

Full Length Research Paper

Extraction and characterization of melanin from *Phomopsis*: A phellophytic fungi Isolated from *Azadirachta indica* A. Juss

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Accepted 11 January, 2011

A phellophytic fungus *Phomopsis* (Endophytic fungi residing in the Bark) of *Azadirachta indica* screened for the presence of melanin on their hyphae using an alkali procedure, yielded a dark brown pigment. The purified pigment was subjected to various chemical and physical tests that are diagnostic for melanin. The pigment responds positively for all the diagnostic tests for melanin. Further study on the nature of melanin was done with a bark fungus. The fungus synthesis and deposits DOPA type of melanin on their hyphae.

Key words: Coelomycetes, dark pigment, DOPA melanin, kojic acid, endophyte.

INTRODUCTION

Melanins are polymeric pigments produced by fungi, plants, animals and other microorganisms (Girivasan et al., 1998; Ravishankar et al., 1995; Ellis and Griffiths, 1974; Thathachari and Bolis, 1969). They protect fungus from osmotic shock, (Ravishankar et al., 1995) microbial attack (Linderman and Toussom, 1966) it aids in the prevention of heavy metal toxicity (Miller, 1991) and in providing hydrostatic pressure to the infection structures of pathogenic fungi (Howard and Ferrari, 1989). Bark and leaf are the two extreme environments which the fungus has to overcome the host barriers. So, it was therefore of interest to know if the dark colored phellophyte *Phomopsis* produce such pigments and their possible role in phellophytic mode of survival.

MATERIALS AND METHODS

The dark colored phellophyte isolated from *Azadirachta indica* was grown on CDA medium. The cultures were incubated for one week at 30°C.

Extraction and purification of melanin

Mycelial plug (1 cm dia) were cut from colonies grown on solid

medium, boiled for 5 min in 5 ml of distilled water and centrifuged for 5 min (5000 g). After washing the pellet melanin was extracted by autoclaving the pellet with 3 ml 1M KOH (20 min at 120°C). No more pigment could be extracted after further treatment with 1 M KOH. Therefore, reference to the pigment means "extractable" melanin. The melanin extract was washed in three changes of distilled water and dried over night at 20°C in a dehumidified atmosphere it was purified by acid hydrolysis (5ml of 7M HCL) in a sealed glass vial for 2 h at 100°C. After cooling, the pigment was washed thrice with distilled water and dried.

UV and IR spectrum of the pigment

A known amount of pigment was dissolved in 1M KOH and the absorption (200 to 600 nm) was read in a Hitachi 220A double beam spectrophotometer. The melanin was recorded as the absorption spectrum of alkaline extract of *Phomopsis* melanin using 1 M KOH as the reference blank (Gadd, 1982). The purified pigment was ground with IR quality potassium bromide (1:10) pressed in to discs under vacuum using spectra lab pelletiser and the spectrum (4000 to 500 cm⁻¹) was recorded in Burker 17S 85 FTIR Spectrophotometer (Ellis and Griffiths, 1974).

Treatment with melanin synthesis inhibitor

In order to confirm the nature of melanin produced by *Phomopsis*, the fungus was grown in medium containing compounds that specifically inhibit melanin synthesis (Elliott, 1995). Tricyclazole (specific inhibitor of DHN melanin) and kojic acid and diethyldithiocarbamate (inhibitors of DOPA melanin) were used. Tricyclazole was added to the medium as an ethanolic solution. The

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Table 1. Diagnostic tests for melanin (Thomas, 1955).

Test	Result
Solubility in water	Insoluble
Color	Black
Solubility in 1 M KOH	Soluble
Precipitation in 3N HCl	Precipitated readily
Solubility in organic solvents Chloroform and Ethanol)	(Acetone, Insoluble.
Reaction to oxidizing agent (H ₂ O ₂)	Decolorized
Reaction for polyphenol test (FeCl ₃ test)	Brown precipitate
Reaction with sodium dithionite and Potassium ferricyanide addition of potassium	Decolorized and turned brown with ferricyanide.
Reaction with ammoniacal precipitate lining the nitrate solution	Formed a gray silver colored silver sides of test tube
Dialysis through cellophane	Negative

DOPA melanin inhibitors were dissolved in water and added to the medium. All the inhibitors were added to autoclaved and cooled medium so as to give a final concentration of 0,1,10 and 100 mg/ml (Wheeler and Stipanovic, 1979). The fungus was inoculated in media with above inhibitors and incubated for one week.

One of the many defense roles postulated for melanin is preventing damage due to ultraviolet radiation. The effect of UV radiation on *Phomopsis* fungus was studied by growing the fungus in CDA medium amended with DOPA and DHN melanin inhibitor (100 ug/ml⁻¹) for a week and then exposed to UV radiation from a 4' Philips (Holland) germicidal UV lamp. The colonies were kept at a distance of 9 cm from the UV light source (without the petridish lid) for 5,10 or 15 min. Treated colonies were wrapped in black paper and observed after 24 h. Colonies growing on CDA medium without DOPA melanin inhibitors and treated with UV light were included as control.

RESULT AND DISCUSSION

The dark pigment extracted from *Phomopsis* a phellophyte of *A. indica* answered positively for melanin. The dark brown pigment from the mycelium could not be extracted with organic solvents such as acetone, chloroform, and ethanol. However, pigment extracted from *Phomopsis* using the alkali procedure of Gadd (1982), responded positively to all the physical and chemical tests (Table 1). The pigment had properties in common with natural melanin studied by others workers (Ellis and Griffiths, 1974). All the extracts were insoluble in water and organic solvents and appeared to have high molecular weight because it could not be dialyzed from an alkaline solution. Similar observation was made in *Cirrenalia pygmea* a marine fungus (Ravishankar et al., 1995). It was decolorized by hydrogen peroxide an

oxidizing agent and gave a positive reaction for polyphenols.

Further characterization of melanin was done using IR and UV spectra. In the spectral study, the absorption showed a characteristic peak in the UV region of wavelength ranging from 200 to 260 nm (Figure 1) but none in the visible region (Bell and Wheeler, 1986; Ravishankar et al., 1995). The absorption of light by melanin is maximum in the UV region and decreased progressively as the wavelength increases this decrease is nearly linear with increasing wavelength. Another procedure normally used by many investigators to characterize melanin is that the log of the optical density of a melanin when plotted against wavelength gave linear curve with negative slope (Bartnicki-Gracia and Reyes, 1964; Ravishankar et al., 1995). The melanin of *Phomopsis* also gave a characteristic straight line with negative slope (Figure 2). Such characteristic straight line with negative slope has been obtained for some terrestrial fungi (Chet et al., 1967; Ellis and Griffith, 1974). The IR spectrum of *Phomopsis* showed absorption peaks near 3 cm⁻¹ (attributed to OH and NH bonds) and near 6 cm⁻¹ attributed to conjugated carbonyl bonds (Bonner and Duncan, 1962) (Figure 3). Similar results were obtained by (Ellis and Griffith, 1974; Ravishankar et al., 1995) they showed that melanin of terrestrial and marine fungus *Cirrenalia pygmea* have principal absorption peaks at these wavelengths. These absorptions are common for melanin from several biological materials (Bonner and Duncan, 1962).

Inhibition of melanin was seen in *Phomopsis* grown in the presence of diethyldithiocarbamate and kojic acid amended media. This was indicated by the lack of dark

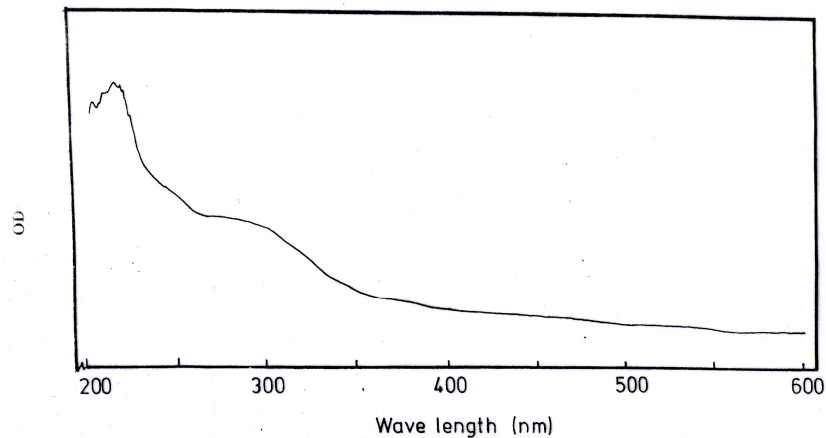


Figure 1. Ultraviolet and visible spectrum of melanin.

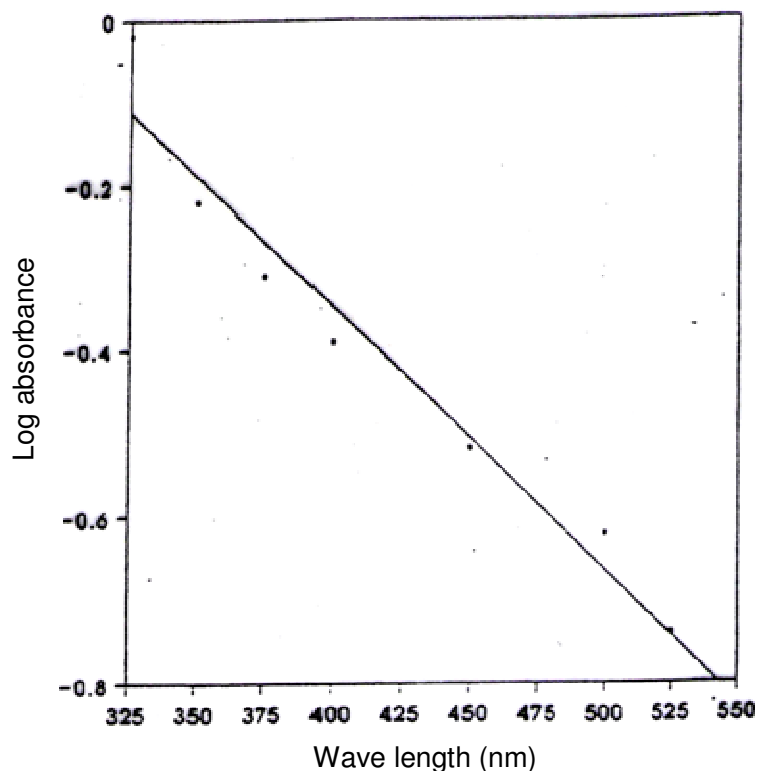


Figure 2. Linear plot with negative slope obtained from extracted melanin.

pigment in the mycelium. However, colonies growing in tricyclazole amended medium produce normal, dark coloured hyphae. These results suggested that *Phomopsis* synthesis and deposits DOPA melanin. Since diethyldithiocarbamate and kojic acid are the specific inhibitor of DOPA type of melanin. The presence of DHN and DOPA type of melanin is several terrestrial marine fungi (Ravishankar et al., 1995; Bell and Wheeler,

1986) and other microbes were reported. To our knowledge this was the first report that *Phomopsis* a phellophyte has been reported to produce DOPA type of melanin.

Thus apart from protecting fungal hyphae from desiccation, microbial attack, UV radiation and osmotic shock (Bell and Wheeler, 1986; Linderman and Toussoun, 1966; Durrel, 1964; Ravishankar et al., 1995)

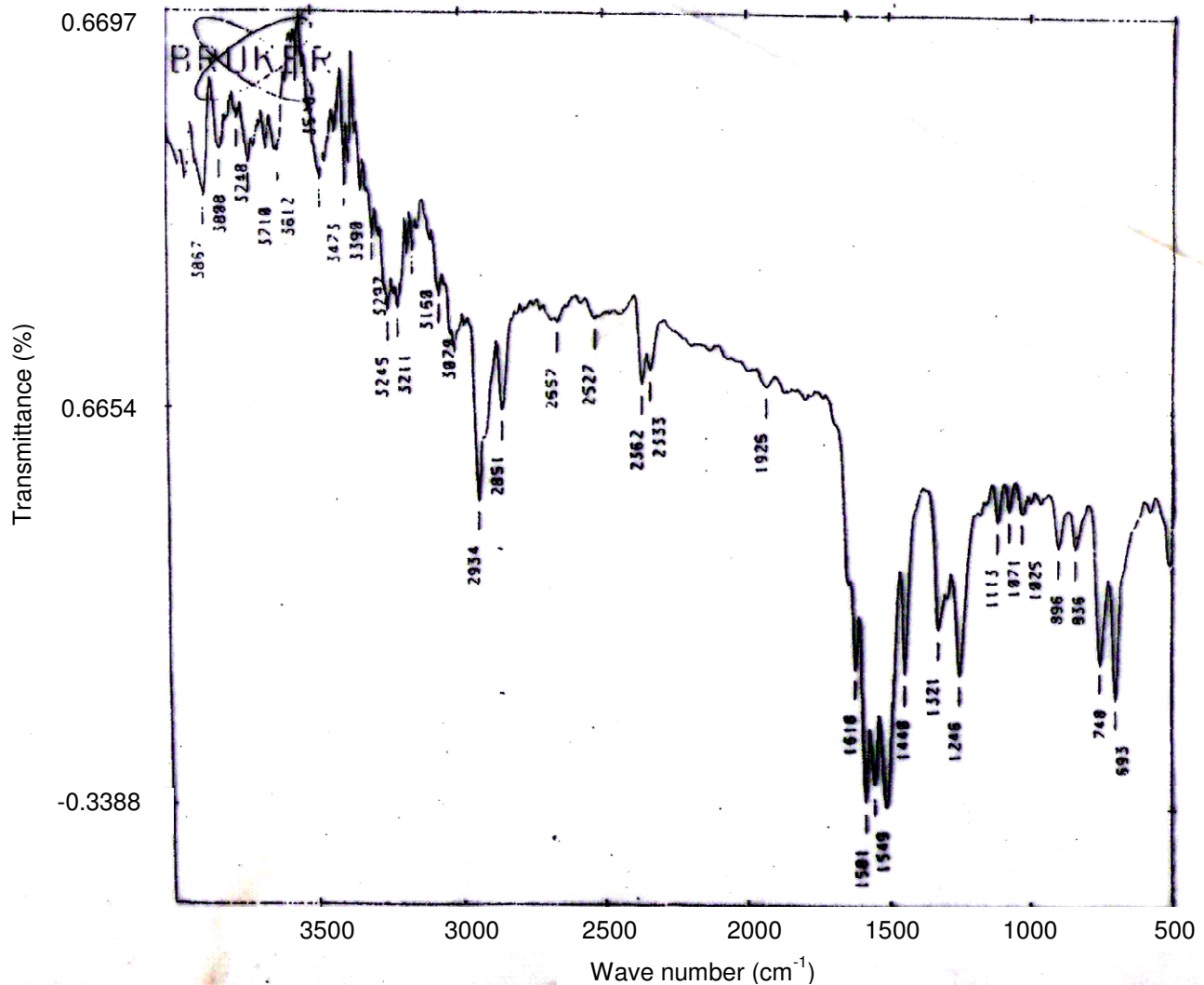


Figure 3. Infrared red spectrum of melanin.

melanin may also protect fungi especially phelloglyte from host defense reactions to overcome host barriers and to survive in harsh environments such as bark and leaf. Considering the multifarious role-played by melanin, it would certainly be advantageous for a phelloglyte to synthesize this pigment. Our finding amply supports this that *Phomopsis* the dark colored phelloglyte isolate had melanin in them. Thus, it is obvious that *Phomopsis* a phelloglyte isolated from bark have evolved panoply of adaptations, which include mechanisms for overcoming host barriers, successful competition with phylloplane fungi and surviving harsh environmental condition such as bark by synthesizing this pigment.

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