- litter, humus, and subsoil of a pine strand. *Pedobiologia* 9:358-365.
27. Yamaguchi, M., W.J. Flocker, and F.D. Howard. 1967. Soil atmosphere as influenced by temperature and moisture. *Soil*

- Sci. Soc. Am. Proc.* 31:164-167.
28. Yugakov, P.S., and Y.E. Popova. 1968. Carbon dioxide regime in soils of the Krasnoyarsk forest steppe. *Sov. Soil Sci.* 6:795-800.

The Persulfate Oxidation of Fungal Melanins¹

F. MARTIN, F. J. GONZALEZ-VILA, AND J. P. MARTIN²

ABSTRACT

Four fungal melanins were degraded by potassium persulfate, a new, mild chemical oxidation method. The percentage of degradation varied from 15 to 50% calculated by weight loss. Analysis of the degradation products by gas chromatography showed numerous fatty acids, both straight and branched chain, in widely varying amounts. The *n*-C₁₆ and *n*-C₁₈ acids were present in greatest abundance. By two-dimensional thin-layer chromatography, several phenols and anthraquinones were noted.

Additional Index Words: melanins, persulfate oxidation, fatty acids, anthraquinones, phenols.

Martin, F., F.J. Gonzalez-Vila, and J.P. Martin. 1983. The persulfate oxidation of fungal melanins. *Soil Sci. Soc. Am. J.* 47:1145-1148.

MANY SOIL FUNGI synthesize dark-colored polymers, called melanins, which are soluble in alkali and precipitated by acid, as are soil humic acids. There is a large variation in the number and kinds of structural units in fungal melanins. The phenolic constituents of some are mostly of the resorcinol type derived from orsellinic acid (Haider et al., 1975), and others contain both orsellinic and *p*-hydroxycinnamic acid-derived phenols. *Eurotium echinulatum* and *Aspergillus glaucus* melanins contain a variety of anthraquinone structures as well as numerous phenols (Linhares and Martin, 1979b; Saiz-Jimenez et al., 1975). During polymerization by enzymatic or autoxidative reactions, proteins, various aliphatic structures including cell wall materials, may be linked into the fungal polymers (Kononova, 1966; Haider et al., 1975; Nelson et al., 1979).

Melanins from *Epicoccum nigrum*, *Aspergillus sydowii*, *Hendersonula toruloidea*, *E. echinulatum* and some *A. glaucus* isolates appear to be similar to soil humic acids with respect to elemental composition, high exchange acidity (Linhares and Martin, 1978), amino acid released upon 6M HCl hydrolysis (Haider et al., 1975), phenols released upon Na-amalgam reductive degradation (Martin et al., 1974), resistance to microbial degradation (Hayes and Swift, 1978; Linhares and Martin, 1979a), fragments released by oxidative degradation (Schnitzer et al., 1973), and pyrolysis techniques (Haider et al., 1977; Meuzelaar et al., 1977; Saiz-Jimenez et al., 1979). The melanin from *Stachibotrys* species appears to contain more aliphatic

structures than that of most other fungi (Schnitzer and Neyroud, 1975), and that from *Aspergillus niger* spores contains > 50% polysaccharide (Saiz-Jimenez et al., 1979).

In view of these properties it has been considered very likely that these fungal melanins could contribute to the soil humus fraction.

Recently, Martin et al. (1981) developed a new, mild oxidative degradation method for soil humic acids that involves the use of potassium persulfate under acid conditions. The method releases 30 to 40% of the starting material and is recommended as a first degradative treatment in a sequence for the characterization of structural units of humic and fulvic acids (Martin and Gonzalez-Vila, 1983). The procedure showed marked differences in the amounts and relative abundance of the different fragments released from humic materials.

The purpose of this paper is to identify structural components released by persulfate oxidation of a variety of fungal melanins to gain a better understanding of the structure of such humic acid-like polymers.

MATERIALS AND METHODS

The melanins employed in this study were obtained from the fungi, *S. atra*, *E. echinulatum*, *A. glaucus*, and *H. toruloidea*. Cultural media for synthesis, methods for isolation of the polymers, and properties of the melanins have been described (Linhares and Martin, 1978).

The oxidation method was described by Martin et al. (1981). Briefly, 200 mg of melanin was placed in a Teflon beaker with 25 mL of quartz distilled water to which 50 mL of a 5% solution of potassium persulfate was added. The beaker was placed in a digestion bomb and heated with stirring at 140°C for 2 h. After cooling, the residue was separated by centrifugation and washed with water several times. The supernatant solution plus washings were extracted with ethyl acetate for 48 h in a liquid-liquid extractor. The extract was dried with purified sodium sulfate, filtered, and the ethyl acetate evaporated in a rotary evaporator. The residue was suspended in methanol and methylated with an excess of diazomethane.

All solvents were HPLC grade and twice distilled in glass. No peaks were observed by gas chromatography after a blank run of every solvent employed.

The methylated degradation products were analyzed using a gas chromatograph-mass spectrometer-computer system (Hewlett Packard, model 5992 B). Separation was achieved by use of a fused silica capillary column 25 m long, 0.2 mm i.d., coated with SP-2100, with the column temperature programmed from 100 to 270°C at a rate of 4°C/min. Mass spectrometer conditions were mass peak detect threshold 20 linear counts, scan speed 690 atomic mass unit second, electron multiplier 1400 V, gas chromatograph detect threshold 500, ionizing voltage 70 eV, and mass range 30 to 400.

¹ Contribution from the Centro de Edafología. C.S.I.C. Apartado 1052, Sevilla, Spain, and Dep. of Soil and Environmental Sciences, Univ. of California, Riverside. Received 24 March 1983. Approved 30 June 1983.

² Soil Biochemists and Professor Emeritus, respectively.

The methylated oxidation products of the melanins were also separated by TLC on Al_2O_3 plates for two-dimensional chromatography. The plates were first developed in 3:1 benzene-ethyl acetate. After a 60-min drying period, the plates were developed in 9:1 dibutyl ether-acetic acid. Identification of anthraquinones and phenols were accomplished as previously reported (Saiz-Jimenez et al., 1975). The anthraquinone spots were scraped off, extracted with methanol, evaporated to dryness, and analyzed by direct insertion in an AEI model MS-30 mass spectrometer.

RESULTS

The degradation percentages calculated by loss of weight upon oxidation were as follows: *H. toruloidea*, 50%; *S. atra*, 15%; *E. echinulatum*, 40%; and *A. glaucus*, 25%.

Figure 1 shows the gas chromatograms of the melanin oxidation products. On each peak is the carbon number of the identified fatty acid. The identification of each compound was achieved by its mass spectra, selected ion monitoring (SIM), and in some cases confirmed on the basis of retention time of standards.

Most of the oxidation products were fatty acids, which show typical fragmentation patterns. Application of the SIM technique made it possible to identify fatty acids present in minute amounts. This was not possible with other types of aliphatic compounds present in similar or greater amounts.

Tables 1 and 2 summarize the identified fatty acids. n-Alkanes in the range C_{24} to C_{30} with a major peak at C_{25} were detected in very small amounts using the SIM technique only in the *H. toruloidea* melanin. In all cases major peaks were those corresponding to C_{16} and C_{18} fatty acids, and as shown by carbon preference index even carbons predominate. Though not shown in the gas chromatograms, by SIM techniques fatty acids up to C_{24} were observed in all four melanins. Also noted were some dialkyl phthalates, ethyl *p*-toluene solphonate, and *n*- $C_9\alpha, \omega$ dicarboxylic acid.

The *E. echinulatum* and *A. glaucus* melanins yielded eight anthraquinones (the same in both cases) and several spots corresponding to compounds of phenolic character. The *H. toruloidea* and *S. atra* melanins showed up to 15 phenolic spots.

Fig. 1—Gas chromatograms of persulfate oxidation products of fungal melanins. n- represents straight chain and b, b', b'' represent different branching.

DISCUSSION

The degradation percentages of the melanins (15–50%) were similar to those of 30 and 40% that have been obtained for a variety of soil humic acids (unpublished data). Aliphatic acids especially $n\text{-C}_{16}$ and $n\text{-C}_{18}$ acids were the most abundant compounds identified and collectively accounted for 70 to 80% of the degraded fraction. Amounts of sugars and amino acids released by persulfate oxidation were small compared with the quantities released from more vigorous acid hydrolysis. *H. toruloidea* contains a higher amount of N and carbohydrates (Lüdemann et al., 1982), and these compounds could contribute to the higher degradation percentage (50%).

Comparing the chromatograms shown in Fig. 1, we can divide the fungal melanins into two groups. In the first group, $n\text{-C}_{16}$ fatty acid is by far the most abundant degradation product, and branched fatty acids as well as other products are present. This group is constituted by *H. toruloidea* and *S. atra*. The second group gave weaker chromatograms, and although $n\text{-C}_{16}$ fatty acid is the major degradation product, the peak amounts of n - and branched fatty acids are relatively small. This group is constituted by *E. echinulatum* and *A. glaucus*.

A more detailed study of the ^{13}C nuclear magnetic resonance spectra of these melanins (Lüdemann et al., 1982) supports the grouping of the melanins since the *H. toruloidea* and *S. atra* polymers showed very high peaks in the area of resonance for aliphatic-protein carbons (10–50 ppm) to which fatty acids could contribute. In contrast the spectra of *E. echinulatum* and *A. glaucus* both showed a single less pronounced peak in this area, which have indicated a lower aliphatic character.

Schnitzer and Neyroud (1975) found that $n\text{-C}_{16}$ and $n\text{-C}_{18}$ fatty acids were major degradation products of certain fungal melanins and that branched fatty acids were present in the alkaline cupric oxide oxidation products. Mendez and Ogner (1979) identified straight and branched fatty acids by potassium permanganate oxidation of algal humic-like substances, but their presence was believed to be related to bacterial synthesis during the humification process.

Aliphatic acids, especially the $n\text{-C}_{16}$ and $n\text{-C}_{18}$ acids, are major degradation products released from the KMnO_4 and alkaline CuO oxidation of soil humic and fulvic acids (Schnitzer, 1977). A comparison of major types of products formed by persulfate oxidation of fungal melanins with those obtained previously from two fulvic acids (Martin and Gonzalez-Vila, 1983) and three humic acids (unpublished data) is of interest. Fulvic acid isolated from a Humic Haplorthod soil gave phenolic acids as major oxidation products, whereas fulvic acid from an Andic Hapludult yielded

Table 1—Straight chain fatty acids released from fungal melanins by persulfate oxidation.

Melanins	n-fatty acid (as methyl esters)†										CPI‡
	13	14	15	16	17	18	19	20	21		
<i>H. toruloidea</i>		22	18	100	7	32	7	2	2	4.6	
<i>S. atra</i>	30	11	4	100	7	42	11	14	7	3.0	
<i>E. echinulatum</i>		48	35	100	24	60	28			2.4	
<i>A. glaucus</i>	28	34	17	100	34	71	8	8	8	1.9	

† Expressed as relative percentage of most abundant component.

‡ CPI = carbon preference index, determined as $\text{CPI} = \frac{\sum \text{odd } C_{13}-C_{21}}{\sum \text{even } C_{14}-C_{20}}$.

benzenecarboxylic acids as the most abundant compounds. Humic acid extracted from an Inceptisol produced fatty, benzenecarboxylic, and phenolic acids in decreasing order. Humic acid from a Typic Chromoxerert gave largely dialkyl phthalates, and humic acid from a Humic Haplorthod almost exclusively straight and branched fatty acids, although small amounts of aromatic compounds were identified. In the fungal melanin degradation products none of the above-cited aromatic compounds was detected.

To further test for the presence of other aromatic substances not detected by the gas chromatograph procedure, the oxidation products were separated by TLC. The anthraquinone spots were scraped off from the TLC plates and studied by direct insertion probe mass spectrometry. Due to the small amount, the mass spectra were not well resolved; however, by comparison with known samples, the anthraquinones found after persulfate oxidation of the melanins were not the same as those reported (Linhares and Martin, 1979b; Saiz-Jimenez et al., 1975) to be present in the culture media of these fungi. An alteration by persulfate oxidation of the original anthraquinones cannot be excluded.

Differences in the relative amounts of various constituents between specific melanins and humic polymers revealed in this and other studies were expected. It appears that soil humic and fulvic acids are characterized by a preponderance of aromatic acids and phenols, whereas fungal melanins have greater quantities of aliphatic structures. The melanins tested have not been in soil. Numerous melanins could be synthesized in the soil and after their release, the more biodegradable parts of the polymers would be slowly utilized by the soil population. Also, parts of the molecules could be linked into existing humic polymers through the action of soil enzymes. With time, the remaining structures could more nearly approach those of the older soil humic polymers. Also, the amounts and types of various fragments released by the oxidation will vary with the C and N sources of the fungi. The quantity and nature of the nitrogen source will influence the peptide content of the melanins (Linhares and Martin, 1978), and when substrates con-

Table 2—Branched fatty acids released from fungal melanins by persulfate oxidation.†

Melanins	14b	14b'	15b	15b'	16b	16b'	16b''	17b	18b	18:1	18:2	19b	10b
<i>H. toruloidea</i>	14	28	18	25	29	19	36		11	9	7		
<i>S. atra</i>	14	4	6	6	2			7	7			4	4
<i>E. echinulatum</i>	32		35										
<i>A. glaucus</i>	28	7	28		20				24			24	

† Expressed as relative percentage of most abundant component. b, b', b'' represent different branching; 18:1 means $n\text{-C}_{18}$, monounsaturated, and 18:2 means $n\text{-C}_{18}$, diunsaturated.

taining lignin are degraded, the melanins may contain lignin-derived units (Martin et al., 1974).

In conclusion, the persulfate oxidation procedure shows structural differences among melanins and between these and humic substance from different origins and may be used as a first step in a sequential degradation. Since it is a mild oxidative reagent and therefore releases only the readily oxidizable parts of the molecule, it shows that fungal melanins are complex polymers whose structural network are very resistant to degradation, but the more oxidizable portions contain primarily fatty acids and some phenolic compounds.

REFERENCES

- Haider, K., J.P. Martin, and Z. Filip. 1975. Humus biochemistry. p. 145-244. In E.A. Paul and A.D. McLaren (ed.). Soil biochemistry, vol. 4. Marcel Dekker, Inc., New York.
- Haider, K., B.R. Nagar, C. Saiz, H.L.D. Meuzelaar, and J.P. Martin. 1977. Studies on soil humic compounds, fungal melanins and model polymers by pyrolysis-mass spectrometry. IAEA, Vienna, p. 211-220.
- Hayes, M.H.B., and R.S. Swift. 1978. The chemistry of soil organic colloids. p. 179-320. In D.J. Greenland and M.H.B. Hayes (ed.) The chemistry of soil constituents. John Wiley & Sons, Inc., New York.
- Kononova, M.M. 1966. Soil organic matter; its nature, its role in soil formation and soil fertility. (2nd ed.) Pergamon Press, Oxford.
- Linhares, L.F., and J.P. Martin. 1978. Decomposition in soil of the humic acid-type melanins of *Eurotium echinulatum*, *Aspergillus glaucus* sp. and other fungi. Soil Sci. Soc. Am. J. 42:738-743.
- Linhares, L.F., and J.P. Martin. 1979a. Carbohydrate content of fungal humic acid-type polymers (melanins). Soil Sci. Soc. Am. J. 43:313-318.
- Linhares, L.F., and J.P. Martin. 1979b. Decomposition in soil of E. Emodin, Chrysophanic acid and a mixture of anthraquinones synthesized by an *Aspergillus glaucus* isolate. Soil Sci. Soc. Am. J. 43:940-945.
- Lüdemann, H.D., H. Lentz, and J.P. Martin. 1982. Carbon-13 nuclear magnetic resonance spectra of some fungal melanins and humic acids. Soil Sci. Soc. Am. J. 46:957-963.
- Martin, F., C. Saiz-Jimenez, and V.J. Gonzalez-Vila. 1981. The persulfate oxidation of soil humic acid. Soil Sci. 132:200-203.
- Martin, F., and F.J. Gonzalez-Vila. 1983. The persulfate oxidation of fulvic acids. Z. Pflanzenernaehr. Bodenk. 146:409-414.
- Martin, J.P., K. Haider, and C. Saiz-Jimenez. 1974. Sodium amalgam reductive degradation of fungal and model phenolic polymers, soil humic acids and simple phenolic compounds. Soil Sci. Soc. Am. Proc. 38:360-364.
- Mendez, J., and C. Ogner. 1979. Permanganate oxidation products of algal humic-like substances. Chem. Geol. 27:265-270.
- Meuzelaar, H.L.C., K. Haider, B.R. Nagar, and J.P. Martin. 1977. Comparative studies of pyrolysis-mass spectra of melanins, model phenolic polymers and humic acid. Geoderma 17:239-252.
- Nelson, D.W., J.P. Martin, and J.O. Ervin. 1979. Decomposition of microbial cells and components in soil and their stabilization through complexing with model humic acid-type phenolic polymers. Soil Sci. Soc. Am. J. 43:84-88.
- Saiz-Jimenez, C., K. Haider, and J.P. Martin. 1975. Anthraquinones and phenols as intermediates in the formation of dark-colored humic-like pigments by *Eurotium echinulatum*. Soil Sci. Soc. Am. Proc. 37:649-653.
- Saiz-Jimenez, C., F. Martin, and A. Cert. 1979. Low boiling point compounds produced by pyrolysis of fungal melanins and model phenolic polymers. Soil Biol. Biochem. 11:305-309.
- Schnitzer, M., M.I. Ortiz de Serra, and K. Ivarson. 1973. The chemistry of fungal humic acid-like polymers and of soil humic acids. Soil Sci. Soc. Am. Proc. 37:229-236.
- Schnitzer, M., and J.A. Neyroud. 1975. Further investigation on the chemistry of fungal "humic acids." Soil Biol. Biochem. 7:365-371.
- Schnitzer, M. 1977. Recent findings on the characterization of humic substances extracted from soils of widely differing climatic zones. p. 117-121. In Soil organic matter studies, vol. II. IAEA, Vienna.

Characteristics of *Rhizobium trifolii* Populations Associated with Subclover in Mississippi Soils¹

C. HAGEDORN, A. H. ARDAHL, AND L. A. MATERON²

ABSTRACT

Indigenous *Rhizobium trifolii* populations that would nodulate *Trifolium subterraneum* L. (cv. Mt. Barker) were isolated and characterized from pastures with and without subclover in five distinct soil regions in Mississippi. Considerable variation in effectiveness was found among isolates from the different areas and from individual sampling sites within areas, and the combined results indicated that subclover production and soil fertility levels were related and were being better maintained within two of the sampling areas. Pastures without clover yielded small *R. trifolii* populations that were poorly effective in N fixation on subclover. Significant correlations were found between the *Rhizobium* population size and soil N levels, soil texture, organic matter content, and exchangeable acidity. Isolate effectiveness was associated with the amount of base

saturation, soil acidity, and N levels. These results are discussed in the context of subclover production as related to *Rhizobium* effectiveness, population size, and soil fertility characteristics.

Additional Index Words: nitrogen fixation, clover, legumes.

Hagedorn, C., A.H. Ardahl, and L.A. Materon. 1983. Characteristics of *Rhizobium trifolii* populations associated with subclover in Mississippi soils. Soil Sci. Soc. Am. J. 47:1148-1152.

INDIGENOUS SOIL POPULATIONS of *Rhizobium* have received little attention compared with efforts on monitoring the survival of introduced strains. This situation is due to both the complexity of native populations that may contain many strains that are difficult to distinguish and an emphasis on legume row crops where inoculum rhizobia may be added at each annual planting. In the forage legumes, however, inoculant rhizobia are added only once in several years (at planting) and must saprophytically compete with naturalized strains while the host plant is either dead or dormant.

In Mississippi, clover (*Trifolium* sp.) has the potential to be a major forage crop even though clover usage

¹Contribution from the Dep. of Agronomy, Mississippi State Univ., and the Mississippi Agric. and For. Exp. Stn. Approved by the Director as Technical Paper no. 5320. Received 11 Nov. 1982. Approved 9 June 1983.

²Soil Microbiologist, Research Technician, and Research Associate, Dep. of Agronomy, P.O. Box 5248, Mississippi State, MS 39762. Research supported by the Science and Education Admin. of the USDA under Grant no. 5901-0410-8-0051-0 from the Competitive Grants Research Office and through Specific Cooperative Agreement no. 58-7B30-8-21 with the Forage Research Unit, USDA-ARS, Crop Science and Engineering Research Lab., Mississippi State, MS 39762.