Stimulation of Rhizomorph Production by Armillaria mellea with Lipid from Tree Roots

A. R. Moody and A. R. Weinhold

Assistant Research Plant Pathologist and Professor, respectively, Department of Plant Pathology, University of California, Berkeley 94720. Present address of first author: Station Federale de Recherches Agronomique de Lausanne, Chateau de Changrins, CH–1260 Nyon, Switzerland.

Accepted for publication 30 May 1972.

ABSTRACT

Lipid extracts from roots of ponderosa pine (*Pinus ponderosa*), Douglas fir (*Pseudotsuga menziesii*), white fir (*Abies concolor*), incense cedar (*Calocedrus decurrens*), and peach (*Prunus persicae*) stimulated rhizomorph production in *Armillaria mellea*. Unsaponifiable lipid from roots of ponderosa pine was inactive, whereas saponifiable lipid was active. Further separation indicated activity due to unsaturated fatty acids and possibly to resin acids. To stimulate rhizomorph production a minimum concentration of 0.05 to 0.1% active fatty acid is required. The amount of unsaturated fatty acid in fresh roots of ponderosa pine (0.23%) is sufficient to stimulate rhizomorph production, whereas the quantity in peach (0.03%) is marginal. Insufficient amounts were found in Douglas fir (0.01%), white fir (0.006%), and incense cedar (0.005%). The quantity of active fatty acid in the roots of these trees correlates with susceptibility to Armillaria root rot. Althought this probably does not govern resistance, it may be important when considering roots as a food source for the fungus after infection. The resin acid fraction and hot water extracts of root tissue also contributed to rhizomorph production, indicating that these materials may also play a role in stimulating growth and rhizomorph production by *A. mellea* during colonization of roots.

Phytopathology 62:1347-1350.

When *Armillaria mellea*(Vahl. ex Fries) Kummer is grown on defined media supplemented with natural plant lipids, abundant rhizomorphs are produced (5, 6). The activity of these natural lipids is due to unsaturated fatty acids such as oleic, linoleic, and linolenic acids. Because these are the major components of lipids from leaves, stems, and roots of higher plants, they may be important to rhizomorph formation in nature. Therefore, an attempt was made to determine if they are present in concentrations great enough to be active in roots and also to determine if they are major stimulating substances in the plant.

MATERIALS AND METHODS.–*Samples.*–Roots of ponderosa pine (*Pinus ponderosa* Laws.), Douglas fir [*Pseudotsuga menziesii* (Mirbel) Franco], white fir [*Abies concolor* (Gord. & Glend) Hoopes], and incense cedar [*Calocedrus decurrens* (Torrey) Florin] were dug at the University of California Experimental Forest at Blodgett. Peach roots [*Prunus persicae* (L.) Batsch 'Nemegard'] were obtained from G. E. Nyland, University of California, Davis.

Extraction and purification.–Roots with bark removed were ground in a Wiley mill with a 4-mm mesh screen and stored at O C until used. Overnight extraction with cold ethyl ether was followed by

three extractions with ether under reflux, changing solutions every 2 hr. Combined extractions were adjusted so that 1 ml of solution contained material from 1 g of root. Measurements were based on fresh weight, since this more nearly approximates natural conditions. Saponification was carried out with 6% KOH in 95% methanol. After saponification, water was added to obtain a 50% methanol-water solution which was extracted with ethyl ether. The saponifiable and unsaponifiable portions were purified by washing with water and ether, respectively. To obtain free resin and fatty acid, the saponifiable fraction was acidified with 10% sulfuric acid and the liberated lipid extracted with ethyl ether. The selective esterification technique of Buchanan et al. (2) was then used to separate resin from fatty acids. This procedure was repeated until gas chromatographic analysis indicated that the resin acid was free from fatty acid.

Bioassay.—The bioassay for activity consisted of growing the fungus on basic medium containing 10 g glucose, 2 g L-asparagine, 1.75 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7 H_2O$, 1 mg thiamine, and 10 g Difco agar/liter of distilled water. One hundred-ml prescription bottles containing 20 ml of medium were laid flat, and the agar surface was seeded with 5-mm diam discs of water agar containing the fungus. On this medium, mycelium but no rhizomorphs were formed. When an active supplement was present, however, abundant rhizomorphs were produced. Incubation was in the dark at 18 C for 5 weeks. We made measurements by melting the agar, rinsing the culture in boiling water, and separating the mycelium from rhizomorphs. After a drying at 90 C for 24 hr, weights were obtained. The fungal isolate used was obtained from R. D. Raabe, who has tested the fungus on four different hosts and found it to be the most virulent and most pathogenic of the isolates in his collection.

Gas chromatography.—Esterification of fatty acids for analysis with gas chromatography was carried out using Boron trifluoride-methanol reagent obtained from Applied Science Laboratories, Inc., State College, Pa. The methylated fatty acids were analyzed with a Varian Aerograph Series 1800 gas chromatograph equipped with a 6 ft by one-eighth inch stainless steel column with 10% EGSS–X (ethylene glycol succinate methyl ester) on 100/120 GAS CHROM. P. The column temperature was 200 C with a nitrogen flow of 25 ml/min. Unknown esters from roots were compared qualitatively to known standards using cochromatography, and quantitative estimations were made with the use of quantitative standards obtained from Applied Science Laboratories.

RESULTS.—*Stimulation of rhizomorph production with crude root lipid.*—The ethyl ether extracts from ground root tissue of five tree species stimulated rhizomorph production (Table 1). In the nonsupplemented controls, no rhizomorphs were formed, and only 12 mg/bottle of mycelial growth developed. The minimum amounts of root containing sufficient lipid to stimulate rhizomorph formation when added to 20 ml of media were 0.5, 0.5, 1, 10, and 25 for peach, ponderosa pine, Douglas fir, incense cedar, and white fir, respectively. Peach contained the greatest concentration of stimulatory materials but extracts were toxic when 5 or more g of root were bioassayed.

TABLE 1. Influence of crude lipid extracts from roots of five tree species on mycelium and rhizomorph production in *Armillaria mellea*

	Average growth/bottle (mg dry weight)[a]					
	Quantity of root tissue extracted (g)					
Source	0.1	0.5	1	5	10	25
Peach						
Rhizomorphs	0	56	114	0		
Mycelium	35	52	10	0		
Ponderosa pine						
Rhizomorphs	0	48	60	184	181	306
Mycelium	34	32	16	X[b]	X	X
Douglas fir						
Rhizomorphs	7	7	28	54	75	139
Mycelium	21	45	26	X	61	20
Incense cedar						
Rhizomorphs	4	9	3	7	26	97
Mycelium	104	99	76	87	67	30
White fir						
Rhizomorphs	3	0	6	1	5	101
Mycelium	57	95	59	94	83	X
Control (no supplement)						
Rhizomorphs	0					
Mycelium	12					

[a]Each figure is the mean of at least 10 replications. Extract from quantity of tissue indicated added to 20 ml media.
[b]X = Separation of rhizomorphs and mycelium not possible.

Rhizomorph production with saponifiable lipid.—The ether extract from ponderosa pine was saponified, and the saponifiable and unsaponifiable portions were bioassayed. The unsaponifiable portion was inactive at all concentrations tested. The saponifiable portion was active, and extract from 0.5 g of ponderosa pine root was the minimum required for rhizomorph production.

Growth on fatty and resin acids.—Selective esterification was used to separate fatty from resin acids. Since it was known that fatty acids would stimulate rhizomorph production, we placed emphasis on obtaining resin acid that was relatively free from fatty acid. We found, using gas chromatographic analysis, that after samples were treated twice there was little fatty acid remaining in the resin acid fraction. A bioassay then showed that resin acid from 1 and 5 g of root tissue stimulated approximately twice as much rhizomorph tissue as fatty acid from the same amount of root (Table 2). Abietic acid, a commercially available resin acid, was bioassayed. Few rhizomorphs were produced when abietic acid was filter-sterilized. When autoclaved, however, abundant rhizomorphs developed at concentrations from 0.1 to 10 g/liter.

TABLE 2. Influence of resin and fatty acid from ponderosa pine roots on mycelium and rhizomorph production by *Armillaria mellea*

	Average growth/bottle (mg dry weight)[a]			
	Quantity of root tissue extracted (g)			
Supplement	0	0.5	1	5
Fatty acid				
Rhizomorphs	0	9	35	67
Mycelium	58	87	76	27
Resin acid				
Rhizomorphs	0		78	144
Mycelium	97		8	X[b]

[a]Each figure is the mean of at least seven replications. Extract from quantity of tissue indicated added to 20 ml media.

[b]X = Separation of rhizomorphs and mycelium not possible.

Quality and quantity of fatty acids.—Gas chromatographic analysis indicated that roots of ponderosa pine contained the most oleic and linoleic acid of the five tree species studied, as well as the largest quantity of total unsaturated fatty acid (Table 3). Peach contained the most linolenic acid. The total amount of unsaturated fatty acids in roots of the five tree species, μg/g fresh wt, was: ponderosa pine, 2,304.9; peach, 331.7; Douglas fir, 141.3; white fir, 59.5; and incense cedar, 52.0. The percent moisture for the root tissue tested was 62, 51, 63, 41, and 54 for ponderosa pine, peach, Douglas fir, white fir, and incense cedar, respectively.

A. mellea was grown on a mixture of unsaturated fatty acids quantitatively like that found in roots of ponderosa pine, and the average rhizomorph tissue produced was 69 mg/culture.

TABLE 3. Quantity of unsaturated fatty acids from roots of five tree species

Species	Fatty acid	μg acid/g root[a]
Ponderosa pine	Oleic	1,321.88±159.11[b]
	Linoleic	847.12±103.72
	Linolenic	135.88± 16.50
	Total	2,304.88
Peach	Oleic	19.33± 3.29
	Linoleic	171.00± 33.44
	Linolenic	141.33± 27.40
	Total	331.66
Douglas fir	Oleic	34.98± 1.29
	Linoleic	64.49± 1.02
	Linolenic	41.82± 1.36
	Total	141.29
White fir	Oleic	16.11± 2.45
	Linoleic	31.37± 1.22
	Linolenic	11.99± 2.28
	Total	59.47
Incense cedar	Oleic	3.18± 0.72
	Linoleic	39.97± 3.61
	Linolenic	8.88± 1.39
	Total	52.03

[a]Based on fresh weight. Percent moisture for the roots used was: ponderosa pine, 62; peach, 51; Douglas fir, 63; white fir, 41; incense cedar, 54.

[b]Each figure is the mean of at least three samples chromatographed at least three times.

Stimulation of rhizomorph production with water extracts.—A hot water extract of ground root tissue from each of five tree species was bioassayed to determine whether other materials might contribute to stimulation of rhizomorph production in nature (Table 4). All of the water extracts stimulated rhizomorph production, with peach the most effective. Peach required extract from only 0.1 g of root tissue, whereas the remaining species required extract from 0.5 g for rhizomorph production.

DISCUSSION.—The amount of unsaturated fatty acid required for rhizomorph production has been found to be 0.05% to 0.1% (6). Based on this, the amount of unsaturated fatty acid in fresh roots of ponderosa pine (0.23%) is sufficient to stimulate rhizomorph production, whereas the quantity in peach (0.03%) is marginal. Douglas fir (0.01%), white fir (0.006%), and incense cedar (0.005%) contain insufficient amounts for rhizomorph stimulation.

Other workers have expressed their results as percent in extract (1), percent in wood (7), or proportions of individual fatty acids (3, 4, 8). Results here are presented as the amount of unsaturated fatty acid in fresh roots so that the importance of these lipids to rhizomorph production in nature can be evaluated.

TABLE 4. Influence of hot water extracts from roots of five tree species on mycelium and rhizomorph production by *Armillaria mellea*

Source	Average growth/bottle (mg dry weight)[a]			
	Quantity of root tissue extracted (g)			
	0.1	0.5	1	5
Peach				
Rhizomorphs	91	90	127	363
Mycelium	66	81	42	X[b]
Ponderosa pine				
Rhizomorphs	1	45	101	157
Mycelium	52	84	48	X
Douglas fir				
Rhizomorphs	0	35	27	167
Mycelium	98	75	76	X
Incense cedar				
Rhizomorphs	0	22	56	154
Mycelium	54	84	84	X
White fir				
Rhizomorphs	4	41	58	155
Mycelium	103	85	85	X
Control				
Rhizomorphs		0		
Mycelium		13		

[a]Each figure represents the mean of at least 10 replications.

[b]X = Separation of rhizomorphs and mycelium was not possible.

Gas chromatographic analysis indicated that fatty acid from 1 to 2 g of ponderosa pine root was required for rhizomorph stimulation. In practice, stimulation resulted when crude ether extract or saponifiable lipid from 0.5 g of root tissue was bioassayed.

The resin acid fraction from 1 g of root tissue also stimulated activity. However, the resin acid, abietic acid, was not active until autoclaved, suggesting that breakdown products were involved. Breakdown products may also have been produced during extraction, purification, and sterilization of the resin acid fraction from ponderosa pine.

It appears that the roots of many woody plant species contain sufficient quantities of unsaturated fatty acids to influence the growth and rhizomorph production of *A. mellea*. It is clear that these are not the only materials involved in the stimulation of *A. mellea* development in nature. Work by Raabe (9, *unpublished data*) and Rehill (10) as well as data presented in this study show that most plants contain water-soluble materials stimulatory to *A. mellea*. In addition, in certain species resin acids may also be involved. The suitability of plant tissue as a substrate for *A. mellea* may depend, therefore, on the presence of a variety of stimulatory materials. One group of these compounds which have been identified are the unsaturated fatty acids.

LITERATURE CITED

1. ANDERSON, A. B., R. RIFFER, & ADDIE WONG. 1969. Monoterpenes, fatty and resin acids of Pinus ponderosa and Pinus jeffreyi. Phytochemistry 8:873-875.
2. BUCHANAN, M. A., R. V. SINNETT, & JOSEPHINE A. JAPPE. 1959. The fatty acids of some American pulpwoods. Tappi 42:578-583.
3. HANSEN, R. P. 1966. A preliminary investigation of the fatty acid composition of the lipids of Pinus radiata and Pinus nigra. New Zealand J. Sci. 9:801-805.
4. HANSEN, R. P., & DERYL F. BODERICK. 1968. The fatty acid composition of the lipids from the seeds of Pinus radiata. Tappi 51:48-51.
5. MOODY, A. R., M. O. GARRAWAY, & A. R. WEINHOLD. 1968. Stimulation of rhizomorph production in Armillaria mellea with oils and fatty acids. Phytopathology 58:1060-1061 (Abstr.).
6. MOODY, A. R., & A. R. WEINHOLD. 1972. Fatty acids and naturally occurring plant lipids as stimulants of rhizomorph production in Armillaria mellea. Phytopathology 62:264-267.
7. MUTTON, D. B. 1958. Hardwood resin. Tappi 41:632-643.
8. PEARL, I. A., & J. A. HARROCKS. 1960. Studies on the chemistry of aspenwood. X. Neutral materials from the benzene extractives of Populus tremuloides. J. Org. Chem. 26:1578-1583.
9. RAABE, R. D. 1962. Wood-based culture media for growing Armillaria mellea. Phytopathology 52:364 (Abstr.).
10. REHILL, P. S. 1968. Stimulation of Armillaria mellea rhizomorphs with alder extracts. Bi-Monthly Res. Notes 24:34.