Mycologia, 75(3), 1983, pp. 562–566.

MELANIN-LIKE PIGMENT IN ZONE LINES OF *PHELLINUS WEIRII*-COLONIZED WOOD

C. Y. Li

U. S. Department of Agriculture, Forest Service, Pacific Northwest Forest and Range Experiment Station, Forestry Sciences Laboratory, Corvallis, Oregon 97331

Phellinus weirii (Murrill) Gilbertson, a serious root pathogen of conifers in the northwestern United States and southwestern Canada, often forms darkly pigmented zone lines in colonized wood. Zone lines are sheets of pigmented fungal tissues composed of characteristic swollen hyphal cells, which, when sectioned, appear as distinct, narrow lines. Zone lines enhance *P. weirii* survival in colonized wood by excluding antagonists (Nelson, **1964**, **1975**).

Melanins are polymeric pigments of unknown chemical structure produced

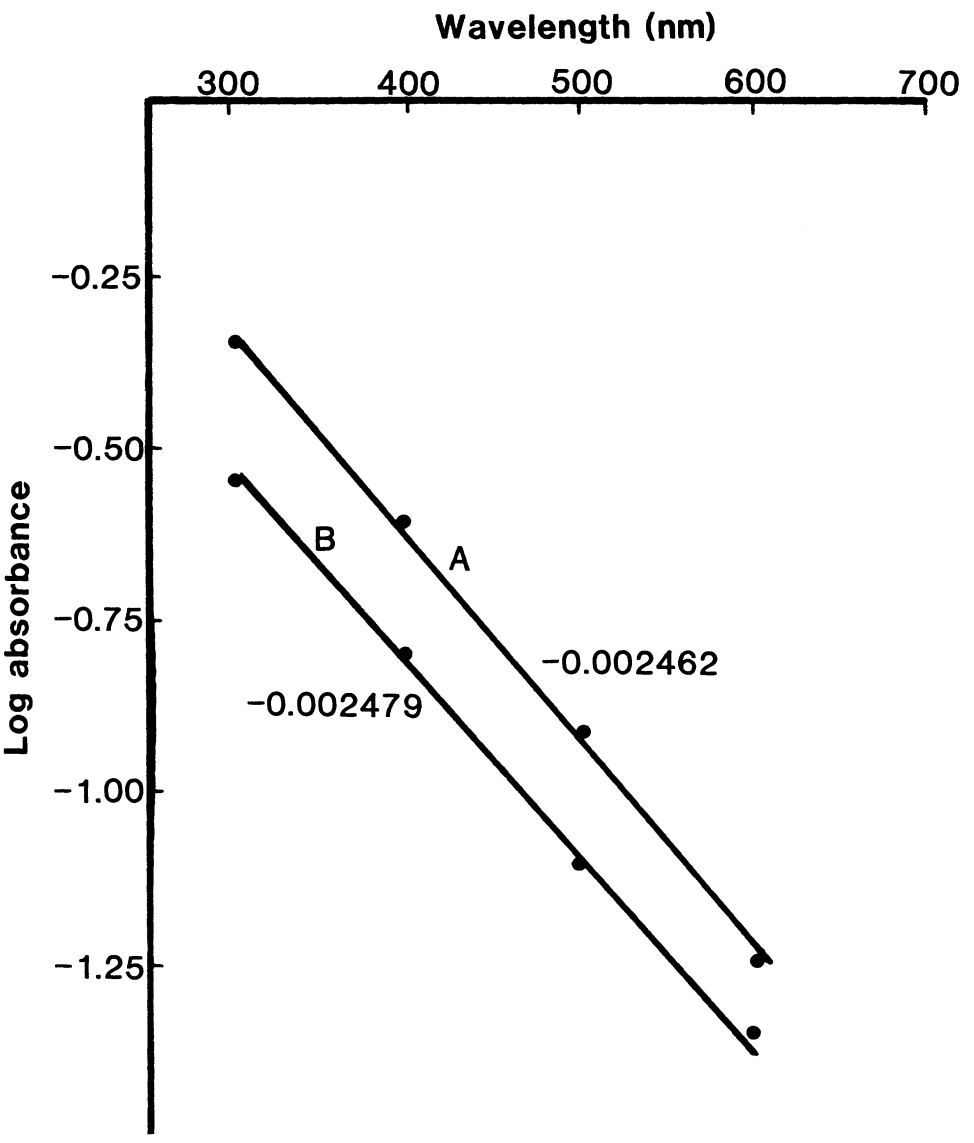

FIG. 1. Absorbance spectra of extracted pigment from zone lines of *P. weirii* (A) and synthetic tyrosine-melanin (B).

by a wide variety of fungi. There are numerous examples of fungal structures particularly resistant to harsh environments. Most of these resistant structures contain black or brownish-black pigments which have been described as melanins. The biological roles of melanins in protecting hyphae against microbial lysis are well-documented (Kuo and Alexander, **1967**; Bull, **1970**; Kubo *et al.*, **1982**). In the present study, the pigment was extracted from the zone lines formed by *P. weirii* in colonized wood, then analyzed to define its physical and chemical properties. These properties were then compared with those which biologists hold to be characteristic of melanins. The effect of the extracted pigment on *in vitro* growth rate of some antagonists of *P. weirii* also was investigated.

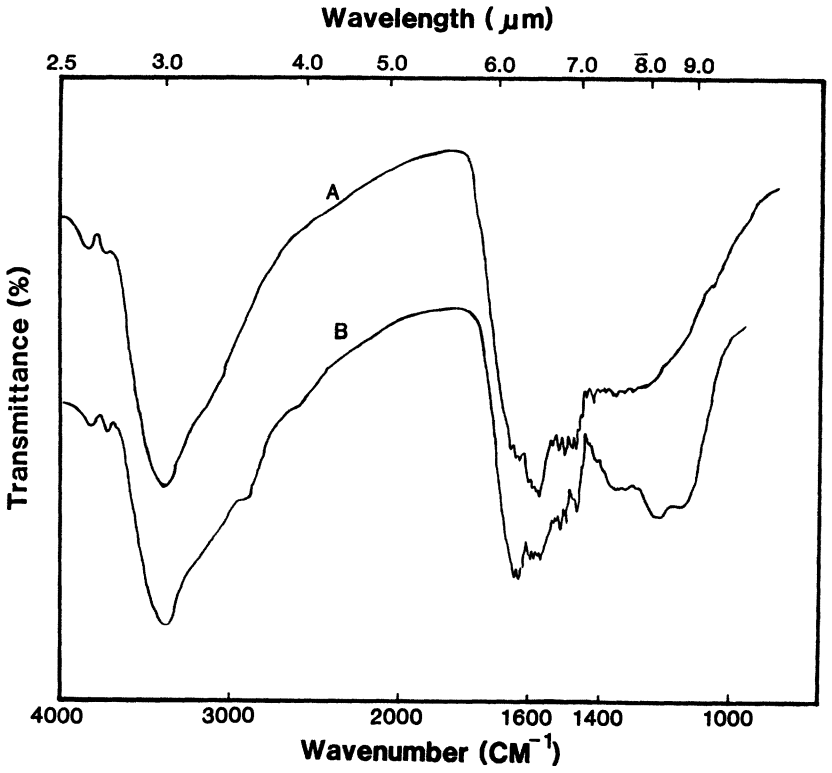

FIG. 2. Infrared spectra of the extracted pigment and synthetic tyrosine-melanin. A. Synthetic tyrosine-melanin. B. Extracted pigment from zone lines of *P. weirii* in wood.

Twenty grams of pigmented fungal tissue (zone lines) of *P. weirii,* separated from colonized Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] stem wood with advanced decay, were homogenized in 150 ml of 1 M NaOH by a Sorvall Omni-mixer at maximum power for 5 min, then sonicated by an ultrasonic disintegrator at maximum power for 10 min. The cellular debris was extracted by a 2-h treatment with hot alkali by boiling water under reflux in an atmosphere of nitrogen. After filtration through three layers of Watman's No. 1 filter paper, the dark brown filtrate was acidified to pH 2. The resultant dark brown precipitate was collected by centrifugation, washed twice in distilled water, and freeze-dried. The dried dark brown powder was hydrolyzed with 7 M HCl for 2 h at 100 C, then washed with water and dried. Visible and ultraviolet spectra of the extracted pigment in 1 M NaOH solution were determined with a Beckman DB recording spectrophotometer. The infrared spectrum was obtained with a Beckman IR-20A spectrophotometer by using 5 mg of the extracted pigment pressed into a pellet with 100 mg of potassium bromide.

Characterization of the extracted pigment in terms of its degradation products was attempted by grinding together 100 mg of pigment material and 1 g powdered NaOH. The mixture was transferred to a small platinum crucible, two drops of water added, and, while a slow stream of nitrogen gas was directed into the crucible, heated for 10 min in a bath of sodium nitrate maintained at its melting point (Hackman and Goldberg, **1971**). The fused mass was extracted with 2.5% acetic

acid and the solution extracted twice with ethyl acetate. The combined ethyl acetate was evaporated and the residue dissolved in 2 ml benzene. The benzene solution was spotted on a cellulose powder thin-layer plate, which was developed in benzene-acetic acid-water (125:72:3) or *n*-butanol-acetic acid-water (72:18:30). The plate was dried and sprayed with 3% $FeCl_3$ in ethanol or diazotized *p*-nitroaniline followed by 2 M NaOH. The same procedures were repeated with the synthetic tyrosine-melanin (Sigma Chemical Co., St. Louis, Missouri). Authentic compounds, commonly found in the degradation products of melanins (Nicolaus *et al.*, **1964**), were run on the same plate for comparison.

Growth of several microorganisms antagonistic toward *P. weirii* was measured in the presence of extracted pigment. The fungi, *Geotrichum* sp., *P. weirii, Scytalidium* sp., *Trichoderma viride* Pers., and other unidentified isolates from *P. weirii*-colonized wood were each placed on 20 ml malt agar thoroughly mixed with 50 or 150 mg of the pigment. A plug of inoculum taken from the edge of each fungal colony was placed inverted on the medium in the center of each plate. Plates were incubated at 21–24 C. Radial growth of the antagonistic fungi was measured. Growth effects on *Bacillus* sp. (Hutchins, **1980**) and *Streptomyces griseoloalbus* (Rose *et al.*, **1980**) were measured on malt-yeast-peptone agar or nutrient agar thoroughly mixed with a bacterial suspension in water after a paper disc 13 mm in diam, with 10 or 15 mg of the pigment on its top, was placed in the center of each agar plate. Clear zones devoid of bacterial growth at the edges of the paper discs indicated inhibition by the pigment.

The pigment extracted from the zone lines of *P. weirii*-colonized wood was dark brown. Its absorption spectrum showed no peaks in the 300 to 600 nm range. Plotting the logarithm of absorbance versus wavelength gave a linear curve with a slope of −0.002462, an identical negative slope to that of the synthetic tyrosine-melanin (FIG. 1). Infrared absorption patterns of the extracted fungal pigment and of the tyrosine-melanin are essentially the same (FIG. 2). The absorbances can be ascribed to the following groups: 2.9 μm to (-OH), 5.9 μm to carboxylic or phenolic groups, 6.1 μm to H-bonded quinone or unsaturated carbonyl groups, and 6.6 μm to (-CH). Diagnostic tests used to identify melanin showed that the extracted pigment had properties in common with tyrosine-melanin. It was only partly soluble in water and formed dark brown flocculent precipitates, was insoluble in both hydrochloric acid and the organic solvents, was decolorized by oxidizing agents (NaOCl and H_2O_2), and gave a positive reaction for polyphenols by producing flocculent brown precipitates with $FeCl_3$.

Attempts to characterize the extracted fungal pigment in terms of its degradation products were only partially successful. Alkali fusion of the extracted pigment or of the synthetic tyrosine-melanin with NaOH resulted in dark-colored solid mixtures. Ethyl acetate extractions, however, yielded similar, but unidentifiable products. One product had an Rf value and color reactions similar to salicylic acid. Spectrophotometric analysis, however, showed the compound to be other than salicylic acid.

Characterization of the extracted pigment in terms of its absorption spectra in UV-visible and IR radiations, degradation products, and other properties indicated that the dark pigment in zone lines of *P. weirii* colonized wood is tyrosine-melanin in nature.

Some of the microorganisms antagonistic toward *P. weirii* were sensitive to the extracted pigment. *Bacillus* sp. and *Streptomyces griseoloalbus,* strongly inhibitory to *P. weirii* and other root rot fungi in culture, were inhibited by the pigment at the concentrations tested. Of all antagonistic fungi isolated from *P. weirii*-colonized wood, only a *Scytalidium* sp. with white mycelium and producing

dark pigment in potato-dextrose agar was inhibited by the pigment at 50 or 150 mg concentration. *Trichoderma viride*, a common antagonist in soil and wood, grew well *in vitro* with the extracted pigment, as did *P. weirii* and other fungi.

From results obtained in the present study, I believe the heavy pigmentation of zone lines in *P. weirii* colonized wood is caused by the accumulation of melanin, which is probably important in the prolonged survival of *P. weirii* in infested stumps. In addition to direct growth inhibition of certain antagonistic microorganisms, melanin might protect *P. weirii* from microbial lysis. The resistance of *Aspergillus nidulans* (Eidam) Wint. to lysis by cell wall degrading enzymes is caused by melanin in the fungal walls; the resistance is directly correlated with the melanin content of the mycelium (Kuo and Alexander, **1967**). Similarly, soil microorganisms decomposing hyphae of *Sclerotium rolfsii* Sacc. cannot attack the melanin-covered sclerotia produced by this fungus (Bloomfield and Alexander, **1967**). Furthermore, cell wall degrading enzymes are susceptible to inhibition by the fungal melanin (Bull, **1970**).

I thank Dr. Everett Hansen, Oregon State University, for providing the bacterial and fungal cultures he isolated from *P. weirii*-colonized wood. I am grateful for the technical assistance of Susan N. Schechter.

Key Words: *Phellinus weirii*, melanin, laminated root rot, zone line.

LITERATURE CITED

Bloomfield, B. J., and M. Alexander. 1967. Melanin and resistance of fungi to lysis. *J. Bacteriol.* **93**: 1276–1280.

Bull, A. T. 1970. Inhibition of polysaccharases by melanin: enzyme inhibition in relation to mycolysis. *Arch. Biochem. Biophys.* **137**: 345–356.

Hackman, R. H., and M. Goldberg. 1971. Microchemical detection of melanins. *Anal. Biochem.* **41**: 279–285.

Hutchins, A. S. 1980. *In vitro* inhibition of root rot pathogens *Phellinus weirii, Armillaria mellea, Fomes annosus,* and *Phytophthora cinnamomi* by a newly isolated *Bacillus* sp. *Microbial Ecol.* **6**: 253–259.

Kubo, Y., K. Suzuki, I. Furusawa, N. Ishida, and M. Yamamoto. 1982. Relationship of appressorium pigmentation and penetration of nitrocellulose membranes by *Colletotrichum lagenarium*. *Phytopathology* **72**: 498–501.

Kuo, M. J., and M. Alexander. 1967. Inhibition of the lysis of fungi by melanins. *J. Bacteriol.* **94**: 624–629.

Nelson, E. E. 1964. Some probable relationships of soil fungi and zone lines to survival of *Poria weirii* in buried wood blocks. *Phytopathology* **54**: 120–121.

———. 1975. Survival of *Poria weirii* in wood buried in urea-amended forest soil. *Phytopathology* **65**: 501–502.

Nicolaus, R. A., M. Piatelli, and E. Fattorusso. 1964. The structure of melanins and melanogenesis—IV. On some natural melanins. *Tetrahedron* **20**: 1163–1172.

Rose, S. L., C. Y. Li, and A. S. Hutchins. 1980. A streptomycete antagonist of *Phellinus weirii, Fomes annosus* and *Phytophthora cinnamomi*. *Canad. J. Microbiology* **26**: 583–587.