Fatty Acids and Naturally Occurring Plant Lipids as Stimulants of Rhizomorph Production in Armillaria mellea

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ABSTRACT

Armillaria mellea was grown on a basic medium consisting of 10 g glucose, 2 g L-asparagine, 1.75 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, 1 mg thiamine, and 10 g Difco agar/1,000 ml distilled water. Abundant mycelia but no rhizomorphs were produced on this medium. Rhizomorphs were formed when the medium was supplemented with natural lipids such as coconut, corn, cottonseed, olive, peanut, safflower or wheat germ oils, lanolin, or vegetable lecithin. Several monocarboxylic acids were then tested to determine the active portions of these naturally occurring lipids. Two groups of free acids stimulated rhizomorph production. The first consisted of propionic, butyric, and valeric acids, whereas the second contained the unsaturated fatty acids oleic, linoleic, and linolenic. The unsaturated fatty acids are major components of the natural plant oils that were active, and activity correlated with the quantity of unsaturated fatty acids in the oils.

Polyoxyethylene sorbitan monolaurate (Tween 20) was highly effective in stimulating rhizomorph production, whereas related compounds were active to a lesser degree. Among additional lipid compounds tested, several sterols and glycerol were found to be inactive.

No rhizomorphs were produced by *A. mellea* when active fatty acids or naturally occurring lipids were the sole source of carbon in the media, indicating that lipids function as growth-promoting substances rather than as sources of carbon.

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Rhizomorphs, the infection structures of *Armillaria mellea* (Vahl ex Fries) Kummer, can be produced easily in culture on many nondefined media such as potato-dextrose agar (PDA). In defined culture, however, they are not produced unless proper nutritional requirements are fulfilled (9, 10). Weinhold (9) reported that ethanol and related compounds would help to meet this requirement. When *A. mellea* was grown on a chemically defined medium containing ethanol at concentrations as low as 100 μg/ml, abundant rhizomorphs were formed. Pentland (5) reported ethanol as the rhizomorph-stimulating substance produced by *Aureobasidium pullulans*, and discussed the possibility that this material might stimulate rhizomorph production under natural conditions. However, stimulating compounds other than low molecular weight alcohols are also present in natural materials. Raabe (6) reported that water extracts of many woody materials such as figwood (*Ficus carica*) will stimulate rhizomorph production. The active ingredient in the water extract of figwood was not a low molecular weight alcohol (10). The types of materials responsible for stimulation of rhizomorph production in nature remain unknown.

Garraway & Weinhold (2) found that when *A. mellea* was grown on a medium containing ethanol-^{14}C, more of the radioactive carbon appeared in the lipid fraction of the fungus than elsewhere. This suggested that lipids may play an active role in the production of rhizomorphs. Also, as lipids are an important component of all plants, they might be involved in rhizomorph production in nature. This study was undertaken to determine the activity of lipids as rhizomorph-stimulating substances.

MATERIALS AND METHODS.—The isolate of *A. mellea* used in these studies was obtained from R. D. Raabe, who has tested the fungus on four different hosts and found it to be both the most pathogenic and the most virulent of the many isolates in his collection (7, 8). Oils and fatty acids were obtained from Nutritional Biochemicals, Cleveland, Ohio. The fungus was grown in 100-ml prescription bottles, each containing 20 ml of media composed of 10 g glucose, 2 g L-asparagine, 1.75 g KH_2PO_4, 0.75 g $MgSO_4$ $7H_2O$, 1 mg thiamine, and 10 g Difco agar, plus the desired experimental supplement per liter of distilled water. The pH was adjusted to 6.8 before autoclaving for 10 min at 15 psi and 120 C. Glucose was sterilized separately and added after autoclaving to prevent the formation of inhibitors which may form when sugars are autoclaved in the presence of phosphate or amino acids (1). After the sterile glucose solution was added to the bottles they were laid flat, forming a layer of medium 5 mm deep.

Lipid supplements were pipetted into the bottles either before or after autoclaving. When added before autoclaving, the pH of the medium was readjusted to 6.8 with NaOH. The monocarboxylic acids were added as free acids unless otherwise stated. All of the supplements except linolenic acid were added before autoclaving. Linolenic acid was sterilized by filtration through a type HA 0.45-μ MF-Millipore filter and pipetted onto the surface of the agar. Oils were diluted for pipetting with peroxide-free diethyl ether. Lipid compounds that were solid at room temperature and sterols were dissolved in ether and pipetted into the bottles. The ether was then allowed to evaporate before introducing the fungus. Agar discs 5 mm in diam were cut from a mycelium which had grown on water agar in petri plates for 10 days to 5 weeks, and were transferred to the surface of the media. Light suppresses growth of *A. mellea*. Therefore, incubation was in the dark at 18 C, the optimum temperature for growth of this isolate on this medium. The cultures were incubated 5 weeks

Table 1. Influence of lipid supplements on growth of mycelia and rhizomorphs of *Armillaria mellea* in culture

	Average growth/bottle (mg dry wt)[a]								
	Concentration of supplement (g/liter)								
Supplement	0.02	0.05	0.1	0.5	1	5	10	25	50
Natural plant oils									
Coconut	74	72	107	131	121	125	166	0	0
Corn	84	68	99	112	119	157	170	322	343
Cottonseed	37	48	105	97	132	86	191	232	470
Olive	88	130	108	118	134	142	188	312	0
Peanut	93	97	100	107	138	161	184	282	389
Safflower	100	91	122	91	121	107	195	174	493
Wheat germ	75	118	102	136	140	125	250	368	447
Lanolin			57	64	100	108	136	255	451
Vegetable lecithin		68	79	192	137	126	244	384	638
Ethanol		68	87	117	124	165	210		0
Control (no supplement)	32								

[a] Figures are the mean of at least three experiments of ten replications each. Measurements made after 5 weeks.

before measurements were made by melting the agar, removing the culture, and placing it in boiling water for ca. 1 min. The tissue was then either separated into mycelium and rhizomorphs or placed whole on tared aluminum foil. After drying at 90 C for 24 hr, weights were obtained.

RESULTS.—*Stimulation of rhizomorph production with supplements of natural lipids.*—When *A. mellea* was grown on a medium supplemented with coconut, corn, cottonseed, olive, peanut, safflower or wheat germ oils, lanolin, or vegetable lecithin, growth was stimulated, and abundant rhizomorphs were formed (Table 1). When no supplement was added to the medium, no rhizomorphs developed, and an average of 32 mg of mycelium was produced.

The natural plant oils tested were similar in their effect. The average dry weight of mycelium and rhizomorphs grown with supplements of 0.1 g/liter was close to 100 mg with all materials tested. As the quantity of natural plant oil was increased from 0.1 g/liter to 50 g/liter, increased rhizomorph production caused an increase in the total dry weight of the culture. Pronounced rhizomorph formation with less than 100 mg of mycelium occurred with all treatments. The maximum amount of growth stimulated by the natural plant oils was obtained with safflower oil at 50 g/liter. Two of the natural plant oils were toxic at higher concentrations. Coconut oil caused death of *A. mellea* at 25 g/liter, whereas olive oil caused death at 50 g/liter.

Lanolin and vegetable lecithin were somewhat different than the natural plant oils in their effect on rhizomorph production in *A. mellea*. Lanolin was not active until a concentration of 1 g/liter was reached. At 50 g/liter, however, lanolin was as active as the natural plant oils. Vegetable lecithin was similar in that 0.5 g/liter of this lipid was required before rhizomorph production became pronounced. Vegetable lecithin at 50 g/liter, however, stimulated more growth than any other material tested.

The lipid supplements tested possessed approximately the same range of activity as ethanol. Total dry weight of cultures grown with an ethanol supplement increased from 68 mg at 0.05 g/liter to 210 mg at 10 g/liter. An ethanol supplement of 50 g/liter was found to be toxic, whereas preliminary studies showed that it was not toxic at 25 g/liter.

Stimulation of rhizomorph production with supplements of free fatty acids.—The natural lipid supplements active in stimulating rhizomorph production contained high amounts of unsaturated fatty acids. Saturated fatty acids and other compounds such as glycerol and sterols also were present. To determine what portion of the natural lipid supplements was active, saturated and unsaturated fatty acids as well as other monocarboxylic acids were tested along with additional lipid-related materials.

Among the active monocarboxylic acids, effectiveness in stimulating rhizomorph production was directly proportional to chain length (Table 2). Acetic acid was only slightly active, whereas propionic, butyric, and valeric acids were increasingly active at 1 g/liter. The saturated fatty acids from caproic to stearic were inactive. Rhizomorph production was also stimulated by the unsaturated fatty acids oleic, linoleic, and linolenic. Rhizomorphs were produced by oleic acid at concentrations greater than 1 g/liter. This material was most effective at 25 g/liter, and caused death of the fungus at 50 g/liter. Stimulation of rhizomorph activity with linoleic and linolenic acids occurred from 0.5 to 5 g/liter, and death of the fungus resulted at concentrations of 10 g/liter or greater. The optimum concentration for both of these fatty acids was 1 g/liter, and linoleic acid was the more active of the two. Linolenic acid required filter sterilization because of decomposition during autoclaving. In comparison with ethanol, a greater concentration of these materials was required for activity, and they were more toxic than ethanol at higher concentrations.

The toxicity of unsaturated fatty acids increased with an increase in the number of double bonds. At 5 g/liter, oleic acid was not toxic, linoleic acid was

TABLE 2. Influence of free monocarboxylic acids on mycelium and rhizomorph production by *Armillaria mellea*

Supplement		Average growth/bottle (mg dry wt)[a]							
		Concentration of supplement (g/liter)							
		0.05	0.1	0.5	1	5	10	25	50
Monocarboxylic acids									
Acetic	Rhizomorphs	0	0	1	18	1	0		
	Mycelium	36	53	67	58	30	9		
Propionic	Rhizomorphs	3	19	83	100	27	12		
	Mycelium	21	30	42	38	42	9		
Butyric	Rhizomorphs	0	0	52	115	82	0		
	Mycelium	26	59	48	X[b]	37	0		
Valeric	Rhizomorphs	2	0	126	131	0			
	Mycelium	22	40	36	8	0			
Oleic	Rhizomorphs	0	0	0	21	158	267	410	0
	Mycelium	57	49	90	93	29	25	X	0
Linoleic	Rhizomorphs	0	0	117	126	45	0		
	Mycelium	34	67	31	X	X	0		
Linolenic	Rhizomorphs	0	0	73	94	9	0		
	Mycelium	46	22	39	11	X	0		
Ethanol	Rhizomorphs	13	32	87	116	170	210		0
	Mycelium	55	50	26	11	X	X		0
Control (no supplement)	Rhizomorphs	0							
	Mycelium	36							

[a] Each figure is the mean of at least 10 replications.
[b] X = separation of mycelia and rhizomorphs not possible.

somewhat toxic, and linolenic acid was most toxic.

Effect of additional lipid compounds on rhizomorph production.—The natural lipids initially examined contained substances other than fatty acids. Some of these compounds were also tested. Glycerol was found to be inactive. The sterols cholesterol, β-sitosterol, ergosterol, stigmasterol, and testosterone were also inactive at concentrations from 1 to 10,000 μg/ml.

Sodium salts of lauric, myristic, palmitic, stearic, oleic, and linoleic acids were also tested. Only sodium linoleate, at 5 g/liter, stimulated rhizomorph production.

Tween 20 (polyoxyethylene sorbitan monolaurate), 40 (polyoxyethylene sorbitan monopalmitate), 60 (polyoxyethylene sorbitan monostearate), and 80 (polyoxyethylene sorbitan monooleate), are the respective lauric, palmitic, stearic, and oleic esters of polyoxyethylene sorbitan. All of the Tween compounds stimulated rhizomorph production at 10 g/liter. However, Tween 20, the lauric ester, was the most effective.

Lipids as sole sources of carbon.—To determine whether rhizomorphs would be produced when natural lipids or fatty acids were the sole source of carbon, *A. mellea* was grown on a medium with these materials as the only carbon source. When glucose was included in the medium with a lipid supplement, abundant rhizomorphs were produced. When no glucose was added and the lipid was the only source of carbon in the medium, no rhizomorphs were produced. This indicates that the role played by lipids in stimulating rhizomorph production involves their functioning as growth-promoting substances rather than as sources of carbon.

DISCUSSION.—The unsaturated fatty acids are major components of the lipid fraction of higher plants as well as fungi. The major components of lipid from leaves, stems, and roots of plants as well as fungi are palmitic, oleic, linoleic, and linolenic acids (3). The unsaturated fatty acid portion of the vegetable oils used in these experiments were: coconut, 2 to 12%; cottonseed, 63 to 81%; corn, 77 to 91%; olive, 73 to 96%; safflower, 87 to 99%; and wheat germ oil, 80 to 100% (3, 4). Coconut oil was lowest in unsaturated fatty acids, whereas safflower and wheat germ oils were highest. If rhizomorph production were due to the unsaturated fatty acid portion of these lipids, coconut oil would be expected to be least effective. Cottonseed oil should be next, followed by corn, olive, and peanut oils with safflower and wheat germ oils being most effective. Results followed these expectations with the exception of coconut oil, which showed average activity, indicating that its activity was due to something other than unsaturated fatty acids. The naturally occurring triglycerides tested were much less toxic than the free fatty acids. The majority of these materials was not toxic at 50 g/liter, whereas all the free fatty acids except oleic were toxic at 10 g/liter or lower. The unsaturated fatty acids appear to be capable of stimulating rhizomorph production in nature when sufficient concentrations exist in the roots of infected plants.

Propionic, butyric, and valeric acids may also contribute to rhizomorph production in nature. *Armillaria mellea* is known to grow deep in the soil where reduced oxygen tensions may favor the metabolic activity of microorganisms capable of producing these materials. If sufficient concentrations

of these acids were synthesized, they could contribute to the production of rhizomorphs.

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