

Growth Stimulation of *Armillaria mellea* by Ethanol and other Alcohols in Relation to Phenol Concentration

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ABSTRACT

The growth rate of 7-day-old *Armillaria mellea* thalli increased and the phenol concentration decreased on glucose media supplemented with various concentrations of ethanol. Similarly, the growth rate of thalli increased and the phenol concentration decreased when an ethanol supplement (500 μ l/liter) was substituted by a comparable concentration of either propanol, butanol or isobutanol. Thus, the physiological responses of *A. mellea* to other low molecular weight alcohols, which promote mycelial growth and rhizomorph development, appear comparable to responses induced by ethanol.

Extracts from 7-day-old thalli incubated for 48 hr on glucose media inhibited *A. mellea* mycelial growth and rhizomorph development. Comparable extracts from thalli incubated on glucose + ethanol (500 μ l/liter) media

were non-inhibitory. Extracts from thalli on glucose media contained two phenolic compounds, (detected by chromatographic separation on polyamide thin-layer plates) which were absent from extracts of thalli on glucose + ethanol media. One spot, at R_F 0.17, revealed ultraviolet absorption maxima at 272 and 232 nm. The other, at R_F 0.61, revealed one maximum at 270 nm. The presence of unique phenols in inhibitory extracts from thalli incubated on glucose media, and their absence from thalli incubated on glucose + ethanol media, suggests that ethanol promotes *A. mellea* thallus growth by altering the phenol composition in addition to reducing phenol concentration.

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Additional key words: growth promoters, growth inhibitors, rhizomorph, morphogenesis.

Rhizomorphs are important in infection of hosts by *Armillaria mellea* (Vahl) Quél (4, 12, 21, 25). They are also important in dissemination and survival of the pathogen (8, 12). Weinhold's discovery (23) that ethanol and other low molecular weight alcohols promote mycelial growth and rhizomorph development when added as a supplement to a glucose medium, was followed by studies (7, 24) which led to the conclusion that growth-promoting concentrations of ethanol did not provide enough carbon to explain the growth observed. Therefore, it was suggested that ethanol promoted growth through its effect on uptake and utilization of glucose.

Studies with glucose- 14 C revealed that ethanol, at a concentration (500 μ l/liter) which stimulated mycelial growth and rhizomorph development, reduced uptake and glycolytic breakdown of glucose (6). Because of this inverse relationship between growth and glucose metabolism in the presence of ethanol, it was suggested that glucose might be converted to inhibitory metabolites in the absence of ethanol and that ethanol might promote growth by suppressing inhibitor production (7).

Further studies with labeled glucose- 14 C indicated the Hexose Monophosphate Pathway (HMP) is a major alternate glycolytic pathway in *A. mellea* (6). These results led to the idea that the proposed

inhibitory metabolites might be synthesized via this pathway. Phenols have been shown to be inhibitory to many fungi and other microorganisms (1, 3, 13). The HMP provides intermediates for the biosynthesis of aromatic compounds including phenols (14). With the HMP being a major alternate glycolytic pathway in *A. mellea*, the proposed inhibitory metabolites might be phenolic in nature.

This study was conducted to determine whether growth-promoting concentrations of ethanol or other low molecular weight alcohols affect (i) the concentration of phenols in *A. mellea* thalli, and (ii) the capacity of extracts from *A. mellea* thalli to inhibit mycelial growth and rhizomorph morphogenesis. A preliminary report has been published (22).

MATERIALS AND METHODS.—The isolate of *A. mellea* used and the liquid medium on which it was grown have been previously described (24). L-asparagine was the nitrogen source used. Water agar plugs (6-mm diam) with the fungus were incubated for 7 days in 8-oz medicine bottles containing 50 ml of medium supplemented with 500 μ l/liter ethanol (24). Groups of fifteen 7-day-old thalli were transferred to petri dishes (15 X 1.5 cm) containing 100 ml basal medium with no glucose, then incubated for an additional 24 hr to deplete endogenous reserves. To replicate dishes containing these thalli,

TABLE 1. Thallus growth and rhizomorph initial production by *Armillaria mellea* after 8 and 12 days incubation on potato-dextrose agar (PDA) supplemented with various extract fractions of *A. mellea* thalli incubated for 48 hr on a glucose medium or one supplemented with 500 μ l/liter ethanol

Extract fraction (ml)	μ g Phenol/ml PDA ^a	Thallus diam (mm) after: ^{b,c}		Number of rhizomorph initials after: ^b	
		8 days	12 days	8 days	12 days
Glucose					
Ethyl acetate					
0.00	0.00	10.5 \pm 0.5	25.0 \pm 3.0	5.0 \pm 2.0	19.0 \pm 1.0
0.25	5.7 \pm 1.0	6.5 \pm 1.0 ^d	14.2 \pm 2.5	0.0 \pm 0.0	6.5 \pm 1.5
0.50	10.2 \pm 2.1	0.0 \pm 0.0	7.0 \pm 1.0	0.0 \pm 0.0	1.5 \pm 1.5
Glucose					
Aqueous					
0.00	0.00	10.5 \pm 1.5	22.3 \pm 2.2	6.5 \pm 1.0	17.5 \pm 3.5
0.25	1.0 \pm 0.0	9.5 \pm 1.0	23.0 \pm 4.0	7.0 \pm 0.5	17.0 \pm 4.0
0.50	2.0 \pm 1.0	11.1 \pm 2.1	22.5 \pm 1.5	6.0 \pm 1.0	18.1 \pm 3.0
Glucose + ethanol, 500 μ l/liter					
Ethyl acetate					
0.00	0.00	9.6 \pm 2.4	24.5 \pm 5.5	4.0 \pm 1.0	15.5 \pm 2.5
0.25	5.0 \pm 1.5	9.0 \pm 3.0	25.5 \pm 5.0	5.0 \pm 2.0	33.0 \pm 3.0
0.50	9.5 \pm 0.5	10.1 \pm 3.7	25.5 \pm 4.0	5.5 \pm 1.0	25.5 \pm 2.0
Glucose + ethanol, 500 μ l/liter					
Aqueous					
0.00	0.00	10.5 \pm 2.2	23.5 \pm 3.0	6.0 \pm 2.0	19.5 \pm 4.5
0.25	1.75 \pm 0.9	10.0 \pm 0.5	23.0 \pm 0.7	6.5 \pm 1.5	19.0 \pm 3.5
0.50	3.50 \pm 1.0	12.0 \pm 0.9	22.1 \pm 2.8	4.3 \pm 1.1	21.5 \pm 4.0

^aAverage of three experiments, four replicates/experiment. Mean deviation is indicated.

^bAverage of three experiments, six-to-eight replicates/experiment. Mean deviation is indicated.

^cThallus diameter includes mycelial plus rhizomorph growth unless otherwise indicated.

^dMycelial growth only.

100 ml of solutions containing the following were added: (i) glucose (5 g/liter), (ii) glucose (5 g/liter) + ethanol (50, 100, 250, 500 or 1000 μ l/liter), and (iii) glucose (5 g/liter) + 500 μ l/liter of ethanol, n-propanol, n-butanol or isobutanol. Growth (dry wt) of thalli after 0, 24, 48 and 72 hr of incubation on each medium was compared with the phenol content of comparable thalli similarly incubated.

Thalli to be extracted were homogenized in cold 80% ethanol (100 thalli/15 ml) in a Virtis "45" homogenizer set at "high" for 3 min. Protein was precipitated with 20% trichloroacetic acid (15 ml/30 ml homogenate). The homogenate was clarified by centrifugation at 37,000 g for 30 min at 0 C in a Sorvall RC2-B refrigerated centrifuge, then the pH of the supernatant solution was adjusted to 2.5 with 1N HCl. Total phenols, as chlorogenic acid equivalents, were estimated in the supernatant solution according to Swain & Hillis (19).

To prepare extracts for thin-layer chromatography, the acidified supernatant solution was partitioned four times into equal volumes of ethyl acetate. The ethyl acetate extracts were combined and reduced on a rotary evaporator at 35 C to a volume which corresponded to 30 thalli/ml ethyl acetate. The aqueous extracts were reduced as above and redissolved in double-distilled water. Thin-layer chromatography of phenol-containing ethyl acetate

extracts was performed on 20- X 20-cm glass plates coated with a 250- μ layer of either MN-Polyamide CD11 (Macheray, Nagel, and Co.) impregnated with 8% by weight plaster of Paris or Avicel Microcrystalline Cellulose (Brinkmann). Solvent systems used were benzene-methanol-acetic acid (90:16:8, v/v/v) for polyamide, and n-butyl ether(water saturated)-acetic acid (90:9, v/v) for cellulose. Fluorescence of compounds was visualized with short and long wave ultraviolet lamps (UVS-11 and UVL-22; Ultra-Violet Products, San Gabriel, Calif.). Phenols were visualized with either diazotized sulfanilic acid, diazotized p-nitroaniline, or ferricyanide-ferric chloride reagents. Compounds were eluted for spectrophotometry by scraping sorbent from thin-layer plates and suspending particles in 80% ethanol in four-dram vials. The particles were removed by centrifugation at 3,000 g and the ultraviolet spectrum of the supernatant solution was monitored using a Beckman DB-G recording spectrophotometer.

Ethyl acetate and aqueous extracts from 7-day-old thalli incubated for 48 hr on a basal medium with glucose or glucose + ethanol (500 μ l/liter) were bioassayed using *A. mellea* as the assay fungus. Five ml of melted (40 C) potato dextrose agar (PDA) was added to petri dishes (5 X 1.5 cm) then mixed with the aqueous thallus extracts or aqueous solutions of

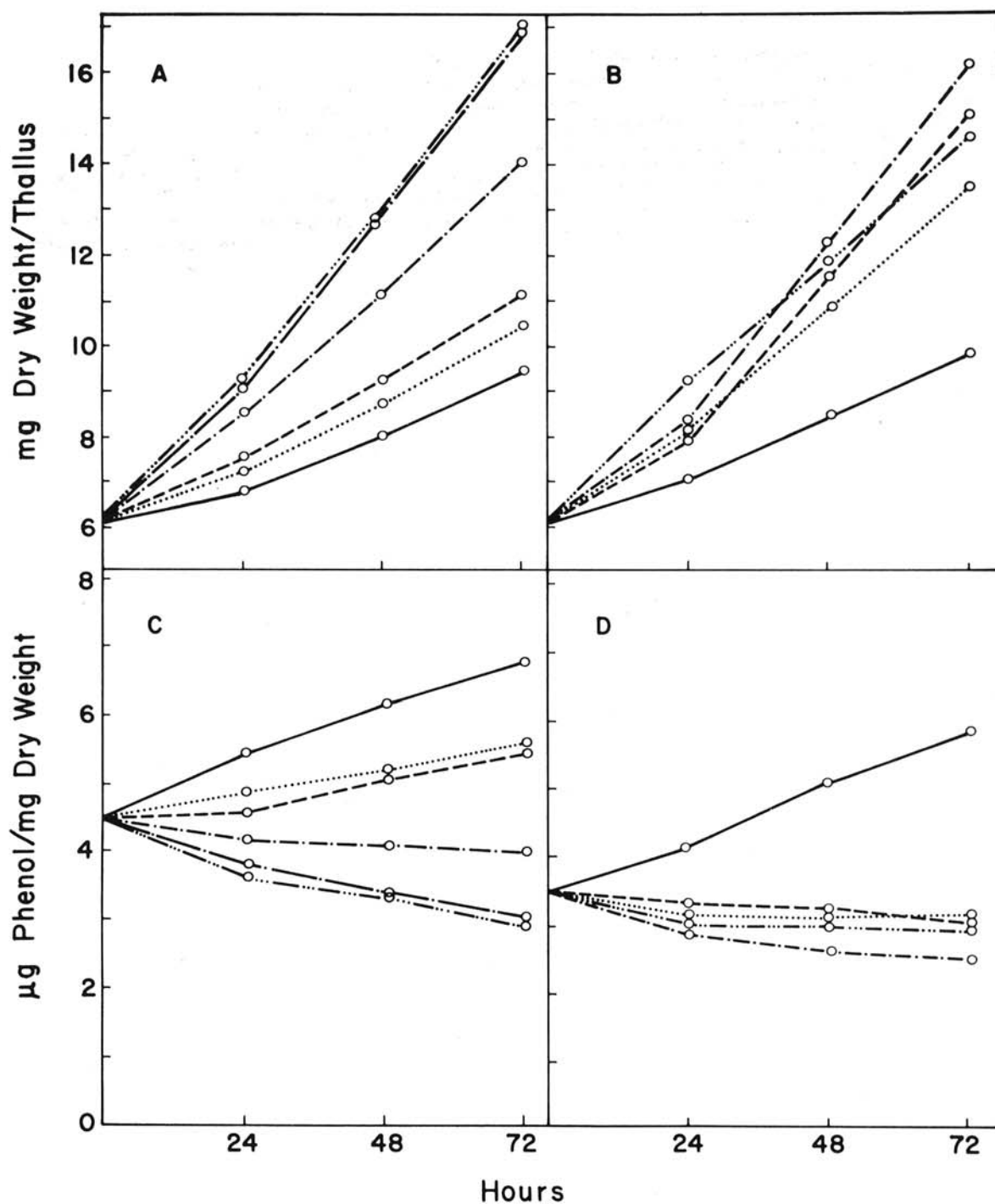


Fig. 1. (A-D) Growth of *Armillaria mellea* thalli on liquid media in relation to phenol concentration: A) Growth after various incubation times on media containing glucose (—) or glucose supplemented with 50 (.....), 100 (----), 250 (-·-·-·-·), 500 (- - - - -), or 1000 (···-···-···) µl/liter ethanol; B) growth after various incubation times on media containing glucose (—) or glucose supplemented with 500 µl/liter of ethanol (-·-·-·-·), propanol (···-···-···), n-butanol (- - - - -) or isobutanol (·····); C) phenol concentration in *A. mellea* thalli after various incubation times on media containing glucose (—) or glucose supplemented with 50 (.....), 100 (----), 250 (-·-·-·-·), 500 (-·-·-·-·) or 1000 (···-···-···) µl/liter ethanol; D) phenol concentration in thalli after various incubation times on media containing glucose (—) or glucose supplemented with 500 µl/liter of ethanol (-·-·-·-·), propanol (···-···-···), butanol (- - - - -) or isobutanol (·····). Each dry wt and phenol value is the mean of five replications.

the ethyl acetate extracts, prepared by reducing the ethyl acetate fractions to dryness then redissolving the residue in an equal volume of double distilled water. The media were seeded with plugs cut from water agar cultures of *A. mellea* with a No. 2 (6-mm diam) cork borer. The linear growth and number of rhizomorph initials of thalli incubated for 8 or 12 days on PDA supplemented with the appropriate extract (Table 1) were compared with those of thalli incubated on PDA alone. All cultures were incubated in the dark at 25 C.

RESULTS.—Effect of ethanol concentration on phenol content of thalli.—A reduced conversion of glucose to various metabolites has been observed (6, 7). In view of this, the effect of various concentrations of ethanol supplemented to glucose media on growth of *A. mellea* thalli was studied in relation to the effect on phenol concentration. Seven-day thalli prepared as above were incubated for various times (0 to 72 hr) on liquid media containing glucose or glucose + ethanol (50, 100, 250, 500, or 1,000 μ l/liter). At 24-hr intervals replicate groups of 15 thalli were removed from the various media and dry weights were determined. Comparable groups of thalli were extracted with ethanol and assayed for total phenols.

The dry wt increase per thallus was linear with time for each concentration of ethanol used (Fig. 1-A). Least growth occurred on media with glucose alone. Increasing the concentration of ethanol in the medium up to 500 μ l/liter produced concomitant increases in dry wt. The increase in growth at 1,000 μ l/liter ethanol was comparable to that at 500 μ l/liter over 72 hr.

The phenol concentration in thalli progressively decreased with increasing concentrations of ethanol (Fig. 1-C). Thalli incubated on glucose alone had higher concentrations of phenol than thalli of any other treatment. As the concentration of ethanol supplemented was increased, the phenol concentration in thalli decreased. With 50 and 100 μ l/liter ethanol in the medium the phenol concentration of thalli actually increased but at a less rapid rate than on glucose alone. With 250 μ l/liter or more of ethanol there was a decrease in phenol concentration in thalli. The decrease in phenols was greater at 500 and 1,000 μ l/liter than at 250 μ l/liter. The decrease in phenols at 1,000 μ l/liter ethanol was comparable to the decrease at 500 μ l/liter.

A positive correlation was found between growth and quantity of phenol at all concentrations of ethanol tested. The correlation coefficient (26) expressed as the critical value of F (FCV) at one and 78 degrees of freedom was 3.95. The regression coefficients expressed as F values for growth vs. phenol concentration for glucose and glucose plus ethanol (50, 100, 250, 500, or 1,000 μ l/liter) were, respectively, 735.21, 508.0, 16.31, 321.72, 119.5, and 117.43. Thus, the concentrations of ethanol which stimulate growth of *A. mellea* thalli suppress phenol accumulation. The growth of *A. mellea* thalli on media containing glucose or glucose plus various concentrations of ethanol appears to be inversely

proportional to their phenol content.

Comparison of ethanol with various alcohols.—Weinhold (23) noted that in addition to ethanol, other low molecular weight alcohols promoted mycelial growth and rhizomorph production in *A. mellea*. Therefore, some of these alcohols were studied to determine whether they resulted in an inverse relation between growth and phenol accumulation. The basal medium was supplemented with glucose or glucose + 500 μ l/liter of either ethanol, propanol, butanol, or isobutanol. The dry wt and phenol concentrations of thalli were determined after 0, 24, 48 and 72 hr of incubation on the various media.

Growth was more rapid on media supplemented with alcohols than on glucose alone (Fig. 1-B). Ethanol appeared to be comparable to butanol in stimulating growth, but was more effective than either propanol or isobutanol. Phenols decreased in thalli incubated on the different alcohols (Fig. 1-D), and increased in thalli incubated on glucose. Thus, the inverse relation observed between growth and phenol accumulation on ethanol occurs with other low molecular weight alcohols.

Thin-layer chromatography of phenolic extracts of *A. mellea*.—The possibility that qualitative changes in phenols accompany the ethanol-induced quantitative changes was investigated. Extracts of 7-day thalli which were incubated on media containing glucose or glucose + ethanol (500 μ l/liter) for 48 hr were analyzed using thin-layer chromatography and ultraviolet (UV) spectrophotometry.

Twenty-five μ l of ethyl acetate extract, 1 ml of which contained phenols extracted from 30 thalli, were spotted on polyamide and cellulose plates. Chromatograms using polyamide as a sorbent revealed five distinct spots based on fluorescence and absorbance characteristics and reactions with phenol detecting reagents (Fig. 2). Three of these were present in extracts of thalli incubated on either glucose or glucose + ethanol. The two at R_F 0.17 and 0.61 were found in extracts of thalli incubated on glucose alone. Both of the latter spots gave positive reactions with diazotized sulfanilic acid and p-nitroaniline, indicating that they contained phenolic compounds. The compound(s) eluted from the R_F 0.17 spot revealed UV absorption maxima at 272 and 232 nm (Fig. 3), and those from the R_F 0.61 spot revealed one absorption maximum at 270 nm.

Chromatograms using cellulose as a sorbent revealed four spots. Three of these were present in extracts from both treatments, whereas one at R_F 0.4, which gave a phenolic reaction, was present only in extracts from thalli incubated on glucose. The compound(s) eluted from this latter spot revealed absorption maxima at 272 and 232 nm.

Bioassay of phenols extracted from *A. mellea*.—Aqueous and ethyl acetate extracts of *A. mellea* thalli incubated on media containing glucose or glucose + ethanol (500 μ l/liter) were supplemented to PDA to find out whether compounds, including phenols, which accumulated in thalli were responsible for inhibition of growth of the fungus.

Thallus growth and the number of rhizomorph initials of *A. mellea* were significantly inhibited (Table 1) on PDA supplemented with the ethyl acetate fraction of an extract prepared from thalli incubated on media with glucose alone. No inhibition occurred with the ethyl acetate fraction prepared from thalli incubated on media containing glucose + ethanol (500 μ l/liter). This occurred even though the measurable phenol concentration of the two extracts was comparable. Neither thallus growth nor the number of rhizomorph initials of *A. mellea* was affected by aqueous extracts from thalli incubated on media containing glucose or glucose + ethanol (500 μ l/liter).

Armillaria mellea thalli, when grown on media with glucose, produce self-inhibitors of mycelial growth and rhizomorph development. Thalli grown on media with ethanol do not produce inhibitors. Thus, ethanol stimulation of growth may be the result of suppression of inhibitor production.

DISCUSSION.—Stimulation of growth of *A. mellea* on a basal glucose medium supplemented with ethanol was accompanied by a concurrent suppression of phenol accumulation in the thallus. A supplement of propanol, butanol, or isobutanol at a concentration which promoted thallus growth also suppressed phenol accumulation. Thus, it appears that other low molecular weight alcohols may produce physiological responses similar to that produced by ethanol.

Phenols extracted from *A. mellea* thalli, incubated on a glucose medium, inhibited mycelial growth and rhizomorph development. This is evidence for a causal relationship between phenol accumulation and

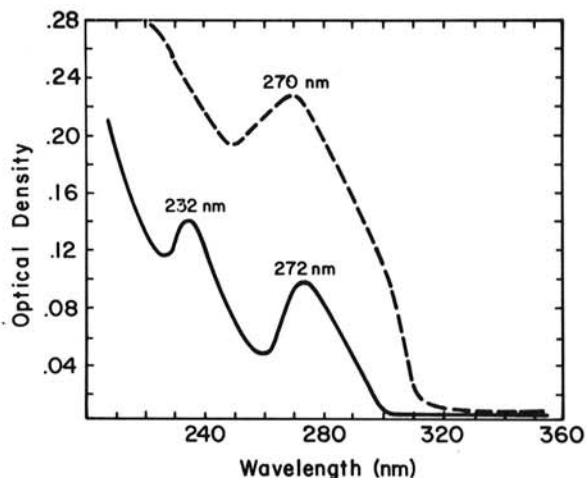


Fig. 3. Ultraviolet spectrum of compounds found at R_f 0.17 (—) and R_f 0.61 (---) in extracts from thalli incubated on liquid media with glucose for 48 hr. Compounds were separated on thin layers consisting of polyamide. No corresponding compounds were found in thalli incubated for 48 hr on liquid media with glucose + ethanol (500 μ l/liter).

poor growth on a glucose medium. Moreover, since thin-layer chromatograms revealed the presence of unique phenols in extracts of thalli incubated on a glucose medium and their absence from thalli incubated on a glucose + ethanol medium, it appears that ethanol promoted growth by altering the composition and concentration of phenolic compounds in thalli. Preliminary assays of the separated phenols eluted from thin-layer plates (C. P. Vance, unpublished) support this conclusion.

Several mechanisms are known which might explain how phenols which accumulate in *A. mellea* cause limitation of mycelial growth and rhizomorph development. Phenols can be oxidized to melanins and lignins (15, 17). These, in turn, can be incorporated into cell walls, cause increased rigidity, prevent cell wall expansion and, thus, limit growth. The recent studies of Smith & Griffin with *Armillariella elegans* Heim (18) suggest that polymers formed by condensation of oxidized phenols may act in the manner proposed above to inhibit rhizomorph growth. In higher plants where an inverse relation has been shown between growth and phenol accumulation (5, 10), it has been hypothesized that phenols polymerize to form compounds which limit cell wall expansion and growth. Phenols may also inhibit enzymes which could be important in mycelial growth and rhizomorph morphogenesis. Enzymes inhibited by phenols include phosphorylases (16), cellulases (11), transaminases (2), and decarboxylases (9).

From this study it can be inferred that chemical and/or biological factors in the environment which promote accumulation of toxic endogenous phenols in *A. mellea* thalli are likely to inhibit mycelial growth and rhizomorph production. Naturally

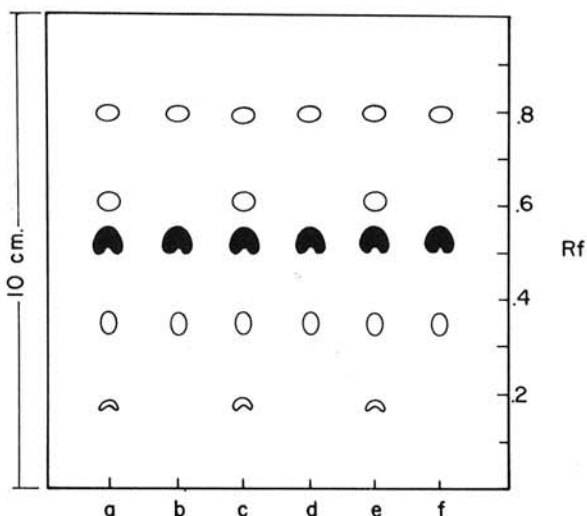


Fig. 2. Facsimile of polyamide thin-layer chromatograms of phenolic extracts from *Armillaria mellea* thalli. Letters a, c, and e represent extracts from three separate batches of thalli incubated for 48 hr on liquid media with glucose. Letters b, d, and f represent extracts from three separate batches of thalli incubated for 48 hr on liquid media with glucose + ethanol (500 μ l/liter).

occurring soil chemicals with the properties of the toxic endogenous phenols identified in the present study should act similarly. In this connection, Swift's studies (20) are significant. He found that *A. mellea* rarely produced rhizomorphs in Rhodesian forest soils because of the presence of an unidentified inhibitory compound. This compound was water extractable and could completely inhibit rhizomorph development in vitro. Thus, the foregoing study may provide the basis for formulating more effective approaches to the control of *A. mellea* in the field.

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