2.004 and linewidth ΔH = 3 Oe, agree well with those reported in the literature. All treatments

Table 1
Composition (percent of dry weight) of the extracellular matrix from germlings of two isolates of *Botrytis cinerea*

Component of ECM	ATTC No. 204446	ATTC No. 11542
Protein	28±2[a]	33±3[d]
Carbohydrate	20±1[a]	14±5[d]
Lipid	6.2±1.1[a], 6.7±0.4[b]	13±3[d]
Melanin	43±6[c]	27±3[c]

Values are mean ±standard-error-of-the-mean.
[a] Data taken from Doss (1999).
[b] Data taken from Cooper et al. (2000).
[c] $N = 5$.
[d] $N = 4$.

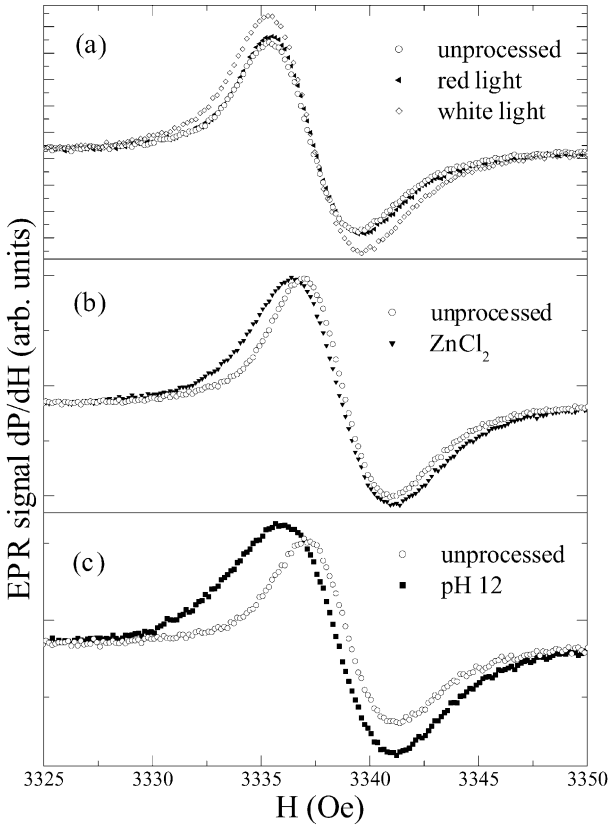

Fig. 1. EPR spectra in aqueous suspension of the dark pigment present in the extracellular matrix of *B. cinerea* germlings ($\Delta H \sim 3.0$ Oe, $g = 2.004$ for the unprocessed pigment). Enhancement of signal intensity was noted after (a) irradiation with red laser light and white light (b) upon suspension in $ZnCl_2$ and (c) upon suspension at pH = 12.

yielded an increase of signal intensity, a shift of the resonance field and a broadening of the line consistent with the standard identification procedure for melanin (Enochs et al., 1993). The enhancement of the intensity (I) compared to the intensity of the unprocessed pigment was 8% for red light, 47% for white light, 40% for $ZnCl_2$, and 110% for alkaline pH 12.0 (NaOH). The process of illumination proved to be reversible, as after 24 h the signal properties showed no difference compared with the signal before illumination, a fact that confirms the stability of the population of free radicals in the substance. Melanin is unique in being "...one of the few known stable free radicals..." (Bell and Wheeler, 1986).

If the dark pigment was allowed to dry prior to carrying out EPR measurement the signal enhancement observed upon suspension in $ZnCl_2$ was no longer observed. The lack of signal enhancement seen when the dried pigment was suspended in $ZnCl_2$, as compared to the enhancement seen upon suspension of the hydrated pigment, has been noted by others (Prota, 1992; Sealy et al., 1980). The data shown were obtained with a preparation from isolate ATCC No. 11542, but virtually identical results were seen with isolate ATCC No. 204446.

The "purified pigment" from both isolates exhibited a filamentous or fibrous nature when examined using scanning electron microscopy (Fig. 2A). A filamentous structure, with a filament diameter (assuming a circular cross section) of 40–50 nm was also seen with transmission electron micrographs made using thin sections through particles of the pigment (Fig. 2B). Images such as that shown in Fig. 2B were seen only when sectioning had occurred in the same plane as the long axes of the filaments.

Purification of melanins from pigment containing matrices typically involves prolonged treatment with a strong mineral acid (Sealy et al., 1980; Wheeler and Bell, 1988). One reviewer of the manuscript describing the work reported here suggested that the filaments of purified melanin that were observed using electron microscopy could be artifacts resulting from this harsh treatment. We doubt that this is the case, as the extracellular matrix seen in scanning electron micrographs of intact germlings on both natural and artificial substrates sometimes exhibits regions possessing a fibrillar structure with filaments of the same diameter as those seen with the purified melanin (data not shown). We speculate that the filaments of melanin may provide a framework upon which the other components of the extracellular matrix are associated.

It is noteworthy, however, that scanning electron micrographs of "purified" melanin show a structure suggesting braids of filaments (Fig. 2A). We feel that these braids, but not the filaments themselves, are artifacts, formed from individual melanin filaments during preparation.

Wall-bound fungal melanins, when present, are usually found as granules in the outer cell wall layer of various fungal structures (e.g., sclerotia, conidia) (Bell and Wheeler, 1986). These melanins are derived through enzymatic (oxidative) polymerization of melanin precursors synthesized in and exported from the fungal cytoplasm (Bell and Wheeler, 1986). In this regard, it is noteworthy that laccase, an enzyme involved in the polymerization step with other fungal melanins, is present in the ECM of *B. cinerea* (Doss, 1999; Hodson et al., 1988), and that when laccase activity is inhibited the virulence of the fungus is reduced (Bar-Nun et al., 1988; Staples and Mayer, 1995).

In addition to wall-bound melanins, extracellular melanins are produced in fungal culture (Bell and Wheeler, 1986; Wheeler and Bell, 1988). Polymerization of melanin precursors, either by the action of a phenol

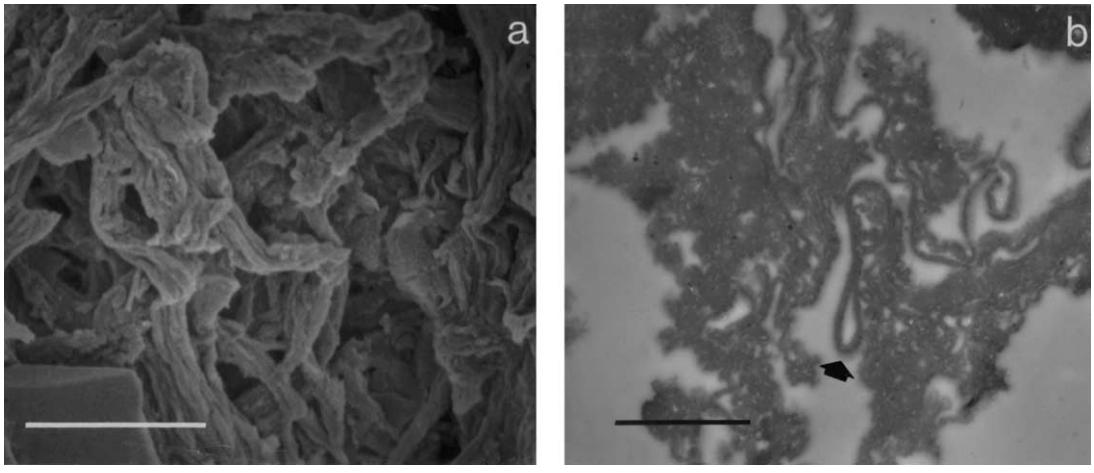

Fig. 2. Electron micrographs of the dark pigment prepared from the extracellular matrix of germlings of *B. cinerea*. (a) Scanning electron micrograph of a particle of the dark pigment. Bar = 10 μm. (b) Transmission electron micrograph of a thin section through a particle of the dark pigment. Arrow indicates a filament. Bar = 100 nm.

oxidase or by autooxidation, occurs in the medium, apart from the cells. The melanins produced as part of the ECM of *B. cinerea*, although filamentous rather than granular (Fig. 2), are at least loosely associated with the fungal cell wall and, thus, can be considered wall-bound.

Here we show that a melanin comprises a significant portion of the extracellular matrix of germlings of the fungus *B. cinerea*, an important plant pathogen. We believe this to be the first demonstration of melanin in a fungal matrix. With this work we can account for virtually all of the mass of the extracellular matrix produced by the germlings (Table 1) What function, if any, melanin may serve in the ECM is unknown.

A number of plant pathogenic fungi require that melanin synthesis be able to occur in order to express their full virulence (Bell and Wheeler, 1986; Wheeler and Bell, 1988). Melanin deficient mutants, and fungi cultured under conditions where melanin biosynthesis has been inhibited by specific inhibitors (e.g., tricyclazole) are sometimes nonpathogenic (Bell and Wheeler, 1986). There is good evidence that inhibition of melanin biosynthesis can decrease virulence of some fungi by reducing the rigidity of the specialized infection cells (i.e., appressoria), whose walls are ordinarily strengthened by melanization, thereby preventing the development of sufficient turgor for host tissue penetration (Bell and Wheeler, 1986; Wheeler and Bell, 1988; Money and Howard, 1996).

Some have speculated without direct evidence that melanins may serve to protect cells against ultraviolet light, as well as against hydrolytic enzymes that could be harmful to the fungi, or antimicrobial substances produced by the host (Bell and Wheeler, 1986; Wheeler and Bell, 1988; Money and Howard, 1996). In this regard, with *B.* cinerea, Gil-ad et al. (2001) have suggested that one role of the extracellular matrix (referred to by them as ''glucan sheath'') is to protect the fungus from toxic defense compounds produced by the host plant. It has also been suggested, again without evidence, that melanins may function in the attachment process or in protecting the fungi from dessication (Bell and Wheeler, 1986; Wheeler and Bell, 1988; Money and Howard, 1996).

3. Experimental

3.1. Fungal culture

An isolate of *B. cinerea* Pers:Fr. (formerly referred to as Bc-1, available from the American Type Culture Collection, ATCC No. 204446) used in earlier studies of adhesion and composition of the extracellular matrix (Cooper et al., 2000; Doss, 1999; Doss et al., 1993, 1995) was used for preparation of the ECM from which the dark pigment was isolated. In addition, to ensure that results were not unique to this isolate, a second isolate (ATCC No. 11542) was also used. Culture of these isolates was as described (Doss, 1999).

3.2. Preparation and composition of the ECM

ECM was prepared using a washing procedure as described previously (Doss, 1999). Tricyclazole [5-methyl-1,2,4-triazolo (3,4-B) benzothiazole], when used, was added to the conidial suspensions from an ethanolic stock solution (2 mg/ml). Weights of dried ECM and of the dark pigment were determined using a Cahn 31 microbalance. Estimates of percent protein, carbohydrate, and lipid were made as described (Doss, 1999).

3.3. Melanin preparation

Samples of ECM (250–500 μg) were heated under reflux in 6 N HCl (155–165 °C) for 24 h to hydrolyze the carbohydrate and polypeptide components. The dark material remaining after hydrolysis was collected in a microcentrifuge tube by centrifugation (14,000*g* for 20 min). The pellet was then washed twice with methanol, once with chloroform:methanol (1:1, v:v) and twice more with methanol. Washing was accomplished by dispersing the pellet in the solvents and collecting the dark pigment by centrifugation. (Note: As the dark substance was difficult to collect from 6 N HCl, in most cases methanol was added prior to the removal of the entire volume of the hydrolysis solution. This enabled a pellet to form readily, and washing to proceed without difficulty.) After overnight drying at room temperature the weight of the dark brown to black material remaining was determined using a microbalance. When this material was to be used for Electron Paramagnetic Resonance (EPR), the drying step was omitted, and the washed preparation was stored at −20 °C in double-deionized water.

3.4. Electron microscopy

Particles of the dark material were subjected to scanning electron microscopy using an AmRay 1000A Scanning Electron Microscope operated at 5 keV. Thin sections of the substance were placed on copper grids and examined using a Philips (CM12-STEM) Transmission Electron Microscope operated at 60 keV.

3.5. Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) was measured in an aqueous suspension of the dark substance. A Bruker ELEXSYS E500 CW-spectrometer at X-band frequencies ($\nu \approx 9.2$ GHz) equipped with continuous gas-flow cryostats for He (Oxford Instruments) and N_2 (Bruker) in the tem-

perature range between 4.2 K and 680 K was used. All spectra were obtained at 150 K with a field-modulation amplitude of 2.0 Oe and a modulation frequency of 100 kHz with a sweep time of 80 s. Due to the Lock-In technique the spectra represent the field derivative of the microwave absorption dP/dH. In order to avoid saturation effects we carefully checked the power dependence of the signal amplitude and finally used a microwave power of 20 μW in the linear regime. The black pigment was investigated after illumination both with red laser light from a HeNe laser ($\lambda = 633$ nm, ~ 10 mW) for 20 min and afterwards with bright white light for 25 min (150 W swan-neck lamp) through an opening in the resonator cavity thereby avoiding any effects that could occur by removing and thawing the sample. Moreover, we studied the EPR spectra in alkaline pH (NaOH) and in the presence of Zn^{2+} ions (0.1 M $ZnCl_2$). Conditions for analysis were those used to allow identification of unknown pigment as a melanin (Enochs et al., 1993).

Acknowledgements

Tricyclazole was provided by Dr. Brian L. Bret of Dow AgriSciences. We thank Dr. Meike Lohmann for preliminary measurements. Two of us (J.D., K.v.N.) were supported by the by the Bundesministerium fuer Bildung and Forschung (BMBF) under Contract No. 13N6917/0.

References

Albersheim, P., Nevins, D.J., English, P.D., Karr, A., 1967. A method for analysis of sugars in plant cell polysaccharides by gas chromatography. Carbohydrate Res. 5, 340–345.

Bell, A.A., Wheeler, M.H., 1986. Biosynthesis and functions of fungal melanins. Ann. Rev. Phytopathol 24, 411–451.

Bar-Nun, N., Tal Lev, A., Harel, E., Mayer, A.M., 1988. Repression of laccase formation in *Botrytis cinerea* and its possible relation to phytopathogenicity. Phytochemistry 27, 2505–2509.

Cooper, L.L.D., Oliver, J.E., De Vilbiss, E.D., Doss, R.P., 2000. Lipid composition of the extracellular matrix of *Botrytis cinerea* germlings. Phytochemistry 53, 293–298.

Doss, R.P., 1999. Composition and enzymatic activity of the ensheathing film secreted by germlings of *Botrytis cinerea*. Appl. Environ. Microbiol. 65, 404–408.

Doss, R.P., Potter, S.W., Chastagner, G.A., Christian, J.K., 1993. Adhesion of nongerminated *Botrytis cinerea* conidia to several substrata. Appl. Environ. Microbiol. 59, 1786–1791.

Doss, R.P., Potter, S.W., Soeldner, A.H., Christian, J.K., Fukunaga, L.E., 1995. Adhesion of germlings of *Botrytis cinerea*. App. Environ. Microbiol. 61, 260–265.

Enochs, W.S., Nilges, M.J., Swartz, H.M., 1993. A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy. Pigment Cell Res. 6, 91–99.

Hodson, MJ., Bar-Nun, N., Mayer, A.M., 1988. Cytochemical localization of laccase in *Botrytis cinerea*. Trans. Br. Mycol. Soc. 89, 572–575.

Gil-ad, N.L., Bar-Nun, N., Mayer, A.M., 2001. The possible function of the glucan sheath of *Botrytis cinerea*: effects on the distrubution of enzyme activities. FEMS Microbiol. Lett. 199, 109–113.

Jarvis, W.R., 1977. *Botryotinia* and *Botrytis* Species: Taxonomy, Pysiology, and Pathogenicity. Monograph No. 15. Canadian Department of Agriculture, Ottawa, Ontario, Canada.

Loganathan, P., Kalyanasundaram, I., 1999. The melanin of the Myxomycete *Stemonitis herbetica*. Acta Protozool. 38, 97–103.

Money, N.P., Howard, R.J., 1996. Confirmation of a link between fungal pigmentation, turgor pressure and pathogenicity using a new method of measurement. Fung. Gene. Biol. 20, 217–227.

Prota, G., 1992. Melanins and Melanogenesis. Academic Press, San Diego, CA.

Sealy, R.C., Felix, C.C., Hyde, J.S., Swartz, M.H., 1980. Structure and reactivity of melanins: influence of free radicals and metal ions. In: Pryor, W.A. (Ed.), Free Radicals in Biology, Vol. IV. Academic Press, New York, pp. 209–259.

Staples, R.C., Mayer, A.M., 1995. Putative virulence factors of *Botrytis cinerea* acting as a wound pathogen. FEMS Microbiol. Lett. 134, 1–7.

Wheeler, M.H., Bell, A.A., 1988. Melanins and their importance in pathogenic fungi. In: McGinnis, M.R. (Ed.), Current Topics in Medical Mycology. Vol. 2. Springer-Verlag, New York, pp. 338–387.

Zeun, R., Buchenauer, H., 1985. Einfluss von Tricyclazol auf die Entwicklung und Melaningehalte der Sklerotien von *Botrytis cinerea*. Phytopath. Z. 112, 259–267.