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THE EFFECT OF ETHANOL AND OTHER LOW MOLECULAR
WEIGHT ALCOHOLS ON PHENOLS AND PHENOL OXIDIZING
ENZYMES IN ARMILLARIA MELLEA (VAHL) QUEL.
IN RELATION TO GROWTH AND RHIZOMORPH DEVELOPMENT.

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ON PHENOLS AND PHENOL OXIDIZING ENZYMES IN ARMILLARIA MELLEA
(VAHL) QUÉL. IN RELATION TO GROWTH AND RHIZOMORPH DEVELOPMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirement for the
Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

By
Carroll Phillip Vance, B.Sc.

********
The Ohio State University

Approved by

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VITA

I, Carroll Phillip Vance, was born in Johnson City, Tennessee on September 20, 1945. I received my elementary and secondary education in the city school system of Johnson City. In September of 1963, I enrolled in East Tennessee State University and received a Bachelor of Science degree in Biology from this institution in August, 1967. In September, 1967, I enrolled in the Graduate School, Department of Plant Pathology, The Ohio State University, and was in residence at this university until December, 1971.

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I. INTRODUCTION

*Armillaria mellea* (Vahl) Quél. is a Basidiomycete in the family Agaricaceae (124). The fungus is pathogenic on a wide range of hosts throughout the world and is very common in forested areas (98). The primary mode of penetration into host and dissemination is through organized hyphal filaments called rhizomorphs (21, 28, 45, 119). Since rhizomorphs play such an important role in the disease cycle, the growth and development of these structures have received much study. Most studies of rhizomorph development and growth have dealt with the descriptive and morphological aspects (1, 18, 21, 45, 48, 56, 57, 70, 119). A number of studies have been involved in the physiology of morphogenesis of rhizomorphs (40, 41, 42, 43, 44, 125, 127). These morphological and physiological studies are dealt with in detail in the literature review.

Since the early 1960's it has been possible to culture *A. mellea* on a completely synthetic liquid medium and stimulate rhizomorph production by adding ethanol and other short chain alcohols to the medium. In the absence of ethanol and other short chain alcohols, rhizomorphs did not develop. These observations prompted an investigation of physiological processes affected by alcohols which were significant for rhizomorph initiation and development.

The objective of the research presented in this thesis was to explain the stimulatory effect that ethanol and other short chain
alcohols have on rhizomorph development and growth. This objective was met by comparing certain physiological parameters in *A. mellea* thalli incubated in the presence and in the absence of ethanol. The parameters investigated were (i) quantitative and qualitative changes in phenols in thallus extracts, (ii) biological activity of phenols in extracts, and (iii) peroxidase and polyphenol oxidase activity of extracts. The effect of ethanol and other low molecular weight alcohols on these parameters provided experimental support for the proposed mechanisms of ethanol stimulation of rhizomorph initiation and growth of *A. mellea*. 
II. REVIEW OF LITERATURE

Introduction

*Armillaria mellea*

Phenols and phenol oxidizing enzymes in relation to growth and morphogenesis
**Introduction:**

A number of papers concerning 1) the pathogen *Armillaria mellea*, and 2) the role of phenols and phenol oxidizing enzymes in growth and morphogenesis, are summarized in this literature review. An attempt has been made to review the most informative papers concerning biology, physiology, control, and importance of *A. mellea*. In regard to phenols and phenol oxidizing enzymes, an attempt has been made to review their importance in morphogenesis and growth in fungi. This review was undertaken to provide the author with some knowledge of the organism used in the research undertaken and reported in this paper. The review also provided a base of knowledge which gave some insight into the problem researched and techniques used in undertaking the research.

**Armillaria mellea:**

_Armillaria mellea_ (Vahl ex Fr.) Quel. is a Basidiomycete in the family Agaricaceae (124). The pathogen was first described by Vahl in 1777 (123). Hartig (56) was the first to establish the importance of *A. mellea* (*Agaricus melleus*) as an important root rot pathogen. He was first to observe that basidiocarps arise from rhizomorphs. The tribal name, the honey agaric, was coined by Hartig. At a later date Hartig (57) studied the penetration and spread of the pathogen. He concluded that the fungus could penetrate uninjured roots of pine and fir trees, but injury was required for penetration of hardwoods such as oak. He found that the pathogen could be transmitted through root grafts between adjacent trees. The apex of the rhizomorph was first described by Hartig and he concluded that rhizomorph growth was the result of
apical cell division. The first techniques for obtaining pure cultures of *A. mellea* were developed by Brefeld in the 1870's (13).

In 1911 Kusano (68) studied the relationship of *A. mellea* to the Japanese orchid, *Gastrodia elata*. He was the first to show that the fungus could grow in a symbiotic relationship with a herbaceous plant. He observed that penetration of the host was through a single hypha and not an intact rhizomorph, and penetration was accompanied by a dissolution of the host walls. The observation that *A. mellea* could become parasitic on *Gastrodia* was also noted.

Little work was done on the development of the basidiocarp of *A. mellea* prior to 1914. Hartig's work, mentioned previously, made some mention to fruiting structure development but no detailed study. In 1914 Atkinson (1) did a thorough study of the development of the basidiocarp of *A. mellea*. Basidiocarps have three developmental zones, differentiation of hymeniophore is endogenous and epinastic. The stipe or stalk is usually dark colored and has an annulus. The pileus or cap is honey colored, gills are white and become darker with age. Spores are generally hyaline, elliptical or slightly reniform, 6 x 9 (124).

Between 1926 and 1934 the major studies of the penetration of *A. mellea* into host plant root systems appeared. In 1926 Zeller (132) observed the penetration of apple and prune roots by the pathogen. He observed individual hypha penetrate root cortex. He recognized three modes of penetration—through wounds, through root grafts, and through injury caused by emergence of lateral roots. He suggested that penetration which occurred at the point of contact of healthy and diseased roots was preceded by the action of a toxic substance produced by the
fungus which acted upon the healthy root. Zeller essentially concluded that the pathogen could not penetrate unless there was some type of injury.

In 1927 more concrete evidence on the mode of penetration of the pathogen was published. Day (28), in a classic study of the pathogen in relation to conifers, gave a complete description of the penetration of roots. Day's work indicated that the pathogen could penetrate uninjured trees. Stages of infection, according to Day, are 1) attachment of the fungus to the bark of the host, appearing to be solely by means of rhizomorphs, 2) the exertion of a toxic influence on the tissue of the host, 3) invasion of the host tissue and penetration of an intact rhizomorph in cork and between the bark and wood. Day was the first to observe the defense reaction of the host at the cellular level. He found that the host responded to the penetration of the pathogen by producing layers of cork cells in the area preceding the rhizomorph tip. These secondary cork layers were almost always broken through by the pathogen. In those trees resistant to the pathogen, a canker formed due to secondary cork layers. Resistance varied among varieties of the same species. In 1934 another classic work was published by Thomas (119). His study was similar to Day's but was in relation to hardwoods and herbaceous plants. The penetration of the pathogen into parsnips, carrot, and black walnut is described. Thomas's work is the most complete study of penetration done by anyone working with A. mellea. He observed rhizomorphs grow along the root and attach through a mucilaginous sheath. Branching and penetration occur at the point of attachment. The rhizomorph penetrates as a single unit, no mycelium
Penetration is both mechanical and chemical. The fungus precedes it. Penetration is both mechanical and chemical. The fungus appears to have a suberin-dissolving enzyme. Death of cells precedes advance of the rhizomorph. In susceptible roots rhizomorphs grow rapidly and cause general destruction, while in resistant roots the fungus gains entry but is soon walled off by a secondary cork layer. The fungus grows well on the sap of some host roots but not at all on others. There is no correlation between inhibition of growth from sap and natural resistance. These studies seem to indicate that 1) rhizomorph penetrates as a unit, 2) penetration involves both chemical and physical mechanisms, 3) resistance is associated with secondary cork formation.

Recent cytological studies with the electron microscope by Motta (84) have given new insight into the structure of growing rhizomorph tips and their development. He found that the rhizomorph was composed of concentric zones of morphologically distinct tissues derived from a sub-apical meristematic region designated as the apical center. He found two types of meristematic activity, 1) primary, localized in the apical center in which new hyphal elements are formed from initials; and 2) secondary, localized in the lateral regions of the apex, in which elongation and secondary crosswall formation occurs. From these zones the tissues of the mature rhizomorph are formed. These include a) peripheral hyphae, b) cortex, c) subcortex, d) primary and secondary medulla.

Variation in fungi is well noted and *A. mellea* is no exception. In 1929 Childs and Zeller (21) observed that there are apparently two physiologic strains (races) of the pathogen. One strain inhabits
conifers, while the other inhabits hardwoods. There is no morphological differences between the two strains. A study in 1941 by Benton and Ehrlich (8) of 27 isolates indicated there was a great variation in colony growth and rhizomorph development between isolates. Variation in rhizomorph type is also found. Hamada (55) found in studying nine different strains of A. mellea that two different types of rhizomorphs occurred. He found that some isolates gave rise to flat, white rhizomorphs, which grew between bark and wood of roots. These were designated \textit{Rhizomorpha subcorticalis}. The other type of rhizomorph was smooth, round, black and grew beneath the soil. This was designated \textit{Rhizomorpha subterranea}. A. mellea and A. tabascens (formerly \textit{Clitocybe tabescens}) were studied by Rhoads (101), also by Gibson and Corbett (51). These workers developed criteria for differentiating the two species on the basis of rhizomorph presence or absence, growth rate \textit{in vitro}, and field symptoms. As with many other pathogens, different isolates of A. mellea vary in pathogenicity. Raabe (97) studied several isolates of the pathogen and found that some isolates killed all test plants, some killed a few, and still others had no effect.

The primary means of field dissemination of the pathogen and penetration of the host apparently occurs through rhizomorphs (18, 21, 45, 57, 70, 119). Due to the importance of these structures in the disease cycle, there have been many studies on their development and growth. As mentioned earlier, Hartig (56) showed that rhizomorphs are well organized structures and grow by division and elongation of the apical cells of the rhizomorph. In 1934 Campbell (18) studied the development of black zone lines in A. mellea. He gave a detailed account of their
development in culture and in wood. The "black lines" of *A. mellea* are made up of a medulla of hyaline hypha surrounded by a black resistant rind of bladder hyphae. He considered "black lines" to be the outer layer of tissue of sclerotia-like development in wood, or the outer layer of tissue of the rhizomorph. Campbell concluded that the differentiation of black lines was not a true sclerotium but a pseudo-sclerotium. His reasoning was that the structure was immersed in wood and as a consequence contained considerable amount of host tissue. Campbell also observed apical growth of rhizomorphs.

Rhizomorphs in the Basidiomycetes are of two types (122), differentiated as in *Armillaria*, and undifferentiated as in *Hymenogaster*. The observation of the morphogenesis of rhizomorphs in culture was followed by Snider (110), who recognized five stages in the development of rhizomorphs. He described these stages as: 1) undifferentiated growth of mycelium, 2) formation of rhizomorph initials, 3) emergence of rhizomorphs and initial growth, 4) linear growth of rhizomorphs—the rhizomorph extends beyond the central mycelium, 5) terminal stages of rhizomorph development as lateral branches develop.

Rhizomorph formation and penetration of host tissue depends on nutritional conditions to a large extent. Townsend (122) found that the type of rhizomorph which developed was correlated with the food base. She also showed a C/N ratio of more than one stimulated rhizomorph development. The logistics of infection of *A. mellea* rhizomorphs was studied by Garrett (39). He found that infection of other hosts by rhizomorphs was a direct function of food base size and
the distance the food base was from other host. Infection occurred through root grafts or rhizomorphs growing through soil to new host material. The farther the root is from inoculum, the less chance for contact or infection because of a decrease in the growth rate of rhizomorphs. Apparently, this decline occurs because of the reduced nutrient supply associated with the lowered efficiency of translocation from the food base to the growing rhizomorph tips and competition from other microorganisms in the soil. In a later paper Garrett (45) concluded that the pathogen had the ability to decompose lignin and cellulose. It can grow ectotrophically or between wood and bark. He also found that the fungus could grow through soil to new host sites as long as it was attached to a food base.

In more recent studies, Weinhold (125) found that low molecular weight alcohols stimulated rhizomorph development in *A. mellea*. Further studies by Weinhold and Garraway (127) showed rhizomorphs were not produced by the fungus with any of the nitrogen sources added to various defined media if ethanol was absent. When such media were supplemented with ethanol, rhizomorph development occurred. Suitable nitrogen sources included amino acids, amides, casein, and ammonia. Fructose, sucrose, or glucose were suitable carbon sources for rhizomorph growth only when present in ethanol supplemented media. They found that partially purified figwood extract would induce rhizomorph development. They concluded that nutritional requirements for mycelial growth differ from those for rhizomorph development and a growth promoting factor is supplied by ethanol or figwood extract. In a later paper Garraway and Weinhold (42) found that the continuous presence of
ethanol was necessary for the early development of rhizomorphs on a basal medium containing glucose, thereafter glucose without ethanol could serve as a carbon source for continued rhizomorph growth. This finding was in contrast to that of Pentland (93) who found that the continuous presence of ethanol was needed for rhizomorph growth.

Other substances which have been found to promote rhizomorph initiation and development are o-aminobenzoic acid, p-aminobenzoic acid, indole acetic acid, and lipids (40, 79).

The physiology of growth and rhizomorph development in A. mellea is an area in which extensive studies have not been completed. A great many studies concern nutrition and optimum conditions for growth (8, 40, 45, 48, 79, 122, 125). Studies of physiology directly related to growth are limited to those by Garraway and Weinhold (40, 41, 42, 43, 44, 127). Using radioactive glucose and ethanol, they found concentrations of ethanol which promoted growth and rhizomorph production of A. mellea on a synthetic glucose medium did not provide enough carbon to explain the increase in growth observed (43, 44, 127). Therefore, it was concluded that ethanol promoted growth through its effect on the uptake and utilization of glucose.

Studies with glucose-^{14}C (43, 44) revealed that ethanol at a concentration (500 ppm) which stimulated growth and rhizomorph production inhibited uptake and glycolytic breakdown of glucose. Because of the 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.
Further observations with specifically labeled glucose-$^{14}$C showed the Hexose Monophosphate Pathway (HMP) is a major alternate glycolytic pathway for glucose breakdown in *A. mellea* (43, 44). These observations led to the idea that the proposed inhibitory metabolites might be synthesized via this pathway.

Other physiological studies concerning *A. mellea* have shown that the pathogen produces in culture a wilt-inducing protein. This protein is, however, not a part of the disease syndrome (120). Greathouse and Rigler (53) have shown that certain alkaloids inhibit growth of the pathogen. In 1968 Swift (115) found in Rhodesian forest soils a water soluble inhibitor of growth which was effective against *A. mellea*.

The importance of *A. mellea* as a pathogen is well documented (28, 57, 70, 98, 119). The fungus is considered a facultative parasite (124). It is found throughout the temperate and tropic world on a wide range of hosts. Raabe (98) has compiled a complete and concise host list of the pathogen. The disease cycle has been described by several authors—Walker (124), Leach (70), Thomas (119), Marsh (80). The importance of basidiospores in the disease cycle is still questionable. However, Rishbeth (102) has shown that spores may be an effective means of dissemination. Most workers have felt rhizomorphs to be the most important source of inoculum (10, 18, 28, 45, 57, 70, 119).

Several studies have been made concerning control of the pathogen. Biological control studies have dealt mainly with the use of *Trichoderma viride*. Bliss (10) found that *T. viride* suppressed growth of *A. mellea* on agar but failed to destroy it after formation of a pseudo-sclerotium. Later work by Bliss showed that *T. viride* developed
rapidly in fumigated soils and had strong antibiotic action against *A. mellea*. Garrett (46, 47) concluded in his work that *A. mellea* was inhibited by *T. viride* in fumigated soil.

Stem girdling (49, 70, 100) has been attempted in control of *A. mellea*. This might be considered a related form of biological control. The results have been conflicting, however. Leach (70) found that ringing of trees a year before felling produced a decrease in *A. mellea* infections in subsequent plantings. He hypothesized that the root system of ringed trees died early and were invaded by saprophytic wood-decomposing fungi. Their occupancy in and on the roots prevented the invasion of the roots by *A. mellea*. Garrett (49) found similar results working with willow. Redfern (70), however, found that ringing of English hardwoods (oak in particular) produced no significant decrease in *A. mellea*. Garrett (49) explains this paradox by concluding that *A. mellea* is more prevalent in English woodland soils and produces abundant rhizomorphs from its food base in English soils. In East Africa, where Leach worked, the pathogen does not have these characteristics and is usually spread through root grafts. Garrett, therefore, concludes that *A. mellea* is so abundant in English woodland soils that it can colonize dead or decaying roots ahead of saprophytic fungi. Thomas (119) found that trenching the soil around diseased trees gave some control.

Another form of control has been through the use of fumigants. Bliss (10) indicated *CS₂*, chloropicrin, and ethylene oxide could control the pathogen in the soil. Garrett (46) confirmed that *CS₂* could control the pathogen. Neither of the authors was sure whether the
control by CS$_2$ was due to the chemical or due to increase of T. viride in CS$_2$ treated soil. Munnecke et al. (86) studied the effect of methyl promide on A. mellea. They found 3000 ppm methyl bromide gave LD 95 in 1.6 days, whereas 500 ppm gave LD 95 in 9.5 days.

The most efficient control to date seems to be through removal of infested soil, soil disinfestation, removal of infested stock, in general, good silviculture practices, according to Walker (124), Leach (70), Hartig (57), Zeller (132), and Marsh (80).

One aspect of control not yet mentioned is control through resistant varieties. At present, this needs more study. Day (28), in his studies, found differences in susceptibility to the pathogen. He found pines most susceptible, while those least susceptible were larch and fir. He also concluded external factors affect the susceptibility of trees to the pathogen. He hypothesizes that races within susceptible species may show resistance. Christensen (22) found resistance varied with density of stands and age of trees. Thomas et al. (118) found the resistance to the pathogen varied with species. He found black walnut, Spanish chestnut, fig, and others resistant to the pathogen, while quince, apricot, prune, and pear were very susceptible to the pathogen.

Phenols and phenol oxidizing enzymes in relation to growth and morphogenesis:

Phenolic compounds are considered to be secondary products of plant metabolism (59, 87, 103). These compounds are involved in aromatic metabolism, cell wall polymer formation, and other secondary metabolic pathways.
The effect of phenols on growth of organisms in the plant kingdom is well documented. The literature is so extensive that the author will refer those more interested in the topic to some selected reviews. The author will also present some information from recent papers to document the importance of phenols in growth.

The role of phenolics in host response to infection has been a major area of interest in plant pathology in recent years. Several extensive reviews have been published, the most recent by Kosuge in 1970 (64). Other major reviews are by Cruickshank (24), Kuc (66), Rubin (104), and Tomiyama (121).

Antimicrobial compounds produced by a host plant in response to infection are generally termed phytoalexins (85). These compounds, many of which are phenols, have been shown to be potent inhibitors of growth of many plant pathogens (25, 64). These compounds are thought to provide a source of resistance to the host by inhibiting the fungus at the site of penetration, thus limiting the pathogen to a discrete area and reducing severity of the disease (25, 64, 66, 121). The mechanisms by which this occurs are still in dispute (26). The possible mechanisms for induction of phytoalexins are highly speculative (26). Generally, one of three mechanisms is thought to be involved. These are 1) gene activation in the host by some product of the fungus (54), this closely resembles Jacob-Monod scheme in bacteria; 2) activation or control of some preformed enzyme (26); 3) allosteric control of enzymes at some regulatory point in the biosynthesis of phytoalexins (26).

Phenolic compounds and their oxidation products have been shown to be inhibitory in vitro and in vivo to a number of organisms. Phenolic
compounds inhibit spore germination of *Diplodia zea* (27). *Fusarium oxysporum f. lycopersici* has been shown to be inhibited by an unknown phenol from tomato (82). Hathaway (60) showed that tannins from myrobalan (*Terminalia chebula Retz.*) inhibit endopolygalacturonase of several fungi. Bell (7) found that the compounds, hydroxybenzaldehyde and vanillin, which form in leaf wound sap of *Phaseolus vulgaris*, were toxic to uredospores of *Uromyces phaseoli*, *Puccinia sorghi*, *U. avenae*, and *U. nuda*. Masuko et al. (81) have isolated an antifungal phenol, plumbagin, from *Ceratastigma willmottianum*. This compound has been shown to inhibit *Cochleobolus miyabeanus* (leaf blight of rice). These are only a few examples of fungi being inhibited by phenolic compounds.

Phenolic compounds have also been shown to inhibit bacteria. Cruickshank (25) and Cardoso (20) have shown phaseollin, a phenolic phytoalexin, has antibiotic properties against several bacterial species. Strezelezyk (112) screened several phenolic compounds for antibiotic properties and found that coumarin, gallic acid, t-cinnamic acid, vanillin, and o-coumaric acid were very potent inhibitors of bacterial growth.

Endogenous accumulation of phenolic materials within fungi and bacteria, to the author's knowledge, has never been explored. It is not known whether phenolic compounds can accumulate endogenously in lower plants to levels which would inhibit growth or differentiation. There have been two reports of this phenomenon occurring in higher plants. Forrest (34), working with tissue culture of tea plants, found an inverse relationship between growth and phenol accumulation. Henshaw
and Pearce (61) found similar results with Acer pseudoplatanus. Phenols accumulate as growth is inhibited.

The study of morphogenesis is the study of those aspects of development related to morphological changes (116). Most studies of morphogenesis have dealt with descriptive and nutritional aspects, although some studies have dealt with the biochemical changes which occur during morphogenesis. The actual mechanisms of morphogenesis in fungi are poorly understood (19, 33, 116, 131).

Some of the major studies in morphogenesis include those of Neiderpruem and Wessels (88) with Schizophyllum commune, Bartnicki-Garcia and co-workers (3, 4, 5, 6) with Mucor rouxii, Garraway and Weinhold (41, 42, 43, 44) and Snider (110) with Armillaria mellea, de Terra and co-workers (30) with Neurospora crassa, and Cantino and co-workers (19) with Blastocladiella emersonii.

The majority of work on the biochemistry of morphogenesis has involved studies of cell wall changes (3, 43, 30, 88). The underlying mechanisms which produce these changes are still unknown.

The role of phenols in morphogenesis is not clearly defined. Few reports are directly related to phenols in morphogenesis. There are some reports, however, concerning the involvement of oxidation products of phenols in fungal morphogenesis (19, 32, 130).

Cantino (19) hypothesized that phenols oxidized to melanins by polyphenol oxidase played an important role in formation of resistant sporangia of Blastocladiella emersonii. His work showed that if you exposed actively growing thalli of the fungus to HCO$_3$ for 3/5 or more of its generation time, resistant sporangia formed. In the absence of
HCO$_3$ ordinary colorless sporangia formed. Resistant sporangia formation was accompanied by the accumulation of $\alpha$-ketoglutarate and polyphenol oxidase. He hypothesized that if $\alpha$-ketoglutarate oxidase and succinic oxidase were inhibited, $\alpha$-ketoglutarate was converted back to isocitrate and excess NADP was left. Polyphenol oxidase caused oxidation of polyphenols and the H$^+$ ions liberated bind with the NADP left from conversion of $\alpha$-ketoglutarate to isocitrate. The oxidized polyphenols then condense forming melanins. He found inhibitors of polyphenol oxidase prevented melanin formation.

Melanins have been reported in other fungi—Aspergillus nidulans (15), Rhizoctonia solani (95), and Cladosporium herbarum (95). Bull (15, 16) and Kuo (67) found that differences in growth and susceptibility to lysis by soil microbes were directly related to melanin content of hyphal filaments of A. nidulans. Potgeiter (95) reported similar results with other fungi. Bull (15) showed that the inhibition of lysis by high melanin content hypha was due directly to the melanin.

To obtain a clear understanding of the role of phenols in morphogenesis, attention should be given concurrently to the enzymes which oxidize the phenols. There is a voluminous literature concerning peroxidase and polyphenol oxidase (46, 75) but only limited information concerning their role in morphogenesis.

Peroxidase is an enzyme with a non-heme iron cofactor (128). This enzyme carries out the H$_2$O$_2$ dependent oxidation of phenylpropane compounds, yielding lignin-like substances (108). This enzyme also oxidizes phenolic compounds and aromatic amines in the presence of
$H_2O_2$. Polyphenol oxidase is an enzyme with a copper cofactor (128). It catalyzes the oxidation mono- and polyphenol to quinones (52).

Cantino showed the importance of polyphenol oxidase in the morphogenesis of resistant sporangia in Blastocladiella emersonii. Esser (32, 33) showed in Podospora auserina that increase in size of perithecia was directly related to increased polyphenol oxidase activity. He also found different polyphenol oxidase isozymes predominate in different morphological mutants. Singh (109), working with Colletotrichum falcatum, found that polyphenol oxidase activity was higher in light colored, highly sporulating strains than in darker, low sporulating strains of the fungus when they grew in tannic acids. It appears from the reports of Singh and Cantino that high polyphenol oxidase activity can be associated with either dark or light spores. The reasons for this apparent contradiction were not clear from the reports of either researcher. Sekhon and Colotelo (106) found in a snow mold organism (unnamed cold-temperature Basidiomycete) that as autolysis occurred with age, there was decreased peroxidase activity. Wilson, studying Hypomyces solani f. sp. cucurbitae (130), found increased number of perithecial primordia was directly associated with increased tyrosinase activity. He found commercial tyrosinase induced perithecial development. He suggested increased tyrosinase may make tyrosine unavailable for protein synthesis or may alter cell permeability or may inactivate certain enzymes by oxidizing protein bound tyrosine. In a later paper (131) he showed increased tyrosinase was associated with altered permeability of cellular membranes as vegetative growth stopped and perithecia formed.
In higher plants the role of peroxidase in morphogenesis is better understood. Peroxidase can inactivate auxin (37, 38, 65, 69, 83). The enzyme oxidizes hydroxyphenyl propanes to lignin precursors, and it is also important in oxidizing reduced nicotinamide adenine dinucleotide and its phosphate (108, 129). In pea stem and geranium tissue culture, peroxidase activity is inversely proportional to growth and auxin content of cells (37, 83). It has also been found that in genetically dwarf plants the peroxidase activity is very high. If the plants are treated to relieve dwarfism, the peroxidase activity drops rapidly as growth is promoted (50).

Whether or not peroxidase plays a similar role in fungi is not well understood. One of plant pathology's most rewarding areas of research in the future may be in the study of phenols, polyphenol oxidase, and peroxidase and their relationship to morphogenesis in fungi and physiology of host parasite relations.
III. GROWTH STIMULATION OF *ARMILLARIA MELLEA* BY LOW MOLECULAR WEIGHT ALCOHOLS CORRELATED WITH INHIBITION OF PHENOL ACCUMULATION.
INTRODUCTION

Ethanol and other low molecular weight alcohols have been shown to promote mycelial growth and rhizomorph development of A. mellea (125). Concentrations of ethanol which promoted growth and rhizomorph production of A. mellea on a synthetic glucose medium did not provide enough carbon to explain the growth observed (127). 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 of 500 ppm, which stimulated growth and rhizomorph production, caused a reduction in uptake and glycolytic breakdown of glucose. Because of the inverse relationship between growth and glucose metabolism in the presence of ethanol, it was suggested that glucose might be converted to inhibiting metabolites in the absence of ethanol and that ethanol might promote growth by suppressing inhibitor production (43, 44).

Further studies with specifically labeled glucose-\(^{14}\)C indicated the Hexose Monophosphate Pathway (HMP) is a major alternate glycolytic pathway for glucose breakdown in A. mellea (43, 44). 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 (7, 20, 25, 75, 81, 89). Phenolic compounds are synthesized via intermediates of the HMP (87, 103). With the HMP a
major alternate glycolytic pathway in *A. mellea*, perhaps the proposed inhibitory metabolites might be phenolic in nature.

The purpose of these first studies was to find out if ethanol and other low molecular weight alcohols stimulation of growth could be associated with suppression of phenolic compounds. These studies were also done to see if phenols which accumulate on ethanol are qualitatively different from phenols which accumulate in thalli grown in the absence of ethanol.
MATERIALS AND METHODS

Organism used and culturing techniques:

A pathogenic isolate of *A. mellea* originally obtained from Dr. R. D. Raabe at University of California, Berkeley, was used for all studies. The fungus was maintained by two methods: 1) By seeding the fungus on potato dextrose agar (PDA) slants sealed for long term storage. This method also provided rhizomorph tips for use in the second method of maintaining the cultures. 2) Cultures were maintained for short term storage on 1.5% water agar. These cultures were used when they were between 4-6 weeks old. The water agar cultures produced no rhizomorphs and the mycelium was very fine. Rhizomorph tips from cultures grown on PDA were used to seed the water agar cultures.

Culture techniques of *A. mellea* for experimental purposes was as follows: The fungus was cultured on a liquid synthetic medium consisting of 5 g D-glucose, 1.75 g KH₂PO₄, 0.75 g MgSO₄·7H₂O, 2 g L-asparagine, 1 mg thiamine, and 0.5 g (500 ppm) ethanol/liter of double distilled water. The pathogen was grown in 8 oz medicine bottles containing 50 ml of the media (109). The medium was seeded by cutting out discs of fungus from water agar cultures with a No. 2 (10 mm) cork borer and transferring these plugs onto one inch nylon gauze (tulle) squares which were floated on the liquid medium (Fig. 1). After seven days on the above system, the thalli were transferred to 15 x 1.5 cm petri plates containing liquid medium with no carbon (ethanol or glucose). Each plate contained 15 thalli (Fig. 2). After 24 hr equilibration
Figure 1. Photograph of technique used to culture *Armillaria mellea* in 8 oz medicine bottles.

Figure 2. Technique used to culture *Armillaria mellea* in 15 cm petri plates. Various treatments added while thalli are in these plates.
period on the no-carbon medium, appropriate treatments were supplemented to the thalli for a time period of from 0-72 hr. The 24 hr equilibration time on no-carbon was used in order for the fungus to utilize endogenous carbon sources.

The treatments to which thalli were exposed consisted of 1) 0 glucose/liter, 2) 5 g glucose/liter, 3) 5 g glucose + 0-500 ppm ethanol/liter, and 4) 5 g glucose + ethanol, propanol, butanol, or iso-butyl alcohol, 500 ppm/liter. After appropriate treatment with ethanol, different alcohols or glucose, thalli were harvested at 0, 24, 48, and 72 hr. Thalli were removed from plates and then were removed from nylon gauze by scraping with a knife into 10 dram vials. The cultures were stored at 0°C until use or were used immediately. Throughout all experiments contaminated thalli were discarded.

Dry weight determinations:

Dry weight determinations were made by placing thalli, selected at random from respective treatments, on tarred aluminum pans and drying for 24 hr at 100°C. Weight determinations were made on a Mettler H20 Analytical Balance.

Estimation and extraction of phenols:

The fungal tissue from appropriate treatments was macerated in 80% ethanol (15 ml/100 thalli) in a Virtis "45" Homogenizer at 45,000 rpm for three minutes. Protein was precipitated by adding 20% trichloroacetic acid (TCA) (15 ml/30 ml extract). The homogenate was centrifuged at 37,000 g for 30 min in a Sorvall RC-2B Refrigerated Centrifuge. The residue was saved and the supernatant was assayed for total phenols.
with a modified Folin-Denis technique adapted from Swain and Hillis (113). Total phenols were estimated as chlorogenic acid equivalents from a standard curve prepared with concentrations of chlorogenic acid from 0-200 μg/ml.

Extraction of phenols from the supernatant was performed to use for Thin Layer Chromatography (TLC) and column chromatography. The supernatant was adjusted to pH 2.5 by addition of 0.1 N HCl or 0.1 N NaOH. The acidified supernatant was then partitioned four times into equal volumes of ethyl acetate. The ethyl acetate fractions were combined and dried with CaCl₂ to remove water. The ethyl acetate fractions were then reduced to a volume which corresponded to 30 thalli/1 ml. (See Fig. 3 for flow diagram).

**Thin layer and column chromatography of extracts:**

TLC of extracts was performed on 20 x 20 cm glass plates, coated with various layers 250 μ thick, prepared with a Brinkmann-Desaga apparatus. Layering materials for TLC were MN-polyamide-DC11 (Macheray, Nagel, and Co.) impregnated with 8% by weight plaster of Paris (15 g polyamide/45 ml methanol), Silica Gel G according to Stahl (111), and Avicel Microcrystalline Cellulose by Brinkmann (25 g/150 ml water). Solvent systems used for TLC butanol-acetic acid-water (4-1-5, v/v) and benzene-methanol-acetic acid (90-16-8, v/v) for polyamide layers. For silica gel and cellulose plates the solvent system was N-butyl ether (water saturated)-acetic acid (90-9, v/v). Fluorescence of compounds was visualized with UV-11 and UV-22 mineralite lamps, phenols were visualized with diazotized sulfanilic acid, diazotized p-nitroaniline, and FeCl₃.
Macerate tissue in Vertis Homogenizer (45,000 rpm for 5 min) in 80% ethanol, 15 ml/100 cultures.

Add 20% TCA (5 ml/10 ml extract)

Centrifuge homogenate at 27,000 x g for 30 minutes.

Residue, save

Supernatant assay for phenols

Acidify to pH 2.5 with 0.1 N HCl or 0.1 N NaOH

Partition 4 times with equal volumes of ethyl acetate

Combine ethyl acetate fractions dry with CaCl₂, reduce to volume which corresponded to 30 thalli/ml

Save aqueous fraction

Figure 3. Flow diagram of phenol extraction.
Column chromatography was attempted using Sephadex LH-20 and G-10 gel filtration systems. Ethanolic solutions of the ethyl acetate extract were passed through the column while using 80% ethanol as a solvent. Column specifications were 1) for LH-20, 300 mm x 20 mm with a bed depth of 200 mm, a void volume of 14 ml, and flow rate 20 drops/min; 2) for G-10, 400 mm x 15 mm with a bed depth of 250 mm, a void volume of 9 ml, and flow rate of 20 drops/min. The optical density (OD) of the eluate was monitored at 254 nm and 280 nm with an Isco Model UA-2 ultraviolet analyzer. Each fraction was analyzed with Folin-Denis to see if UV peaks corresponded to phenol peaks. TLC was done of fractions to detect which fractions corresponded to specific spots on TLC.

Ultraviolet spectra of spots from TLC:

Compounds which appeared to respond to specific treatments were eluted from TLC plates and UV spectra were obtained. Compounds were collected by two methods, 1) by scraping layers off of plates, which corresponded to specific spots, with a spatula onto glassine paper and then placing this into 4 dram vials to be eluted with 95% ethanol; 2) the second method consisted of vacuuming spots off of plates (Fig. 4) into tubes, then eluting the material with 95% ethanol. Material from both methods of collection was bathed in eluting solution for 24 hr. If material was eluted for less than this period, percent recovery was reduced. At 24 hr elution, 90%+ recovery was obtained. Appropriate blanks were obtained by removing an equivalent amount of layering material which had been exposed to solvent and elution of this material. The eluate was millipore filtered to remove all traces of layering material. UV spectra were obtained by placing samples with
Figure 4. Apparatus used to elute substances from thin layer chromatograms.
corresponding blanks in a Beckman DB-G Recording Spectrophotometer and scanning from 340 to 220 nm.
RESULTS

Growth and phenol accumulation in A. mellea after various incubation times on media with and without a supplement of ethanol:

For this study, 7-day old thalli which were growing on a glucose medium supplemented with ethanol (500 ppm) were transferred to fresh media with no carbon source. After 24 hr on no-carbon media, the thalli were transferred to glucose media with ethanol (500 ppm) supplemented (hereafter called G + E media) or thalli were not supplemented with ethanol (hereafter called G media). At 24 hr intervals from zero to 72 hr, replicate groups of 15 thalli were removed from either medium for dry weight determinations. Comparable groups of thalli were extracted with ethanol and assayed for total phenols with Folin-Denis reagent. The results of this study are presented in Fig. 5 and Fig. 6.

The dry weight per thallus increased linearly with time on either medium (Fig. 5). The increase was considerably more rapid on G + E than on G. The dry weight per thallus was 6.0 ± 0.2 mg at the beginning of the experiment. After 72 hr the dry weight on G + E and G were, respectively, 16.7 ± 0.3 mg and 8.9 ± 0.2 mg. The growth on G + E was twice as great as growth on G.

The concentration of phenol in the fungus (Fig. 6) decreased rather linearly with time on G + E but increased on G. The phenol concentration was 4.5 ± 0.3 µg/mg dry weight at the beginning of the experiment. After 72 hr the phenol concentration decreased to 2.9 ± 0.2 µg/mg on
Figure 5. Growth of Armillaria mellea after various incubation times on liquid media containing glucose (---) or glucose supplemented with ethanol (500 ppm) (-----). Each value is the mean of five replications with four weights per replication. Mean deviation shown by vertical bars.
Figure 6. Changes in the concentration of phenols in *Armillaria mellea* thalli after various incubation times on liquid media containing glucose (-----) or glucose supplemented with ethanol (500 ppm) (------). Each value is the mean of five replications with four weights per replication. Mean deviation shown by vertical bars.
G + E, while on glucose the concentration increased to 6.5 ± 0.3 μg/mg dry weight. The phenol concentration on G was about twice as great as on G + E after 72 hr incubation.

These data indicate that the concentration of ethanol supplement which stimulates growth of *A. mellea* suppresses phenol accumulation. The percentage increase in growth on G + E appears to be proportional to the percentage decrease in phenols.

A similar study was done with 7-day thalli which were transferred to a no-carbon medium and incubated for 24 hr prior to incubation. The ethanol and glucose were directly supplemented to the no-carbon medium. Thalli were not transferred to a fresh medium. The results in these experiments were identical to those reported above.

**Growth and phenol accumulation in A. mellea after various incubation times on media containing different concentrations of ethanol supplement:**

This study was undertaken to determine whether the inverse relationship between growth and phenol concentration observed on media containing 500 ppm ethanol was consistent over a range of ethanol concentrations. The experimental methods were identical to those described above. The results of these studies are presented in Fig. 7 and Fig. 8.

The dry weight per thallus increased rather linearly with time for each concentration of ethanol (Fig. 7). The dry weight increase coincided with increase in ethanol concentration up to 500 ppm. The rate of growth at 1000 ppm ethanol was comparable to that at 500 ppm over 72 hr.
Figure 7. Growth of Armillaria mellea after various incubation times on liquid media containing glucose (---) or glucose supplemented with 50 (--......), 100 (-----), 250 (-----), 500 (-----), or 1000 (-----) ppm ethanol. Each value is the mean of five replications with four weights per replication.
Figure 8. Changes in phenol concentration in Armillaria mellea after various incubation times on liquid media containing glucose (---) or glucose supplemented with 50 (-----), 100 (-----), 250 (-----), 500 (-----), or 1000 (-----) ppm ethanol. Each value is the mean of five replications with four phenol determinations per replications. Phenols were measured as chlorogenic acid equivalents.
The phenol concentration in thalli was progressively reduced with increasing concentrations of ethanol (Fig. 8). At 50 and 100 ppm ethanol the phenol concentration actually increased but at a less rapid rate than on G. At 250, 500, and 1000 ppm the phenol concentration decreased. The magnitude of the decrease was greater at 500 and 1000 ppm than at 250 ppm ethanol. The decrease at 1000 ppm ethanol was comparable to the decrease at 500 ppm. Data related to the effect of various concentrations of ethanol on growth and phenol accumulation at 72 hr are summarized in Table 1.

These data indicate that the inverse relationship between growth and phenol concentration on ethanol observed previously is valid over a range of ethanol concentrations. Apparently, 500 ppm ethanol is the optimum concentration for stimulation of growth and suppression of phenols.

Growth and phenol accumulation in A. mellea after various incubation times on a glucose medium compared with that on a similar media supplemented with different alcohols:

Weinhold (125) noted that in addition to ethanol, other low molecular weight alcohols promoted growth and rhizomorph production in A. mellea. Therefore, these alcohols were studied to determine whether they produced an inverse relationship between growth and phenol accumulation when added as a supplement to a glucose medium in place of ethanol. The experimental methods were the same as those described above. The glucose medium was supplemented with 500 ppm of either ethanol, propanol, butanol, or iso-butanol. Growth and phenol
Table 1. Growth and phenol content of young *Armillaria mellea* thalli following 72 hr of incubation on liquid media containing various concentrations of ethanol.

<table>
<thead>
<tr>
<th>Ethanol Conc. (ppm)</th>
<th>Growth$^a$ (mg. dry wt.)</th>
<th>Phenol Conc.$^a$ ($\mu$g/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.2 ± 0.88</td>
<td>6.7 ± 0.20</td>
</tr>
<tr>
<td>50</td>
<td>10.4 ± 1.20</td>
<td>5.6 ± 0.35</td>
</tr>
<tr>
<td>100</td>
<td>11.1 ± 0.60</td>
<td>5.5 ± 0.15</td>
</tr>
<tr>
<td>250</td>
<td>14.1 ± 0.75</td>
<td>4.0 ± 0.72</td>
</tr>
<tr>
<td>500</td>
<td>17.0 ± 0.90</td>
<td>3.0 ± 0.23</td>
</tr>
<tr>
<td>1000</td>
<td>17.2 ± 0.50</td>
<td>3.2 ± 0.38</td>
</tr>
</tbody>
</table>

Dry wt. of thalli at Ohr = 6.2 mg; phenol content 4.5 $\mu$g/mg.

$^a$Average of 5 replicates in 5 experiments and mean deviation.
accumulation were compared with that on a non-supplemented (G) medium after various incubation times. The results of these studies are presented in Fig. 9 and 10.

Increase in growth was more rapid on media supplemented with alcohols than on glucose (Fig. 9). After 72 hr incubation the dry weight per thallus on glucose or glucose supplemented with either ethanol, propanol, butanol, or iso-butanol was 8.5 ± 0.2, 16.2 ± 0.4, 13.9 ± 0.5, 15.1 ± 0.2, and 13.3 ± 0.3 mg, respectively. Ethanol appeared to be more effective than propanol and iso-butanol but was comparable to butanol.

The phenol concentration decreased with all alcohols tested (Fig. 10). The phenol concentration after 72 hr incubation on glucose or glucose supplemented with either ethanol, propanol, butanol, or iso-butanol was 5.8 ± 0.3, 2.9 ± 0.1, 3.1 ± 0.3, 3.2 ± 0.2, and 3.4 ± 0.4 μg/mg dry weight, respectively. The decrease in phenol with ethanol was greater than with other alcohols. These data indicate that the inverse relationship between growth and phenol accumulation on ethanol occurs with other low molecular weight alcohols.

Phenol composition of extracts of A. mellea thalli incubated for 48 hr on glucose media with or without ethanol:

The possibility that qualitative change in phenols in A. mellea extracts may also have accompanied the ethanol induced quantitative changes reported above was investigated. For these studies, extracts of 7-day thalli which were incubated on either G or G + E for 48 hr were analyzed using thin layer chromatography (TLC) ultraviolet (UV) spectrophotometry, and gel filtration.
Figure 9. Growth of Armillaria mellea after various incubation times on liquid media containing glucose (---) or glucose supplemented with iso-butanol (----------), propanol (--), butanol (--), or ethanol (----). Each value is the mean of five replications with four weights taken in each replication.
Figure 10. Changes in phenol concentration in Armillaria mellea thalli after various incubation times on liquid media containing glucose (— — — — ), or glucose supplemented with iso-butanol (———), propanol (— — —), butanol (— — —), or ethanol (— — —). Each value is the mean of five replications with four phenol determinations per replicate. Phenols were measured as chlorogenic acid equivalents.
For TLC studies of phenolic extracts of *A. mellea*, 25 µl of ethyl acetate, 1 ml of which contained the phenols extracted from 30 thalli incubated on either G or G + E, were spotted on either silica gel G, polyamide or cellulose thin layer plates. The developing solvents were n-butyl ether-acetic acid, benzene-methanol-acetic acid, and n-butyl ether-acetic acid, respectively. Following development of the chromatograms, fluorescent or absorbent spots were visualized with long or short wave UV light and phenolic spots were detected by spraying with either diazotized p-nitroaniline or diazotized sulfanilic acid. Facsimiles of the thin layer chromatograms are presented in Figs. 11, 12, and 13.

Chromatograms developed on a polyamide layer revealed five distinct spots on the basis of fluorescence or absorbence characteristics and reaction with phenol detecting reagents (Fig. 11). Three of the spots were present in extracts of either G or G + E and two at Rf 0.17 and 0.61 were found in extracts from G only. Both of the latter spots found in G extracts gave positive reactions with phenol detecting reagents. The ultra violet absorption spectrum of these compounds is presented in Fig. 14. The compound at Rf 0.17 had UV absorption maxima at 272 and 232 nm. The 272/232 ratio of this compound was 0.781. The compound at Rf 0.61 had an absorption maximum at 270 nm.

Chromatograms developed on cellulose revealed four spots (Fig. 12). Three of these spots were present in extracts from G or G + E, while one at Rf 0.40 was found only in G extract. The latter spot gave a positive reaction with phenol detection reagents and had UV absorption maxima at 272 and 232 nm (Fig. 15). The 272/232 ratio of this compound was 0.705.
Figure 11. Facsimile of polyamide thin layer chromatogram of phenolic thallus extracts of Armillaria mellea. Letters a, c, and e represent phenolic extracts of thalli incubated for 48 hr on liquid media with glucose. Letters b, d, and f represent phenolic extracts of thalli incubated for 48 hr on liquid media with glucose + ethanol (500 ppm).
Figure 12. Facsimile of cellulose thin layer chromatogram of phenolic extracts of *Armillaria mellea*. Letters a, c, and e represent phenolic extracts of thalli incubated for 48 hr on liquid media with glucose. Letters b, d, and f represent phenolic extracts of thalli incubated for 48 hr on liquid media with glucose + ethanol (500 ppm).
Figure 13. Facsimile of silica gel G thin layer chromatogram of phenolic extracts of Armillaria mellea. Letters a, c, and e represent phenolic extracts of thalli incubated 48 hr on liquid media with glucose. Letters b, d, and f represent phenolic extracts of thalli incubated for 48 hr on liquid media with glucose + ethanol (500 ppm).
Figure 14. Ultraviolet spectrum of compounds found at Rf 0.17 (---) and Rf 0.61 (-----) in 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 ppm). (See Fig. 11).
Figure 15. Ultraviolet spectrum of compound at Rf 0.40 on cellulose plates. This compound was found in extracts of thalli of Armillaria mellea incubated on liquid media with glucose for 48 hr. No corresponding compound was found in extracts of thalli incubated on glucose + ethanol (500 ppm). (See Fig. 12)
The spot at Rf 0.17 on the polyamide plate and the spot at Rf 0.40 on cellulose were eluted and respotted on a silica gel G plate. Using n-butyl ether-acetic acid as the developing solvent, the Rf of the compound from either spot was 0.33. Using 70% ethanol as the developing solvent, the compound from either spot remained at the origin. These data suggest a similarity between the compound at Rf 0.17 and 0.40 on polyamide and cellulose thin layer plates, respectively.

Chromatograms developed on a silica gel layer (Fig. 13) revealed five distinct spots. Four of these spots were present in extracts of both G and G + E, while one, at Rf 0.65, was found only in G + E extract. This compound was found to be phenolic as revealed by its reaction with Folin-Denis, diazotized p-nitroaniline, and sulfanilic acid.

These data indicate that the presence of ethanol in an incubation medium containing glucose affects not only the amount of phenols, but also the kinds of phenols which accumulate in A. mellea thalli.

Gel filtration of phenolic extracts of A. mellea was performed using Sephadex LH-20 and G-10. The 280 nm and 254 nm absorption profiles of fractions eluted from LH-20 gel are given in Fig. 16 and 17, respectively. The void volume of the columns was collected in fractions 1-6. The fractions from glucose extracts had much higher optical density (OD) values than comparable fractions from G + E at both 280 nm and 254 nm. The OD 280 nm (Fig. 16) of fraction 14 from G extracts was 0.22 and that from G + E extract was 0.10. Similarly, the OD 254 nm (Fig. 17) of fraction 15 from G extracts was 0.39, while that from G + E extract was 0.20. The tracings revealed poor resolution of the component substances in the extracts. However, with extracts from glucose
the material(s) in fractions 20-22 (Fig. 17) appeared to be clearly separated from the other compounds. Comparable fractions from G + E extracts contained no detectable UV absorbing material.

Fractions 19-22 (Fig. 17) were collected, reduced in volume, and then chromatographed on silica gel and cellulose plates. The fractions from glucose contained a compound which had an Rf similar to the compounds described earlier. On cellulose the Rf was 0.40, on silica gel it was 0.31. The fractions taken from G + E did not have a coincident spot when TLC was performed. The compound in glucose extracts gave positive phenol test with Folin-Denis and diazotized p-nitroaniline and fluoresced under long wave UV light.

Gel filtration with Sephadex G-10 was inconclusive. No separation was obtained, however, the OD 254 nm was measured for G extracts and G + E extracts, and again there was approximately twice as much absorbance at 254 nm in extracts of thalli grown on G as compared to extracts of thalli grown on G + E. In fraction 11 the G extract had an OD of 0.30, while the G + E extract had an OD of 0.09. There was approximately three times more 254 absorbing material in this fraction of G extract as compared to G + E extract.

Folin-Denis phenol measurements were made of fractions collected from LH-20 columns. This was done to see if UV peaks corresponded to phenol positive compounds in the eluate. The results are presented in Table 2. The highest OD with Folin-Denis were obtained in fractions 11-23 for extracts of thalli grown on G. This corresponds to the higher OD 254 as monitored in these fractions. The higher OD values for Folin-Denis of G + E extracts were found in fractions 9-18. These correspond
Figure 16. A comparison of the OD 280 of fractions from gel filtration of phenolic extracts of Armillaria mellea incubated on liquid media for 48 hr containing glucose (-----) or glucose + ethanol (500 ppm) (--->). Extracts were fractionated on Sephadex LH-20 with 80% ethanol as eluate.
Figure 17. A comparison of the OD 254 of fractions from gel filtration of phenolic extracts of Armillaria mellea incubated on liquid media for 48 hr containing glucose (— — — — —) or glucose + ethanol (500 ppm) (— — — — — —). Extracts were fractionated on Sephadex LH-20 with 80% ethanol as eluate.
to the higher OD 254 as monitored in these fractions. When monitored at 280 nm the higher OD's were obtained in fractions 10-18 for G extracts and fractions 12-17 for G + E extracts. These data indicate that the fractions absorbing at OD 254 and OD 280 correspond closely to Folin-Denis absorbing fractions of the eluate. This gives further support that more and different aromatic compounds accumulate in thalli incubated on G as compared to thalli incubated on G + E.
Table 2. Phenol concentration in eluate fractions of *Armillaria mellea* incubated for 48 hr on glucose media or glucose + ethanol, 500 ppm, media compared to OD 254 nm and OD 280 nm of eluate fractions.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Glucose</th>
<th></th>
<th>G + E, 500 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol Conc. aµg</td>
<td>OD 254</td>
<td>OD 280</td>
</tr>
<tr>
<td>6</td>
<td>0.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>9</td>
<td>2.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>10</td>
<td>2.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>11</td>
<td>2.5</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>12</td>
<td>5.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>13</td>
<td>15.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>14</td>
<td>35.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>15</td>
<td>37.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>16</td>
<td>35.0</td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td>17</td>
<td>13.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>18</td>
<td>10.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>19</td>
<td>8.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>20</td>
<td>14.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>21</td>
<td>13.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>22</td>
<td>13.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>23</td>
<td>5.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>24</td>
<td>0.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*aPhenols measured as chlorogenic acid equivalents by Folin-Denis Technique at 725 nm.*
CONCLUSIONS

The foregoing study showed that there is an inverse relation between growth of *A. mellea* thalli and phenol accumulation. Stimulation of growth of thalli by the addition of an ethanol supplement to a glucose medium was accompanied by a decrease in phenol concentration. Decrease in phenols was proportional to the ethanol supplement added to the medium. Other low molecular weight alcohols appeared to produce the same effect as ethanol. Butanol was comparable to ethanol in stimulating growth and depressing phenol concentration. Propanol and iso-butanol were less effective. Ethanol, at concentrations which stimulated thallus growth, also affected the kinds of phenols present. Thus, the mechanism by which ethanol and other low molecular weight alcohols stimulate growth of *A. mellea* thalli may be related to their effect on the quantity and quality of phenols present.

Ethanol and/or products of ethanol metabolism probably affect phenol levels through regulation of primary metabolic pathways. Metabolic effects of ethanol and/or its metabolites which could alter phenol concentrations and kinds of phenols in *A. mellea* are mentioned briefly below.

Ethanol has been shown to stimulate incorporation of glucose into cell wall polysaccharides (43). Gancedo (39) showed ethanol stimulated gluconeogenesis in yeast by stimulating fructose-1-6-diphosphatase activity. This enzyme is a major regulatory enzyme in gluconeogenesis.
Garraway (43), measuring release of C14O2, concluded ethanol slowed glucose breakdown. Ethanol could be acting to produce a Pasteur type of effect. As ethanol is utilized via the glyoxylate cycle (76), excesses of adenosine triphosphate (ATP) might be produced. This would slow glucose breakdown in both the HMP and Embden-Meyerhof-Parnas (EMP) pathways. Excess ATP inhibits allosterically phosphofructokinase in the EMP. This would decrease glycolysis via the EMP (78, 128). Excess ATP could also control glucose breakdown via HMP by allosterically controlling enzymes in this pathway (78, 128).

All the mechanisms mentioned above working in the presence of ethanol would decrease or utilize precursors of phenol biosynthesis. Therefore, in the presence of ethanol phenol biosynthesis is reduced and phenols do not accumulate as extensively as they do in the absence of ethanol.

If phenol accumulation is a primary mechanism in limiting growth and morphogenesis in A. mellea when incubated on G, several explanations could show how phenols control the morphogenetic process. Phenols can be oxidized to melanins and lignins (16, 19, 52, 78, 89, 108). These could be incorporated into cell walls, giving the wall more rigidity. This could prevent cell wall expansion and thus limit growth. Forrest (34) and Henshaw (61) have found in higher plants an inverse relation between growth and phenol accumulation. They hypothesize phenols polymerize to form compounds which limit cell wall expansion.

Phenols which accumulate on G may act to inhibit many enzyme systems in the fungal thallus. The phenolic compounds may inhibit cell
wall synthesis by inhibition of phosphorylation of cell wall precursors. Phosphorylated compounds are known to be important as intermediates in cell wall synthesis (3, 78, 128). Schwimmer (105) has shown phenol inhibition of phosphorylases, enzymes involved in phosphorylation of compounds. Phenols have also been shown to inhibit other reactions and enzymes. Evidence has been reported which indicates phenols inhibit cellulases (16, 52, 54), pectinases (29, 82, 89), transaminases (12), and decarboxylases (58). With phenolic compounds being involved in inhibition of enzymes, this could explain why growth is inhibited on G as phenols accumulate.

Another aspect to consider is that inhibition may be the result of one or two compounds. Thin layer chromatography and column chromatography indicate that there are at least two phenolic compounds which are found in thalli cultured on G which are not found in thalli cultured on G + E. Either of these compounds, or both, may be the active components in suppression of growth. Ethanol may act to prevent their synthesis or prevent oxidation of the compounds to toxic entities.

It was noted in this author's studies that butanol and ethanol were more effective in stimulating growth than iso-butanol or propanol. This might be the result of preincubation for seven days on ethanol. On this system the enzymes for utilization of even numbered straight chain alcohols would be induced and then when transferred to ethanol or butanol, both even numbered and straight chain, no further adaptation of enzymes would be needed. While, if thalli are transferred to odd numbered carbon alcohols (propanol) or alcohols which are not straight chain (iso-butanol), new enzymes would have to be synthesized before
the compound could be used. This system is known to occur for lipid utilization via Beta-oxidation (78, 128). An analogous system could be working with alcohols.
IV. ANTIMICROBIAL ACTIVITY OF PHENOL–CONTAINING EXTRACTS FROM _ARMILLARIA MELLEA_ AND OF AUTHENTIC AROMATIC COMPOUNDS.
INTRODUCTION

Two observations from the foregoing study provided the impetus for the present investigation. First, there was an inverse and quantitative relationship between ethanol stimulation of growth of *A. mellea* and ethanol suppression of phenol accumulation. Second, the kinds of phenols in extracts from thalli incubated on a G medium differed from those of thalli incubated on G + E. These observations led to the idea that quantitative and qualitative differences in phenols of thalli incubated on G might be the cause of the reduced growth rate on G as compared to that on G + E. In view of this, the antimicrobial properties of the phenol extracts and component phenols from *A. mellea* thalli were investigated. For comparison, the antimicrobial properties of several known (authentic) aromatic compounds were investigated. The known compounds tested were precursors of lignin.
MATERIALS AND METHODS

Organisms used, culture techniques, extraction procedures:

The organisms used were A. mellea, Botrytis cinerea, and several genera of bacteria. Culture techniques and extraction procedures for A. mellea have been previously described (see p. 22 to 24).

Bioassay of Armillaria mellea:

For this bioassay the rate of linear growth of A. mellea colonies on PDA was compared with that of colonies on PDA to which phenolic extracts of variously treated A. mellea thalli or known aromatic compounds were added. Five ml of melted PDA (40°C) were added to small petri dishes (5 cm dia) which contained aqueous solutions of A. mellea phenolic extract or of known aromatic compounds. The aqueous extracts were prepared by evaporating an ethyl acetate extract of A. mellea to dryness, then redissolving the residue in an equal volume of sterile double distilled water. With this technique, over 80% of the phenols in the ethyl acetate extract were recovered in the aqueous extract. The controls consisted of PDA to which neither fungal extract nor aromatic compound was added.

PDA plates were seeded with plugs cut from water agar cultures of A. mellea with a No. 2 cork borer (6 mm dia). The data were recorded as the colony diameter resulting from mycelial growth after eight days, and the colony diameter resulting from rhizomorph and mycelial growth after 12 days. If no rhizomorphs had developed in 12 days, colony
diameter was measured as mycelial growth. Data were also recorded as the number of rhizomorph initials following eight and 12 days. All plates were incubated in the dark at 25°C.

Bioassay of Botrytis cinerea:

*B. cinerea* was grown on 30 ml PDA in 250 ml Erlenmeyer flasks. Spores from the resultant cultures were harvested after ten to 12 days by flushing the surface of the media with 50 ml of sterile double distilled water. The spore suspension formed was passed through a fine wire mesh to remove large cellular fragments. Spores were then washed three times with double distilled water. After each washing, spores were centrifuged (5000 rpm/5 min), and then resuspended in sterile double distilled water. After the final wash, spores were resuspended in germination media which consisted of 10 g D-glucose, 3 g NaN₃, 1.5 g KH₂PO₄, 0.75 g MgSO₄·7H₂O, 1 mg CuCl₂, 1 mg MnSO₄·7H₂O, 1 mg MoO₃, and 1 mg FeSO₄/liter of double distilled water. The concentration of spores was determined with a hemacytometer and adjusted to a final concentration of 2 x 10⁵ spores/ml. One ml of this spore suspension was incubated for 12 hr with various amounts of *A. mellea* extract in 4 dram screw cap vials.

*A. mellea* extract was prepared by homogenizing thalli in 80% ethanol. The homogenized ethanolic thalli were acidified and partitioned against three volumes of ethyl acetate as described earlier (p. 25). The ethyl acetate fractions were combined, evaporated to dryness and redissolved in 80% ethanol. This fraction was termed the ethyl acetate fraction. The ethanol fraction of the homogenate was taken to dryness and redissolved in double distilled water. This is
called the aqueous fraction. Fifty μl of extract was then added to each vial with spore suspension. Controls were 50 μl 80% ethanol to vials and 50 μl of double distilled water added to vials.

Preparation and bioassay of bacteria:

Cultures of Bacillus subtilis, Corynebacterium michiganense, Escherichia coli, and Xanthomonas pruni, kindly supplied by Dr. P. O. Larsen, were used to test the antibiotic activity of extracts from A. mellea. Bacteria were cultured on agar slants of nutrient broth-sucrose medium which consisted of 8 g nutrient broth, 1 g yeast extract, 1 g sucrose, 15 g agar/liter of double distilled water. After 72 hr on this medium, 5 ml of sterile double distilled water was added to slants. Slants were then shaken slightly to suspend bacteria in water. The bacterial suspension was decanted into sterile 8 dram vials and stored at 4°C until used in bioassay studies.

The bioassay consisted of a method described by Johnson (63) as the cylinder, cup or plate method. Nutrient broth-sucrose agar medium was poured into petri plates. After solidification of media, three sterilized stainless steel cylinders (8 mm dia) were placed equidistant on the surface of the medium. Into two of these appropriate concentrations of extract from variously treated A. mellea thalli were added, the other cylinder was used for an appropriate control. Treatments consisted of 15 μl, 30 μl, or 45 μl of ethyl acetate extract which had been taken to dryness and redissolved in 95% ethanol. The control consisted of 95% ethanol. Extract was allowed to diffuse into the media. The diffusion time varied from three to 24 hr, depending upon the volume of extract. After the extract had diffused into the media,
the plates were seeded with bacteria which had been resuspended in water agar.
RESULTS

Growth of Armillaria mellea on media containing phenolic extracts of A. mellea thalli.

This study was undertaken to test the hypothesis that the phenols which accumulate in A. mellea thalli incubated on glucose media are responsible for inhibition of growth of the fungus.

Aqueous and ethyl acetate extracts of A. mellea were tested. The aqueous extract is that fraction of homogenized thalli which was partitioned against ethyl acetate. This fraction was taken to dryness and resuspended in double distilled water as described earlier. The ethyl acetate fraction was taken to dryness and redissolved in an equal volume of double distilled water. The results of these experiments are in Table 3.

Colony growth and number of rhizomorph initials of A. mellea were significantly inhibited on PDA supplemented with phenols from the ethyl acetate fraction of thalli incubated on G but not G + E media. This occurred even though the measurable phenol concentration of the two extracts were comparable. Neither colony growth nor the number of rhizomorph initials of A. mellea was affected by aqueous extracts of thalli incubated on either medium.

These data indicate that unique phenols or other unidentified inhibitory compounds accumulate in thalli of A. mellea incubated on G which inhibit rhizomorph initiation and growth, while phenols from
thalli grown on G + E had little effect on rhizomorph development and growth.

Growth of A. mellea on an unidentified phenol separated from phenolic extracts of A. mellea thalli:

It was observed previously that an unidentified phenol could be separated from phenolic extracts of thalli incubated on G which was not detected in extracts of thalli incubated on G + E. This observation and the above report, that comparable amounts of phenol in extracts of thalli incubated on G were more inhibitory than those on G + E media, prompted a study of the antimicrobial activity of the unidentified phenol mentioned. This phenol had an Rf 0.40 on cellulose TLC plates with N-butyl ether-acetic acid (90-9) and 0.17 on polyamide TLC plates developed with benzene-methanol-acetic acid (90-10-8). The UV spectrum revealed absorption maxima at 232 and 272 nm. Samples of the compound were eluted from cellulose thin layer chromatograms by the methods described previously (see p. 25 and Fig. 4). Rhizomorph initiation and growth of A. mellea on PDA supplemented with two concentrations of the unidentified phenol were compared with that on non-supplemented PDA.

Rhizomorph growth and the number of rhizomorph initials produced appeared to be significantly inhibited after eight and 12 days of incubation on PDA supplemented with the higher concentration of the unidentified phenol (7.2 ± 2.5 μg phenol/ml PDA) (Table 4). The amount of phenol and the extent of inhibition was comparable to that of 0.25 ml of ethyl acetate extract from thalli incubated on G (Table 3).

These data suggest that the antimicrobial activity of ethyl acetate extracts from A. mellea grown on G may be due to the presence of
Table 3. Colony growth and rhizomorph production by *Armillaria mellea* after 8 and 12 days incubation on PDA supplemented with various fractions of *A. mellea* thalli incubated for 48 hr on a glucose medium or one supplemented with 500 ppm ethanol.

<table>
<thead>
<tr>
<th>ml Extract</th>
<th>µg Phenol/ml PDAa</th>
<th>Colony dia., mmb,c after:</th>
<th>Number of Rhizomorph Initialsb after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 days 12 days</td>
<td>8 days 12 days</td>
</tr>
<tr>
<td>Glucose Ethyl Acetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>10.5 ± 0.5</td>
<td>25.0 ± 3.0</td>
</tr>
<tr>
<td>0.25</td>
<td>5.7 ± 1.0</td>
<td>6.5 ± 1.0d</td>
<td>14.2 ± 2.5</td>
</tr>
<tr>
<td>0.50</td>
<td>10.2 ± 2.1</td>
<td>0.0 ± 0.0</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>Glucose aqueous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>10.5 ± 1.5</td>
<td>22.3 ± 2.2</td>
</tr>
<tr>
<td>0.25</td>
<td>1.0 ± 0.0</td>
<td>9.5 ± 1.0</td>
<td>23.0 ± 4.0</td>
</tr>
<tr>
<td>0.50</td>
<td>2.0 ± 1.0</td>
<td>11.1 ± 2.1</td>
<td>22.5 ± 1.5</td>
</tr>
<tr>
<td>Glucose + Ethanol, 500 ppm, Ethyl Acetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>9.6 ± 2.4</td>
<td>24.5 ± 5.5</td>
</tr>
<tr>
<td>0.25</td>
<td>5.0 ± 1.5</td>
<td>9.0 ± 3.0</td>
<td>25.5 ± 5.0</td>
</tr>
<tr>
<td>0.50</td>
<td>9.5 ± 0.5</td>
<td>10.1 ± 3.7</td>
<td>25.5 ± 4.0</td>
</tr>
<tr>
<td>Glucose + Ethanol, 500 ppm, Aqueous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>10.5 ± 2.2</td>
<td>23.5 ± 3.0</td>
</tr>
<tr>
<td>0.25</td>
<td>1.75 ± 0.9</td>
<td>10.0 ± 0.5</td>
<td>23.0 ± 0.7</td>
</tr>
<tr>
<td>0.50</td>
<td>3.50 ± 1.0</td>
<td>12.0 ± 0.9</td>
<td>22.1 ± 2.8</td>
</tr>
</tbody>
</table>

a Average of three experiments, 4 replicates/experiment. Mean deviation is indicated.
b Average of three experiments, 6-8 replicates/experiment. Mean deviation is indicated.
c Colony diameter includes mycelial plus rhizomorph growth unless otherwise indicated.
d Mycelial growth only.
the unidentified phenol. Figures 18 and 19 represent graphically the data in Table 4. These data indicate that colony diameter and rhizomorph initials are reduced by at least one-half after 12 days of incubation on PDA supplemented with the unidentified phenol at concentrations of 8-11 \( \mu \)g phenol/ml PDA. A photograph (Fig. 20) comparing the growth of \textit{A. mellea} with that grown on non-supplemented PDA clearly shows the inhibition.

Growth of Armillaria mellea on various concentrations of authentic aromatic compounds:

This study was undertaken to investigate the inhibitory effect of various concentrations of authentic compounds on \textit{A. mellea} rhizomorph initiation and growth. Standard aqueous solutions of the aromatic compounds were sterilized using millipore filtration, then added to melted PDA (40°C). The test compounds used and the concentration added to PDA are indicated in Table 5. Plugs cut from water agar cultures of \textit{A. mellea} with a No. 2 cork borer (6 mm dia) were seeded to PDA in petri plates (5 cm dia) with or without the concentration of test compounds indicated. The results were recorded as colony diameter (linear growth of either mycelia or rhizomorphs) and the number of rhizomorph initials after eight and 12 days of incubation.

The colony diameter decreased with increased concentrations of 3,5-dimethoxycinnamic, 3,5-dimethoxybenzoic acid, and 4-hydroxy-3-methoxycinnamic acid after either eight or 12 days of incubation (Table 5). For either incubation time, growth inhibition on media with 300 ppm 3,5-dimethoxycinnamic acid was significantly greater than on media containing the same concentration of either 3,5-dimethoxybenzoic
Table 4. Colony growth and rhizomorph production by *Armillaria mellea* after 8 and 12 days of incubation on PDA supplemented with an unidentified phenol separated from ethyl acetate extract of *A. mellea* thalli incubated for 48 hr on a glucose medium.

<table>
<thead>
<tr>
<th>ml Extract</th>
<th>μg Phenol/ml PDA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Colony dia., mm&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt; after:</th>
<th>Number of Rhizomorph Initials&lt;sup&gt;b&lt;/sup&gt; after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 days</td>
<td>12 days</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>11.1 ± 1.3</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>Unknown</td>
<td>2.3 ± 0.6</td>
<td>11.1 ± 2.1</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>0.50</td>
<td>7.2 ± 2.5</td>
<td>8.1 ± 1.1</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>1.00</td>
<td></td>
<td></td>
<td>7.1 ± 1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of two experiments, 4 replicates/experiment. Mean deviation is indicated.

<sup>b</sup> Average of two experiments, 6-8 replicates/experiment. Mean deviation is indicated.

<sup>c</sup> Colony diameter includes mycelial plus rhizomorph growth unless otherwise indicated.
Figure 18. Number of rhizomorph initials of Armillaria mellea after 8 and 12 days incubation on PDA with (———) and without (-----) a supplement of unidentified phenol (7.2 ± 2.5 μg/ml) separated from Armillaria mellea thalli. Data derived from Table 4. Vertical bars indicate mean deviation.
Figure 19. Colony diameter of Armillaria mellea after 8 and 12 days incubation on PDA with (-----) and without (------) a supplement of unidentified phenol (7.2 ± 2.5 μg/mL) separated from Armillaria mellea thalli. Data derived from Table 4. Vertical bars indicate mean deviation.
Figure 20. Comparison of growth inhibition of Armillaria mellea on PDA supplemented with 8-11 μg unidentified phenol/ml PDA as compared to growth on non-supplemented PDA. Photograph taken after 12 days.
or 4-hydroxy-3-methoxycinnamic acid. 3,4-Dihydroxycinnamic and 1,3,4,5-tetrahydroxybenzoic acid were not inhibitory at any of the concentrations used.

Rhizomorph initials did not develop after eight days of incubation on media containing 250 or 300 ppm of 3,5-dimethoxycinnamic acid, 3,5-dimethoxybenzoic acid, 3,4-dihydroxycinnamic acid, or 4-hydroxy-3-methoxybenzoic acid. After 12 days of incubation the number of rhizomorph initials decreased with increasing concentrations of 3,5-dimethoxybenzoic acid, 3,5-dimethoxycinnamic acid, and 4-hydroxy-3-methoxycinnamic acid. At this incubation time 250-300 ppm 3,5-dimethoxycinnamic acid and 300 ppm 4-hydroxy-3-methoxycinnamic acid completely inhibited rhizomorph initial formation. The inhibition of initials observed at eight days with 3,4-dihydroxycinnamic acid was completely overcome at 12 days. It would appear that 3,4-dihydroxycinnamic acid causes a delay in the development of rhizomorphs but does not inhibit their growth once developed. The compound 1,3,4,5-tetrahydroxybenzoic acid was ineffective after either incubation time.

The influence of various concentrations of aromatics on growth and number of rhizomorph initials after 12 days is presented graphically in Figures 18 and 19, respectively. A comparison of the inhibitory effect of known aromatics is presented in Figure 20.

These data indicate that 3,5-dimethoxycinnamic acid appears to be significantly more inhibitory to rhizomorph initiation and growth than either 3,5-dimethoxybenzoic or 4-hydroxy-3-methoxycinnamic acid. 3,4-Dihydroxycinnamic acid, on the other hand, causes a delay in the development of rhizomorph initials but once they develop, growth is
Table 5. Colony growth and rhizomorph production by Armillaria mellea after 8 and 12 days of incubation on PDA with or without a supplement of various concentrations of authentic compounds.

<table>
<thead>
<tr>
<th>Compound and conc. (µg/ml PDA)</th>
<th>Colony dia., mm&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt; after:</th>
<th>Number of Rhizomorph Initials&lt;sup&gt;a&lt;/sup&gt; after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 days</td>
<td>12 days</td>
</tr>
<tr>
<td>3,5-Dimethoxybenzoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.5 ± 0.5</td>
<td>22.5 ± 3.0</td>
</tr>
<tr>
<td>150</td>
<td>9.7 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.5 ± 1.5</td>
</tr>
<tr>
<td>250</td>
<td>11.5 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.5 ± 1.2</td>
</tr>
<tr>
<td>300</td>
<td>8.9 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.1 ± 0.9</td>
</tr>
<tr>
<td>3,5-Dimethoxycinnamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.7 ± 0.8</td>
<td>22.0 ± 2.1</td>
</tr>
<tr>
<td>150</td>
<td>9.6 ± 0.7</td>
<td>12.6 ± 1.6</td>
</tr>
<tr>
<td>250</td>
<td>5.0 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.2 ± 0.75&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>300</td>
<td>5.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.5 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,4-Dihydroxycinamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.8 ± 0.8</td>
<td>21.1 ± 1.9</td>
</tr>
<tr>
<td>150</td>
<td>11.2 ± 0.61&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.7 ± 1.6</td>
</tr>
<tr>
<td>250</td>
<td>12.0 ± 0.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.2 ± 1.3</td>
</tr>
<tr>
<td>300</td>
<td>12.5 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.3 ± 2.7</td>
</tr>
<tr>
<td>1,3,4,5-Tetrahydroxybenzoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.2 ± 4.1</td>
<td>25.7 ± 0.7</td>
</tr>
<tr>
<td>150</td>
<td>13.5 ± 1.3</td>
<td>24.6 ± 3.7</td>
</tr>
<tr>
<td>250</td>
<td>13.7 ± 0.3</td>
<td>25.0 ± 2.0</td>
</tr>
<tr>
<td>300</td>
<td>13.9 ± 0.5</td>
<td>25.6 ± 1.4</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxycinamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13.6 ± 3.1</td>
<td>24.2 ± 2.2</td>
</tr>
<tr>
<td>150</td>
<td>12.0 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.7 ± 3.1</td>
</tr>
<tr>
<td>250</td>
<td>8.3 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.2 ± 2.4</td>
</tr>
<tr>
<td>300</td>
<td>6.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.2 ± 0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of four experiments with 6-8 replicates/experiment. Mean deviation is indicated.

<sup>b</sup> Colony diameter includes mycelial plus rhizomorph growth unless otherwise indicated.

<sup>c</sup> Mycelial growth only.
Figure 21. Colony diameter of Armillaria mellea after 12 days incubation on PDA supplemented with 0, 150, 250, and 300 ppm of 3,5-dimethoxybenzoic acid (--), 3,5-dimethoxycinnamic acid (- - - -), 3,4-dihydroxycinnamic acid (---), 1,2,4,5-tetrahydroxybenzoic acid (-----), 4-hydroxy-3-methoxycinnamic acid (-- -- ). Data was derived from Table 5.
Figure 22. Number of rhizomorph initials of Armillaria mellea after 12 days incubation on PDA supplemented with 0, 150, 250, and 300 ppm of 3,5-dimethoxybenzoic acid (— — — —), 3,5-dimethoxycinnamic acid (-- --), 3,4-dihydroxycinnamic acid (— — — —), 1,2,4,5-tetrahydroxybenzoic acid (——— —), 4-hydroxy-3-methoxycinnamic acid (-----).
Figure 23. Comparison of growth inhibition of *Armillaria mellea* on PDA supplemented with varying concentrations of authentic aromatic compound A- 150 ppm, B- 200 ppm 3,5-dimethoxycinnamic acid; C- 150 ppm, D- 200 ppm 3,5-dimethoxybenzoic acid; E- 150 ppm, F- 200 ppm 2,4-dihydroxycinnamic acid. Photograph taken after 12 days incubation.
not inhibited. 1,3,4,5-Tetrahydroxybenzoic acid was ineffective in inhibiting rhizomorph development and growth. There is a striking differential response of *A. mellea* rhizomorph initiation and growth to aromatic compounds (3,4-dihydroxycinnamic compared to 4-hydroxy-3-methoxycinnamic acid) which differ by only one substituent on the ring.

**Spore germination of Botrytis cinerea in media containing phenolic extracts of *A. mellea* thalli:**

The results of this study are presented in Table 6. The percentage of spore germination was inhibited in media containing either ethyl acetate extracted phenols from thalli incubated on G medium for 48 hr or on G + E. The reduction in germination due to extracts of thalli grown on G was significantly greater than the reduction due to extracts from thalli grown on G + E. This was observed even though there was no significant difference in the phenol content (expressed as chlorogenic acid equivalents) of the extracts from either source.

In contrast, the aqueous fraction did produce some inhibition of germination. The inhibition observed, however, was much less than that observed with ethyl acetate extracts. These data indicate that the ethyl acetate extractable phenols of *A. mellea* were considerably more inhibitory than the aqueous extractable phenols. Apparently, the ethyl acetate extract from thalli incubated on G contains unique phenols which are considerably more inhibitory to *B. cinerea* spore germination than those present in ethyl acetate extracts of thalli incubated on G + E.
Table 6. Spore germination of *Botrytis cinerea* in media containing phenolic extracts of *Armillaria mellea* thalli incubated for 48 hr on a glucose medium or on glucose + ethanol, 500 ppm, media.

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Extract&lt;sup&gt;a&lt;/sup&gt; Fraction</th>
<th>Total Phenols&lt;sup&gt;b&lt;/sup&gt; (µg) in Fraction</th>
<th>% Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Ethyl Acetate</td>
<td>16.66 ± 1.6</td>
<td>18.0 ± 2.0</td>
</tr>
<tr>
<td>Glucose + Ethanol</td>
<td>Ethyl Acetate</td>
<td>14.0 ± 1.1</td>
<td>43.0 ± 4.0</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>0</td>
<td>65.0 ± 5.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>Aqueous</td>
<td>4.5 ± 0.7</td>
<td>57.0 ± 3.0</td>
</tr>
<tr>
<td>Glucose + Ethanol</td>
<td>Aqueous</td>
<td>6.2 ± 2.1</td>
<td>54.0 ± 4.0</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>0</td>
<td>69.0 ± 6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> 50 µl of each extract was added and phenols were determined with Folin-Denis and are expressed as chlorogenic acid equivalents.

<sup>b</sup> Mean of four experiments, 3 replicates/experiment. Mean deviation is indicated for each value.
Growth of bacteria on media containing phenolic extracts of A. mellea thalli:

The bacteria used in this study were Xanthomonas pruni, Escherichia coli, Bacillus subtilis, and Corynebacterium michiganense. Preliminary tests revealed (Table 7) that the ethyl acetate extracts of A. mellea thalli inhibited X. pruni and B. subtilis but not E. coli and C. michiganense. Aqueous fractions were ineffective against all bacteria tested. These data indicate that different bacteria respond differentially to inhibitory phenols from A. mellea thalli.

The result of more detailed studies with X. pruni are presented in Table 8, Fig. 24 and 25. Ethyl acetate extracts from thalli cultured on G and on G + E inhibited the bacteria. The extracts from thalli cultured on G alone show, at each volume of extract tested, the capacity to produce twice as much inhibition as the extracts from thalli cultured on G + E. G extracts contained approximately 10% more phenol than G + E extracts. The G extracts, however, produced 80-100% more inhibition than the G + E extracts. This corresponds closely to an observation made earlier with B. cinerea spore germination. The difference in amount of total phenols is not enough to explain the dramatic increase in inhibition G extracts produce as compared to G + E extracts.

A plate depicting the bioassay of X. pruni is shown in Fig. 25. In this plate clear areas occur on the surface of the agar. These are the areas in which inhibition of bacterial growth occurred. In preparing the bioassay, three metal cylinders were placed equidistant on the surface of the agar. Two of the cylinders contained extract of A.
The other cylinder contained the appropriate control. The two areas of growth inhibition are where *A. mellea* extract was placed. The area where the control was located was covered by bacterial growth.

More detailed studies were also done with *B. subtilis*. Results of these studies are shown in Table 9, Fig. 26 and 27. The ethyl acetate extract of thalli of *A. mellea* grown on G had more antibiotic activity than extract of those thalli grown on G + E (Table 9). Extracts from thalli grown on G are 1.5-2.0 times more inhibitory than the extracts from thalli incubated on G + E. The plates in Fig. 26 are comparisons of growth of *B. subtilis* on nutrient broth-sucrose agar to which phenolic extracts of *A. mellea* have been added in different amounts. The inhibition observed is rather linear and the difference between treatments can be easily observed.

The above studies with *X. pruni* and *B. subtilis* revealed that although phenol concentration in thalli extracts incubated on G was about 15-25% greater than that of thalli incubated on G + E, growth inhibition on the former was 50% greater than on the latter. This provides additional support for the conclusions that phenols from extracts of thalli incubated on G were more inhibitory than those from extracts of thalli incubated on G + E.
Table 7. Growth of various bacteria on nutrient broth-sucrose agar media supplemented with phenols from aqueous or ethyl acetate extracts or fraction of *Armillaria mellea* thalli.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µl of Extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Aqu.</td>
<td>E.A.</td>
<td>Aqu. E.A.</td>
</tr>
<tr>
<td><strong>Xanthomonas pruni</strong></td>
<td>+&lt;sub&gt;e&lt;/sub&gt;</td>
<td>-&lt;sub&gt;d&lt;/sub&gt;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Corynebacterium</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>michiganense</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Extracts were obtained from *A. mellea* thalli incubated on glucose medium for 48 hr.

<sup>b</sup> Aqu. = Aqueous extract.

<sup>c</sup> E.A. = Ethyl acetate extract.

<sup>d</sup> + = growth not inhibited; based on absence of an inhibition zone.

<sup>e</sup> + = growth not inhibited; based on absence of an inhibition zone.
Table 8. Growth inhibition of *Xanthomonas pruni* on nutrient broth sucrose agar media supplemented with various amounts of phenol from ethyl acetate fractions or extracts of *Armillaria mellea* thalli.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume of Extract</th>
<th>µg Phenol</th>
<th>Inhibition Zone Dia., mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>15 µl</td>
<td>5.8 ± 0.6</td>
<td>15.75 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>30 µl</td>
<td>10.66 ± 0.3</td>
<td>24.90 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>45 µl</td>
<td>15.20 ± 1.1</td>
<td>34.88 ± 2.0</td>
</tr>
<tr>
<td>Glucose + Ethanol</td>
<td>15 µl</td>
<td>4.33 ± 0.5</td>
<td>7.85 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>30 µl</td>
<td>8.25 ± 1.0</td>
<td>15.14 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>45 µl</td>
<td>12.33 ± 0.7</td>
<td>18.42 ± 1.0</td>
</tr>
</tbody>
</table>

a The medium on which *A. mellea* thalli were incubated for 48 hr prior to extraction.

b Mean of three experiments, 4 replicates/experiment, with mean deviation.

c Mean of three experiments, 9 replicates/experiment, with mean deviation.
Figure 24. Comparison of growth of *Xanthomonas pruni* on media containing various amounts of phenol from *Armillaria mellea* thalli incubated on glucose (-----) or glucose + ethanol (-----) media. Deviations from mean indicated by vertical bars. Data adapted from Table 8.
Figure 25. Comparison of growth inhibition of Xanthomonas pruni on media containing phenolic extracts from glucose treated thalli with those from glucose + ethanol treated thalli. Size of halo indicated extent of inhibition. Note inhibition zones on glucose + ethanol 15 μl is overgrown.
Table 9. Growth inhibition of *Bacillus subtilis* on nutrient broth sucrose agar media supplemented with various amounts of phenol from ethyl acetate extracts of *Armillaria mellea* thalli.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume of Extract</th>
<th>μg Phenol</th>
<th>Inhibition Zone Dia., mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>15 μl</td>
<td>5.80 ± 0.6</td>
<td>12.63 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>30 μl</td>
<td>10.66 ± 0.3</td>
<td>20.40 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>45 μl</td>
<td>15.20 ± 1.1</td>
<td>30.56 ± 2.1</td>
</tr>
<tr>
<td>Glucose + Ethanol, 500 ppm</td>
<td>15 μl</td>
<td>4.33 ± 0.5</td>
<td>5.33 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>30 μl</td>
<td>8.25 ± 1.0</td>
<td>11.36 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>45 μl</td>
<td>12.33 ± 0.7</td>
<td>19.90 ± 1.1</td>
</tr>
</tbody>
</table>

a Medium on which *A. mellea* thalli were incubated for 48 hr prior to extraction.

b Mean of three experiments, 4 replicates/experiment. Mean deviation is indicated.

c Mean of three experiments, 9 replicates/experiment. Mean deviation is indicated.
Figure 26. Comparison of growth of Bacillus subtilis on media containing various amounts of phenol from Armillaria mellea thalli incubated on glucose (-----) or glucose + ethanol (-----) media. Deviation from mean indicated by vertical bars. Data adapted from Table 9.
Figure 27. Comparison of growth inhibition of *Bacillus subtilis* on media containing 15 µl of phenolic extract from glucose treated thalli with those from glucose + ethanol treated thalli. Size of halo indicates extent of inhibition.
CONCLUSIONS

Evidence is presented which shows that phenolic inhibitors accumulate in *A. mellea* thalli incubated on G. Ethyl acetate extracts containing these phenols inhibited rhizomorph initiation and growth of *A. mellea*. These extracts also caused striking inhibition of *B. cinerea* spore germination and growth of *B. subtilis* and *X. pruni*. An unidentified phenol was separated by TLC from ethyl acetate extracts of thalli incubated on G. The unidentified phenol inhibited rhizomorph initiation and growth of *A. mellea*. The inhibitory potency of this phenol to *A. mellea* appeared to be comparable to that of the phenols in the ethyl acetate fraction from which it was obtained.

Although phenols were detected in ethyl acetate extracts of thalli incubated on G + E, these phenols appeared not to be inhibitory to *A. mellea* rhizomorph initiation and growth at the concentration tested. Such extracts contained no detectable unidentified phenol comparable to that detected in extracts of thalli incubated on G. Also, the extracts from thalli grown on G + E were less inhibitory to *B. cinerea* spore germination and growth of *X. pruni* and *B. subtilis* than extracts from thalli grown on G.

The inhibitory potency of the unidentified phenol to *A. mellea* appeared to be comparable to the inhibitory potency of cinnamic acid derivatives to rust uredospores reported by Macko (75, 76). In view of this and in view of the observation that authentic cinnamic acid
derivatives inhibit rhizomorph initiation and growth, the possibility
that the unidentified phenol from A. mellea thalli incubated on G is a
cinnamic acid derivative should be investigated.

In studies with authentic aromatic compounds, it was found that
3,5-dimethoxybenzoic acid, 3,5-dimethoxycinnamic acid, and 3-methoxy-
4-hydroxycinnamic acid were inhibitory to rhizomorph development and
growth, while comparable concentrations of 3,4-dihydroxycinnamic acid
and 1,3,4,5-tetrahydroxybenzoic acid were not inhibitory to A. mellea
growth and rhizomorph development. This could be the result of the
action of oxygenases in fungal thalli. Oxygenases are enzymes, some of
which catalyze cleavage of aromatic rings (78, 128). The cleavage
usually occurs between two adjacent phenolic groups such as in 3,4-
dihydroxycinnamic acid and 1,3,4,5-tetrahydroxybenzoic acid. Cleavage
of the aromatic ring could occur in these two compounds by oxygenase
action, while cleavage would not occur in the three other aromatics
tested. This cleavage might destroy the inhibitory capacity of 3,4-
dihydroxycinnamic acid and 1,3,4,5-tetrahydroxybenzoic acid. In the
other compounds the aromatic ring is presumably not cleaved and
inhibition occurs.

It was observed in bioassays of B. cinerea, X. pruni, and E. coli
that inhibition was approximately twice as great with phenolic ethyl
acetate extracts of thalli incubated on G as compared to extracts of
thalli incubated on G + E. The concentration of phenols in ethyl
acetate extracts of thalli grown on G was, however, not twice as great
as the phenols in extracts of thalli grown on G + E. This would sug-
gest that total phenol accumulation may not be the only factor involved
In inhibition of growth. An unidentified phenol was found in G extracts which was inhibitory to rhizomorph initiation and growth of A. mellea. This compound was not present in extracts of thalli grown on G + E. These observations suggest the possibility of two mechanisms or a combination of two may be involved in inhibition of growth of thalli in G. The mechanism could be 1) the accumulation of total phenols to a toxic level, and/or 2) the differential synthesis of a single compound on G to toxic levels.

The stimulation of rhizomorphs by ethanol may be explained in one of two fashions. Ethanol may promote synthesis of some growth stimulating compound. One compound was found in thalli grown on ethanol which did not appear in thalli grown on glucose. This compound has not been studied for biological activity. It could, however, be a growth stimulator. Garraway (40) has shown growth promoters such as indole-acetic acid (IAA), p-aminobenzoic acid (PABA), and o-aminobenzoic acid (AA) stimulate growth effective at 10 ppm, while optimum ethanol concentration is 500 ppm. Even though ethanol reduces glucose metabolism, as mentioned earlier (Chapter 1), the reduction in metabolism could occur while growth stimulators are concomitantly being synthesized in minute amounts needed for growth. Another explanation, and one better substantiated in view of this author's findings, is ethanol suppresses the accumulation of endogenous self-inhibitors which are phenolic in nature by mechanisms previously discussed in Chapter 3 (p. 52-54).
V. INFLUENCE OF ETHANOL AND OTHER LOW MOLECULAR WEIGHT ALCOHOLS ON THE ACTIVITY OF PEROXIDASE AND POLYPHENOL OXIDASE FROM ARMILLARIA MELLEA THALLI.
INTRODUCTION

The previous studies showed that phenols which inhibit growth and rhizomorph production of *A. mellea* accumulate in thalli incubated on G but not in thalli incubated on G + E. This finding provided experimental support for the hypothesis that ethanol promotes mycelial growth and rhizomorph production by suppression of accumulation of endogenous inhibitors (44). In view of the many reports in the literature concerning the interrelationship between phenols, phenol oxidizing enzymes, and growth (17, 29, 60, 104), this discovery also provided a clue concerning the possible mode of action of endogenous inhibitors of *A. mellea* growth and rhizomorph production. Thus, the oxidation of phenols by peroxidase and/or polyphenol oxidase and their subsequent condensation to polymers which inhibit *A. mellea* growth seemed feasible. Inhibition of fungal growth by phenols and their condensation products is well known in connection with studies concerning their role in plant disease resistance (24, 64, 66). Studies with higher plants have revealed an inverse relationship between peroxidase activity and growth (38, 50, 83), reduced growth being accompanied by increases in peroxidase activity. Possible mechanisms involved include (i) destruction of IAA, which is needed for normal growth, by peroxidase (50, 65, 83), and (ii) involvement of peroxidase with lignin formation and deposition which impedes growth (95). Both mechanisms may be interrelated (36, 37, 38, 117).
In view of the foregoing possibility, that an increase in the concentration of *A. mellea* phenols observed previously might be accompanied by increases in the activity of peroxidase and polyphenol oxidase, the activity of these two enzymes was investigated. Peroxidase and polyphenol oxidase activities in *A. mellea* thalli incubated on G were compared with those incubated on G supplemented with ethanol and other low molecular weight alcohols.
MATERIALS AND METHODS

Organism used and culture techniques:

These materials and methods have been described in a previous chapter.

Fungal enzyme preparation:

Fresh or frozen thalli were homogenized in a Sorvall Omnimix Homogenizer set at high speed at 0°C for three minutes in 0.05 M potassium phosphate buffer, pH 6.0 (2 ml/5 thalli). The homogenate was clarified by centrifugation at 30,000 g for 30 minutes in a Sorvall RC2-B Refrigerated Centrifuge at a temperature of 0-2°C. These clarified extracts are termed the crude extract. They were stored at 0-2°C until enzyme measurements were made.

Acetone precipitated enzyme was prepared by the following method: One volume of crude extract was mixed with two volumes of acetone. The mixture was allowed to stand for 30 min at 4°C and then was centrifuged at 30,000 g for 25 min. The supernatant was discarded and the protein pellet was retrieved. The pellet was dried of acetone with air and resuspended after grinding in a Ten-Broek Homogenizer in 0.1 M potassium phosphate buffer, pH 6.0 (2.0 ml/5 thalli). This solution was called the acetone precipitate.
**Peroxidase assay:**

The crude extract was diluted 1:50 with double distilled water and 0.2 ml were used to determine peroxidase activity. The reaction mixture consisted of 1.85 ml of potassium phosphate buffer, pH 7.0, 0.30 ml of 0.01 M pyrogallol, 0.20 ml crude extract, and 0.05 ml of 0.2 M H$_2$O$_2$. The reaction was initiated by addition of H$_2$O$_2$. The blank consisted of the same reagents with boiled enzyme (15 min). The activity was recorded as change in optical density per minute per mg protein (ΔOD/min/mg prot) at 430 nm. Measurement of the acetone precipitated enzyme activity was carried out in the same manner.

**Polyphenol oxidase assay:**

The activity was determined by using 0.5 ml of crude extract or 0.5 ml acetone precipitate. The reaction mixture contained 1.5 ml of 0.05 M potassium phosphate buffer, pH 6.0, 0.5 ml catechol, 0.02 M enzyme boiled or unboiled. The activity was recorded as ΔOD/min/mg protein by following OD change at 470 nm.

**Protein determination:**

Protein was determined according to the method of Lowry (73).

**Studies of pH optimum for peroxidase and polyphenol oxidase:**

The enzyme assays were carried out as described earlier. However, in these studies the pH of the potassium phosphate buffer varied from 2.0 to 9.0. The enzymes were allowed to equilibrate in the buffer system for 10 min. After this equilibration time, the substrates were added. The peroxidase assay was read at the end of three minutes and
data was plotted as ΔOD/min vs pH. Acetone precipitated enzyme was used for all studies.

**Studies of temperature optimum for peroxidase and polyphenol oxidase activities:**

Enzyme assays were carried out as described earlier. In these studies the enzyme and buffer were exposed to temperatures from 5°C to 65°C. The enzyme buffer system was allowed to equilibrate in a circulating water bath at the desired temperature for seven minutes. After equilibration, catechol or H₂O₂ was added to the system to start the reactions. The peroxidase assay was read after 3 min and the polyphenol oxidase assay after 5 min.

**Column Chromatography:**

Column chromatography of acetone precipitated material was attempted with Sephadex G-75, G-100, and G-200. The column specifications were 15 mm x 400 mm, the bed depth of gel was 300 mm. The solvents used were potassium phosphate buffer, pH 6.0, 0.1 M and 0.05 M; pH 7.0, 0.1 M and 0.05 M. Flow rate of the buffer was 20 drops per minute (1 ml/min).
RESULTS

The effect of ethanol on peroxidase activity in A. mellea:

The peroxidase activity in A. mellea after various incubation times was inversely related to the concentration of ethanol supplemented to the incubation medium (Table 10 and Fig. 28). After various incubation times on G, the peroxidase activity was higher than at zero time. In contrast, peroxidase activity was less than at zero time following various incubation times on glucose media supplemented with various ethanol concentrations. The data indicate that the peroxidase activity of thalli after various incubation times on a glucose medium was 3-5 times higher than that of thalli incubated on a glucose plus ethanol medium. Therefore, it appears that ethanol concentrations which promote growth of A. mellea suppress peroxidase activity in addition to suppressing the concentration of inhibitory phenols.

Tracings from a recording spectrophotometer which show the activity of peroxidase (crude extract and acetone precipitated) from thalli incubated for 24, 48, 72 hr on G or G + E are presented in Fig. 29, 30, and 31. The peroxidase activity of thalli is high at all incubation times on G (Table 10). After 24 and 48 hr on G + E peroxidase activity is very low and increases significantly after 72 hr incubation.

These data support the idea that the inhibition of growth on G may be the result of high peroxidase activity in association with the phenol accumulation which occurs during this period.
Table 10. Peroxidase activity of *Armillaria mellea* after various incubation times on glucose media or on glucose media supplemented with various concentrations of ethanol.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Specific Activity AOD/min/mg proteinb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>1) Glucose</td>
<td></td>
</tr>
<tr>
<td>Crude extractc</td>
<td>14.81 ± 1.80</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>24.73 ± 1.87</td>
</tr>
<tr>
<td>2) Glucose + Ethanol, 100 ppm</td>
<td></td>
</tr>
<tr>
<td>Crude extractc</td>
<td>4.18 ± 0.82</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>6.75 ± 0.10</td>
</tr>
<tr>
<td>3) Glucose + Ethanol, 250 ppm</td>
<td></td>
</tr>
<tr>
<td>Crude extractc</td>
<td>1.76 ± 0.33</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>2.13 ± 0.13</td>
</tr>
<tr>
<td>4) Glucose + Ethanol, 500 ppm</td>
<td></td>
</tr>
<tr>
<td>Crude extractc</td>
<td>0.40 ± 0.40</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>0.60 ± 0.00</td>
</tr>
</tbody>
</table>

a Treatment which thalli were incubated on before peroxidase was assayed.

b Average of three experiments, 6 replicates/experiment, and mean deviation. Specific activity at time 0 is 8.17 ± 0.47 crude extract, and 14.05 ± 0.95 acetone precipitate.

c Peroxidase assay made with crude extract protein.

d Peroxidase assay made with acetone precipitated protein.
Figure 28. Peroxidase activity of *Armillaria mellea* after various incubation times on glucose media (— — —) or glucose media supplemented with 100 ppm ethanol (— — —), 250 ppm ethanol (— — —), or 500 ppm ethanol (— — —). Data taken from acetone precipitate Table 10. Vertical bars indicate deviation from the mean.
Figure 29. Comparison of peroxidase activity of *Armillaria mellea* incubated on glucose media or glucose + ethanol (500 ppm) media for 24 hr. Crude extract protein assay is shown on right, acetone precipitated protein is shown on left.
Figure 30. Comparison of peroxidase activity of *Armillaria mellea* incubated on glucose media or glucose + ethanol (500 ppm) media for 48 hr. Crude extract protein assay is shown on right, acetone precipitated protein is shown on the left.
Figure 31. Comparison of peroxidase activity of Armillaria mellea incubated on glucose media or glucose + ethanol (500 ppm) media for 72 hr. Crude extract protein assay is shown on left, acetone precipitated protein is shown on right.
Peroxidase activity in *A. mellea* after various incubation times on a glucose medium compared to that on a similar medium supplemented with different low molecular weight alcohols:

Weinhold (109) noted that in addition to ethanol, other low molecular weight alcohols promote growth and rhizomorph production in *A. mellea*. Therefore, some of these alcohols were used to determine whether they produced an inverse relationship between growth and peroxidase activity when added as a supplement to a glucose medium. Glucose medium was supplemented with 500 ppm of either ethanol, propanol, butanol, or iso-butanol. Peroxidase activity was compared with that on a non-supplemented glucose medium after various incubation times (Table 11 and Fig. 32).

Peroxidase activity was suppressed in all thalli which were supplemented with alcohol as compared to peroxidase activity in thalli grown on a glucose medium. The peroxidase activity of thalli incubated on G was 3-6 times higher than the peroxidase of thalli incubated on alcohol after 72 hr. After 24 hr, activity could only be detected in thalli incubated on glucose medium. After 72 hr peroxidase was apparent in all treatments. The difference in activity among thalli incubated on different alcohols after 72 hr was not appreciably different except in thalli incubated on iso-butanol.

These data indicate that other low molecular weight alcohols which stimulate growth and rhizomorph development suppress peroxidase activity and, as found earlier, suppress phenol accumulation. This gives support to the idea that growth inhibition may be the result of
Table 11. Peroxidase activity of *Armillaria mellea* incubated on different low molecular weight alcohols as compared to peroxidase activity of thalli incubated on glucose alone.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extractc</td>
<td>9.80 ± 1.00</td>
<td>8.88 ± 0.40</td>
<td>8.31 ± 0.23</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>17.65 ± 2.40</td>
<td>16.38 ± 1.70</td>
<td>15.44 ± 0.73</td>
</tr>
<tr>
<td>2) Glucose + Ethanol, 500 ppm</td>
<td>0</td>
<td>1.45 ± 0.81</td>
<td>1.93 ± 0.12</td>
</tr>
<tr>
<td>Crude Extractc</td>
<td>0</td>
<td>2.22 ± 0.55</td>
<td>4.01 ± 0.70</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>0</td>
<td>1.91 ± 0.35</td>
<td>3.47 ± 0.15</td>
</tr>
<tr>
<td>3) Glucose + Propanol, 500 ppm</td>
<td>0</td>
<td>0.97 ± 0.22</td>
<td>1.91 ± 0.13</td>
</tr>
<tr>
<td>Crude Extractc</td>
<td>0</td>
<td>1.91 ± 0.35</td>
<td>3.47 ± 0.15</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>0</td>
<td>1.06 ± 0.42</td>
<td>2.19 ± 0.12</td>
</tr>
<tr>
<td>Acetone Precip.d</td>
<td>0</td>
<td>2.61 ± 0.30</td>
<td>4.67 ± 0.40</td>
</tr>
<tr>
<td>4) Glucose + Butanol, 500 ppm</td>
<td>0</td>
<td>0</td>
<td>1.11 ± 0.17</td>
</tr>
<tr>
<td>Crude Extractc</td>
<td>0</td>
<td>0</td>
<td>2.45 ± 0.28</td>
</tr>
</tbody>
</table>

a Treatment which thalli were incubated on before peroxidase was assayed.

b Average of three experiments, 6 replicates/experiment, and mean deviation. Specific activity at time 0 is 6.15 ± 0.17 for crude extract and 11.87 ± 0.65 for acetone precipitate.

c Peroxidase assay made with crude extract protein.

d Peroxidase assay made with acetone precipitated protein.
Figure 32. Peroxidase activity of Armillaria mellea thalli incubated on 500 ppm glucose (—), glucose + butanol (-----), glucose + ethanol (-----), glucose + iso-butanol (-----), glucose + propanol (-----). Data derived from acetone precipitate of Table 11. Vertical bars indicate deviation from the mean.
Increased phenol accumulation in conjunction with increased peroxidase activity.

Peroxidase substrate specificity of A. mellea thalli incubated on glucose media as compared to thalli incubated on glucose + ethanol, 500 ppm, media for 48 hr and commercial peroxidase:

The substrate specificity of peroxidase in A. mellea incubated on G or G + E was compared to commercial horseradish peroxidase obtained from Nutritional Biochemical Corp. (Table 12). Guaiacol, pyrogallol, and gallic acid are substrates for commercial peroxidase. A. mellea thalli incubated on either medium can use pyrogallol or gallic acid as substrates but not guaiacol. These data indicate that A. mellea peroxidase(s) may be substrate specific. These data also indicate that A. mellea peroxidase may be somewhat different from horseradish peroxidase. The differences in substrate specificity may indicate differences in active site affinities of the enzyme.

Polyphenol oxidase activity of A. mellea thalli after various incubation times on glucose media compared to thalli incubated on glucose media supplemented with various concentrations of ethanol:

Polyphenol oxidase activity was higher in thalli incubated on G for 24 hr than in any other treatment (Table 13). As thalli were exposed to various treatments over 72 hr, the activity progressively decreased. After 72 hr the activity among all treatments was approximately equal. The relative insensitivity of polyphenol oxidase to the presence of ethanol suggests that polyphenol oxidase may not be
Table 12. Comparison of substrate specificity of peroxidase in *Armillaria mellea* incubated on glucose or glucose + ethanol (500 ppm) with commercial horseradish peroxidase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Commercial Enzyme Source</th>
<th>Glucose + Ethanol Enzyme Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose + Ethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thalli</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 ppm Thalli</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>+a</td>
<td>-b</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Shikimic Acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinic Acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a  + = Activity
b  - = No activity
Table 13. Polyphenol oxidase activity of *Armillaria mellea* thalli after various incubation times on glucose media or on glucose media supplemented with various concentrations of ethanol.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Activity AOD/min/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>1) Glucose</td>
<td></td>
</tr>
<tr>
<td>Crude Extract</td>
<td>1.13 ± 0.20</td>
</tr>
<tr>
<td>Acetone Precip.</td>
<td>2.04 ± 0.30</td>
</tr>
<tr>
<td>2) Glucose + Ethanol, 100 ppm</td>
<td></td>
</tr>
<tr>
<td>Crude Extract</td>
<td>0.85 ± 0.12</td>
</tr>
<tr>
<td>Acetone Precip.</td>
<td>1.53 ± 0.23</td>
</tr>
<tr>
<td>3) Glucose + Ethanol, 250 ppm</td>
<td></td>
</tr>
<tr>
<td>Crude Extract</td>
<td>0.82 ± 0.14</td>
</tr>
<tr>
<td>Acetone Precip.</td>
<td>1.41 ± 0.12</td>
</tr>
<tr>
<td>4) Glucose + Ethanol, 500 ppm</td>
<td></td>
</tr>
<tr>
<td>Crude Extract</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>Acetone Precip.</td>
<td>1.23 ± 0.06</td>
</tr>
</tbody>
</table>

a) Treatment which thalli were incubated on prior to polyphenol oxidase assay.

b) Average of three experiments, 6 replicates/experiment, and mean deviation. The specific activity at time 0 is 0.91 ± 0.30 crude extract and 2.02 ± 0.08 acetone precipitate.

c) Polyphenol oxidase assay made with crude extract.

d) Polyphenol oxidase assay made with acetone precipitated protein.
specifically involved in ethanol stimulation of rhizomorph development and growth.

**Comparison of substrate specificity of polyphenol oxidase in A. mellea incubated on glucose or glucose + ethanol (500 ppm) media with commercial mushroom tyrosinase:**

The polyphenol oxidase activity of *A. mellea* was compared to commercial mushroom tyrosinase obtained from Nutritional Biochemical Corp. (Table 14). It can be seen that that *A. mellea* polyphenol oxidase activity from thalli incubated on G media or on G + E media is not different in substrate specificity. The polyphenol oxidase from *A. mellea* reacts with catechol, hydroquinone, L-DOPA, and p-phenylene-diamine. Commercial tyrosinase (polyphenol oxidase) reacts with all substrates tested except hydroquinone. These data indicate that *A. mellea* is somewhat different in substrate specificity than commercial mushroom tyrosinase. *A. mellea* polyphenol oxidase causes oxidation of o-dihydroxyphenols. These results indicate that the polyphenol oxidase of *A. mellea* is probably a laccase. Laccases have the capacity to oxidize o-dihydroxyphenols and p-dihydroxyphenols but not monohydroxyphenols (52, 65, 71).

**Temperature optimum for A. mellea peroxidase and polyphenol oxidase:**

Studies were undertaken to find out if the temperature optimum varied for peroxidase and polyphenol oxidase of *A. mellea* cultured under different conditions. The experiments were carried out in a constant temperature circulating water bath that could be controlled from 5°C to 75°C.
Table 14. Comparison of substrate specificity of polyphenol oxidase in Armillaria mellea incubated on glucose or glucose + ethanol (500 ppm) with commercial tyrosinase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Commercial</th>
<th>Glucose Thalli</th>
<th>Glucose + Ethanol 500 ppm Thalli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p-Phenylenediamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> + = Activity

<sup>b</sup> - = No activity
There is no difference in the temperature optimum of polyphenol oxidase activity of thalli cultured on G and those thalli cultured on G + E (Fig. 33). The optimum temperature for polyphenol oxidase activity was 25°C. There was a significant decrease in activity at 15°C and 35°C.

The optimum temperature for peroxidase activity of those thalli cultured on G is 35°C (Fig. 34). There was no detectable activity for those thalli cultured on G + E.

**pH optimum for peroxidase and polyphenol oxidase of A. mellea:**

Optimum pH studies were done to see if difference in nutritional conditions caused a variation in pH optima of peroxidase and polyphenol oxidase activity of A. mellea.

The optimum pH for polyphenol oxidase activity of thalli incubated on G was 6.0 (Fig. 35). The optimum pH for peroxidase activity of A. mellea incubated on G was 7.0 (Fig. 36). No activity was detected for thalli incubated on G for any pH tested.

**Column chromatography of polyphenol oxidase and peroxidase of A. mellea:**

Sephadex column chromatography of the acetone precipitated and crude enzyme preparation was attempted. Chromatography was tried with Sephadex G-75, G-100, and G-200. The data obtained indicated that there was no separation of activity on any of the gels worked with. The activity for peroxidase and polyphenol oxidase always appeared in the void volume. The activity always appeared in the same tubes. This indicated that the enzymes may be bound to each other and that the molecular weight of the complex was greater than 200,000.
Figure 33. Effect of varying temperatures on polyphenol oxidase activity of Armillaria mellea. Glucose indicated by --, glucose + ethanol (500 ppm) indicated by .

*Figure 33. Effect of varying temperatures on polyphenol oxidase activity of Armillaria mellea.*

Glucose indicated by –, glucose + ethanol (500 ppm) indicated by ....... .
Figure 34. Effect of varying temperatures on peroxidase activity of *Armillaria mellea*. Glucose indicated by ———, glucose + ethanol (500 ppm) indicated by ————.
Figure 35. Effect of varying pH on polyphenol oxidase activity of Armillaria mellea. Glucose (---), glucose + ethanol (500 ppm) (---------).
Figure 36. Effect of varying pH on peroxidase activity of Armillaria mellea. Glucose indicated by ———, glucose + ethanol (500 ppm) indicated by ————.
CONCLUSIONS

Data have been presented which indicate that ethanol and other short chain alcohols suppress peroxidase activity in A. mellea thalli while growth is stimulated. Thalli on G grew less rapidly and had relatively high peroxidase activity. The data suggest that peroxidase may play an important role in suppression of growth of thalli and rhizomorph development on G. These data also suggest that ethanol suppresses peroxidase activity, and this suppression may be associated with stimulation of growth and rhizomorph development of A. mellea.

The mechanism of ethanol suppression of peroxidase might be, ethanol stimulates synthesis of compounds which control peroxidase by having allosteric effects on the enzyme or by regulating isozyme production.

Several workers have shown IAA to regulate peroxidase activity (37, 38, 69, 117). Ethanol could stimulate the synthesis of IAA or a compound which might act in the same manner in control. In the presence of the growth stimulator peroxidase activity is lowered (38,117). On G little or no growth stimulator is synthesized and peroxidase activity is not suppressed. If the growth stimulator is synthesized on G, the high peroxidase activity acts as an oxidase and destroys the growth promoting effect of the compound. Tang (117), Gaspar (50), Sequeira (107), and McCune (83) have shown that most peroxidases have the ability to act as growth promoter oxidases, particularly IAA oxidases.
Ethanol may also suppress peroxidase by stimulating the synthesis of proteins or other organic compounds which could bind to the enzyme and alter its activity. Ethanol has been shown to affect fructose-1-6-diphosphatase by allosteric control (39).

The increase in peroxidase on thalli incubated on G could explain the inhibition of growth and rhizomorph development observed on G. Peroxidase could oxidize phenols which accumulate to lignin- or melanin-like polymers. These polymers could then bind cell walls, making them more rigid and thus prevent further cell wall expansion and growth. Galston (38) and Siegel (108) have shown peroxidase to be important in oxidation of hydroxyphenylpropane units to lignin-like substances.

Data were also presented which indicate peroxidase of *A. mellea* from thalli grown on G was similar to the peroxidase of *A. mellea* cultured on G + E. Both reacted with the same substrates, both had the same temperature and pH optima. These data suggest the enzyme from different treatments is very similar in certain characteristics.

The peroxidase of *A. mellea* was found to be different from the peroxidase of horseradish in substrate specificity.

Studies with polyphenol oxidase gave no clear cut evidence that the enzyme could be correlated to the inhibition phenomenon. There were differences in activity between G incubated thalli and G + E incubated thalli. These differences in activity were not evident, however, at 72 hr. The most overt differences occurred in thalli after 24 hr incubation. The polyphenol oxidase activity decreased progressively until the activity was equal in both treatments at 72 hr.
Substrate studies and comparison of *A. mellea* polyphenol oxidase with commercial tyrosinase showed that the two enzymes were different. *A. mellea* polyphenol oxidase oxidized o-dihydroxyphenols and p-dihydroxyphenols, but not monophenols or m-dihydroxyphenols. Commercial tyrosinase oxidized monophenols and o-dihydroxyphenols. The oxidation characteristics of *A. mellea* polyphenol oxidase indicate that it has laccase activity. Laccases are able to oxidize ortho- and para-dihydroxyphenols, according to Goodman et al. (52) and Krupasagar and Sequeira (65).

Comparison of polyphenol oxidase from G treated thalli with G + E treated thalli indicated they have the same substrate specificity, pH optimum and temperature optimum. The enzyme from the two differently treated thalli may be very similar.

Column chromatography of the enzymes indicated that the two enzymes were closely associated with each other. They could not be separated on G-75, G-100, or G-200 columns. Activity of both was always found in the same tubes in the void volume. This suggests that both have molecular weights of greater than 200,000. This value is higher than the published value for either of the enzymes (35, 77). It is possible that in the author's system the enzymes are part of a protein complex or that the enzymes are bound to each other. It is also possible that the enzymes are part of a particulate fraction such as mitochondrial membranes (78, 128, 130) and the author could not separate them.

The author has presented data which suggest peroxidase may play an integral part in inhibition of thallus growth and rhizomorph
development. The role of polyphenol oxidase is not as clearly defined. Perhaps it also plays a major role in morphogenesis in *A. mellea*. Further studies need to be undertaken to better understand the role of phenol oxidizing enzymes in morphogenesis of *A. mellea* in relation to the effect of ethanol.
VI. GENERAL DISCUSSION

Evidence has been presented which indicates ethanol and other short chain alcohols suppress phenol accumulation in thalli of *A. mellea*. Thalli incubated on glucose alone produce no rhizomorphs and mycelial growth is inhibited. Phenol concentrations are much higher in these thalli than in thalli incubated on glucose plus either ethanol, butanol, propanol, or iso-butanol. Studies with TLC and column chromatography indicated that ethanol not only affects the amount of phenols synthesized, but also the kinds synthesized. At least two distinct unidentified phenolic compounds were found in thalli incubated on glucose (G) which were not found in thalli incubated on glucose + ethanol (G + E). One unidentified phenolic compound was found in thalli incubated on G + E which did not appear in thalli incubated on G.

A toxic phenol was found in extracts of *A. mellea* incubated on G which produced self-inhibition of rhizomorph development and mycelial growth of the fungus *in vitro*. No comparable phenol was found in thalli incubated on G + E. Consideration of how ethanol can stimulate growth and still produce suppression of a category of metabolites is discussed in the conclusions to Chapter 3 (p.55) and Chapter 4 (p.89). Briefly, it was considered that ethanol and/or products of ethanol metabolism probably affect phenol levels through regulation of primary metabolic pathways. Ethanol stimulates pathways involving cell wall
synthesis and gluconeogenesis. While at the same time ethanol breakdown via the glyoxylate cycle produces excess ATP, which in turn, reduces glucose breakdown via the HMP and EMP through allosteric effects on key enzymes in these systems. These factors working in the presence of ethanol decrease the amount of precursor material available for phenol biosynthesis. Therefore, phenol accumulation is suppressed in the presence of ethanol.

This author also observed that ethanol and other short chain alcohols suppressed peroxidase activity in A. mellea thalli. Peroxidase suppression was inversely related to growth of thalli and rhizomorph development. As ethanol was increased growth increased, and this was accompanied by a decrease in peroxidase activity in thalli. This suppression is discussed in the conclusion of Chapter 5 (p. 117). Briefly, ethanol could stimulate synthesis of a protein or growth regulating compound which might regulate peroxidase activity or synthesis. In thalli incubated on G these compounds are not produced or are produced in an inactive state, thus no effective regulation of peroxidase can occur.

This author has presented evidence which suggests that ethanol suppresses endogenous toxic products phenolic in nature, also ethanol suppresses peroxidase, an enzyme with the capacity to cause oxidation of phenols. These observations are explained through ethanol stimulation of primary metabolic pathways and through stimulation of compounds which regulate enzyme activity. These data and observations support Garraway and Weinhold's (44) hypothesis that ethanol acts to stimulate growth by suppression of endogenous inhibitor accumulation.
Assuming the above observations and conclusions are true, the mode of action of inhibition of growth in the absence of ethanol might be explained. When thalli are incubated in the absence of ethanol, phenol concentrations and peroxidase activity in thalli are higher. The phenols may act as a substrate for the enzyme peroxidase. Phenols could be oxidized to polymers such as lignin and/or melanin. These, in turn, can bind to cell walls, giving them more rigidity, thus limiting cell wall expansion and growth. Siegel (108), Bull (15), Galston (38), Kuo (67), and Potgeiter (95) have all shown lignin and/or melanin to be involved in cell wall expansion. Several reports have shown peroxidase to be important in lignin polymer formation (38, 87, 103, 108).

Phenol accumulation and peroxidase activity have been theorized by several authors as being involved in IAA stimulation of growth (37, 50, 61, 69, 129). Garraway (40) found IAA, AA, and PABA to stimulate rhizomorph development more effectively than ethanol. These compounds were active at 10-20 ppm, while ethanol was effective at 500 ppm. Garraway found IAA to be most effective, AA next, and PABA least effective. He concluded that AA was converted to IAA more readily than PABA, therefore it is more effective than PABA in promoting growth and rhizomorph development. This author suggests that the stimulatory effect of ethanol and IAA are similar. Ethanol is less effective because several metabolic steps are involved in converting ethanol to a growth promoter. This growth promoter may be IAA or a related compound. If this does occur, the mechanism of IAA, AA, PABA, and ethanol stimulation of growth might be one and the same. IAA is known to regulate peroxidase activity (36, 69, 117). Galston (36) and Lavee (69) have
shown as IAA is synthesized, peroxidase activity is lowered. \textit{A. mellea}, when incubated in presence of IAA, PABA, or AA should, then, have decreased peroxidase activity. Forrest (34) theorized IAA reduces phenols in tissue culture. This could occur in \textit{A. mellea}, therefore, in the presence of IAA one would also expect to see not only a reduction in peroxidase, but also phenols. If IAA produces the same effect in \textit{A. mellea} as in higher plants, then it would seem IAA would cause a reduction in the same physiological parameters that this author found ethanol to cause a reduction in. This would give a common element to growth stimulation by IAA and ethanol.

In view of the data presented by this author and mechanism(s) proposed regarding the effect of ethanol on growth and rhizomorph stimulation in \textit{A. mellea}, some prior observations on growth of the fungus \textit{in vivo} and \textit{in vitro} will be considered. This author will discuss other research involving \textit{A. mellea} and give alternative explanations of the phenomena observed. Studies by Pentland (91, 92, 93, 94), Weinhold and Hendrix (126), Leach (70), Garrett (46, 47), and Swift (114) will be considered.

Increases in phenols and/or peroxidase may help explain Pentland's observations concerning the effect of \textit{Aureobasidium pullulans} and ethanol on growth of \textit{A. mellea}. Pentland observed that \textit{Aureobasidium pullulans} stimulated growth of \textit{A. mellea} in pure culture and in sterilized soils. Pentland found the stimulatory substance to be ethanol. She observed \textit{A. mellea} required continuous presence of ethanol for rhizomorphs to develop and continue growth. Garraway and Weinhold (42), in contrast, found after initial exposure of rhizomorphs
to ethanol for 12-13 days, growth continued in the absence of ethanol. This inconsistency may be explained in light of the concentrations of glucose used by the workers in consideration and how this might affect phenol accumulation or peroxidase activity. Pentland used 20 g glucose/liter or 2% glucose, while Garraway and Weinhold used 5 g/liter or 0.5%. On Pentland's system the excess glucose may have been the reason for continuous need of ethanol. The continuous presence of ethanol might prevent excess glucose metabolism to phenols. On the 2% glucose solution, as ethanol is removed excess glucose remaining is converted to phenols and peroxidase increases. The increase in phenols and peroxidase act to inhibit growth. Oxidation of phenols to polymers by peroxidase occurs. These, in turn, bind to cell walls and prevent growth, as described earlier. With ethanol continuously present, this does not occur. On the 0.5% glucose solution, after initial exposure to ethanol initial glucose levels are reduced and when ethanol is removed there is not the excess glucose present to be converted to phenols because the initial level of glucose in this system is much lower.

Forrest (34) and Henshaw (61) have shown increases in sugar levels in media cause increase in phenol production in tissue cultures of plants. Perhaps isolates of the fungus used could also explain the inconsistency. Pentland's isolate may have needed continuous presence of a growth stimulator because the fungus did not have the enzymes for endogenous synthesis of a stimulator, while the isolate used by Garraway and Weinhold had the capacity to synthesize the stimulator after initial growth of the rhizomorph tip occurred as a response to ethanol and/or some metabolite of ethanol.
Phenol accumulation and peroxidase activity are known to be stimulated in higher plants by light. Galston (36) and Tang (117) have shown peroxidase of higher plants to be stimulated by light, also Forrest (34) and Henshaw (61) have shown light to stimulate phenol synthesis in higher plants. Weinhold and Hendrix (126) found *A. mellea* rhizomorph growth to be inhibited by light. They hypothesized that this might be the result of H$_2$O$_2$ formation because H$_2$O$_2$ could produce the same effect as light. In addition, they found catalase prevented the light inhibition. Perhaps these observations could be explained by increased phenols and peroxidase found in thalli exposed to light. If this occurs in *A. mellea*, the peroxidase increase could produce oxidation of phenols to polymers which bind cell walls or peroxidase might cause oxidation of a growth stimulator. This could explain Weinhold and Hendrix's observations. Involvement of H$_2$O$_2$ may have been only to provide the substrate for peroxidase. Catalase prevention of inhibition by light might be the result of catalase utilization of H$_2$O$_2$ to form H$_2$O + O$_2$. Catalase caused oxidation of 2H$_2$O$_2$ → 2H$_2$O + O$_2$ (128).

The finding by this author that peroxidase and phenol are associated with decreased growth and rhizomorph development and utilization of existing information could help explain some phenomena observed in the field regarding *A. mellea*. IAA, as mentioned previously, stimulates rhizomorph growth and development. IAA is inactivated by IAA oxidases. Many workers (37, 65, 83, 107) have shown IAA oxidases are peroxidases. In trees IAA is translocated downward through the cambium to roots (52). As IAA reaches the root it could stimulate rhizomorph development and growth if the tree is infected by *A. mellea*. This would provide
additional inoculum sources for invasion of other hosts. If by some mechanism *A. mellea* could be stimulated to produce more peroxidase, perhaps IAA could be inactivated. This could slow down or halt rhizomorph growth and development. Some foliar applicants have been found which stimulate peroxidase, CCC and AMO-1618 (50). Perhaps compounds such as these could aid in control of the pathogen. The feasibility of this in terms of cost and practicability would have to be studied before it could be applicable.

The effect of peroxidase activity and its effect on IAA should also be considered in studying the effect of ring barking of trees in controlling *A. mellea*. Leach (70) and Garrett (49) found ring barking of trees two years prior to felling reduced the incidence of *A. mellea* in new plantings. They attributed this reduction to depletion of starch reserves in roots and to competition between *A. mellea* and other soil microorganisms. Both of these factors do probably play a significant role in reduction of disease incidence. Ring barking would also prevent transport of IAA and other growth substances to roots. Over a period of time IAA would be depleted or inactivated. This depletion of IAA and other growth factors present in roots might affect the growth of *A. mellea* through increasing peroxidase activity and increasing phenols. The increase in these two physiological parameters could cause inhibition of rhizomorph growth and development by previously described mechanisms. This might prevent spread of the fungus through the soil, thus reducing inoculum. Another aspect to be considered is that perhaps as roots decay they release toxic phenols which inhibit
growth of the pathogen in infected roots or prevents those roots which are not infected from becoming infected.

A consideration of toxic phenols must be taken into account when Swift's work is discussed. Swift (114) found that \textit{A. mellea} did not form rhizomorphs in Rhodesian forest soils. He found a water soluble compound which he could extract from the soil that could completely inhibit rhizomorph development of \textit{A. mellea}. This compound could, perhaps, be a phenolic type compound. Since the compound was found in forest soils, it was probably a product of lignin degradation. The compound may be a benzoic or cinnamic acid derivative. This author found benzoic and cinnamic acid derivatives could inhibit rhizomorph growth and development \textit{in vitro}. This author also found a water soluble endogenous phenolic compound which caused self-inhibition of \textit{A. mellea}. These observations might suggest Swift's compound could be a cinnamic or benzoic acid derivative.

Further support of the endogenous inhibitor in \textit{A. mellea} might be related to cinnamic acid derivatives are the work of Macko (75, 76) and Allen et al. (Phytopathology 61:1382-1390, 1971). Both have found cinnamic acid derivatives to be endogenous inhibitors of rust uredospore germination. This author found cinnamic acid derivatives to inhibit \textit{A. mellea} \textit{in vitro}. These observations would suggest that the endogenous inhibitor of growth when \textit{A. mellea} is incubated on glucose could be a cinnamic acid derivative.

\textit{Trichoderma viride} is thought to be involved in biological control of \textit{A. mellea}. Bliss (10) and Garrett (46, 47) found control of \textit{A. mellea} associated with increased \textit{T. viride} after fumigation with CS2.
T. viride is antagonistic to A. mellea (2, 46). This antagonism could be the result of the action of the antibiotic Viridin produced by T. viride (14). Viridin is a hydroxylated polyphenyl propanoid structure similar to cinnamic acid derivatives. As mentioned previously, this author has shown cinnamic acid derivatives to inhibit A. mellea in vitro. Therefore, it would seem that antagonism of T. viride could be the result of the action of Viridin.

Variation in rhizomorph production and pathogenicity of A. mellea may be associated with phenol oxidizing enzymes and phenol accumulation. Esser (32, 33), Singh (109), Cantino (19) have all found association between phenol oxidizing enzymes and morphogenetic variation in fungi. Galston (38), McCune (83), and Gaspar (50) have shown morphogenetic variation in higher plants associated with peroxidase activity. Forrest (34) and Henshaw (61) have shown phenols associated with morphogenesis in tissue culture of higher plants.

Rhizomorphs of A. mellea are also resting structures and are described as pseudosclerotia (18). The involvement of peroxidase and phenols in morphogenesis indicates that differentiation of resting structures of other organisms should be studied with regard to involvement of peroxidase and phenols. These factors could play important roles in differentiation of chlamydompores of Verticillium spp., sclerotia of Botrytis and Rhizoctonia spp., and strands of Phymatotrichum spp. A study of the physiology of morphogenesis of these type structures could ultimately aid in developing control measures for these organisms.
Several questions have been raised which need further study. Some of these have been mentioned. Direct questions related to this author's work which need to receive further study are: 1) Do other compounds which stimulate growth and rhizomorph development in *A. mellea* produce the same effects as ethanol on phenols and peroxidase? 2) What is the actual identity of toxic compound(s) in phenolic extracts of *A. mellea* incubated in absence of ethanol? 3) What is the actual role of peroxidase in morphogenesis of rhizomorphs? 4) Do growing rhizomorph tips have the capacity to produce IAA or some other growth stimulator? 5) Is the peroxidase of *A. mellea* an IAA oxidase? 6) Can ethanol suppress pure peroxidase *in vitro*? 7) Do phenols which accumulate in the absence of ethanol inhibit cell wall formation or do they inhibit by forming polymers or some mechanism other than these? 8) Could *A. mellea* be induced to produce toxic phenols or peroxidase through some soil or foliar applicant? 9) Is light inhibition the result of peroxidase activation and phenol accumulation? 10) Is *T. viride* inhibition of *A. mellea* the result of Viridin or is it some other mechanism? 11) Can maximum field spread of *A. mellea* rhizomorphs be correlated with seasonal periods when growth stimulators are produced most abundantly? 12) Is the compound which inhibits *A. mellea* in Rhodesian forest soils a phenolic cinnamic acid derivative?
VII. SUMMARY

Ethanol and other low molecular weight alcohols stimulate rhizomorph development and growth in *Armillaria mellea* (Vahl) Quel. The objective of research presented in this thesis was to explain the stimulatory effect that ethanol and other short chain alcohols have on rhizomorph development and growth. This objective was met by comparing certain physiological parameters, listed below, in *A. mellea* thalli incubated in the presence or absence of ethanol. These parameters included (i) quantitative and qualitative changes in phenols in thallus extracts, (ii) biological activity of phenols in extracts, and (iii) peroxidase and polyphenol oxidase activity of extracts.

*A. mellea* was grown for 7 days on a glucose-L-asparagine medium supplemented with ethanol (500 ppm); then groups of thalli were transferred to a medium without ethanol or to a similar medium supplemented with 500 ppm either ethanol, butanol, propanol, or iso-butanol. The dry weight of transferred thalli was determined at various incubation times from zero to 72 hr. Concurrently, similarly treated thalli were extracted with 80% ethanol or 0.05 M KH$_2$PO$_4$ buffer. Phenol content of ethanolic extracts was determined by Folin-Denis technique while peroxidase (using pyrogallol as a substrate) and polyphenol oxidase (using catechol as a substrate) were assayed with protein precipitated with acetone from the buffered extracts. Phenolic extracts of thalli incubated in the presence or absence of ethanol (500 ppm) were compared
by thin layer chromatography, column chromatography, and through biological assays.

The studies indicated ethanol and other short chain alcohols stimulated growth while suppressing phenol accumulation in *A. mellea* thalli. Also, TLC and column chromatography of phenolic extracts of thalli incubated in the presence and absence of ethanol indicated that ethanol affects not only the amount of phenols synthesized in thalli, but also the kinds of phenols synthesized. One unidentified phenol was found in thalli incubated on ethanol, while no comparable compound was found in thalli incubated without ethanol. Also, two unidentified phenolic compounds were found in thalli incubated in absence of ethanol while no comparable compounds were found in thalli incubated in the presence of ethanol. One of the phenolic compounds found in extracts of thalli incubated in the absence of ethanol produced self-inhibition of *A. mellea* in vitro. Phenolic extracts of thalli incubated without ethanol were more inhibitory to growth of *Bacillus subtilis*, *Xanthomonas pruni*, and spore germination of *Botrytis cinerea* than phenolic extracts of thalli incubated on ethanol.

Other studies with ethanol and other short chain alcohols indicated that peroxidase activity in thalli incubated in the presence of ethanol was reduced. In thalli incubated without ethanol peroxidase was increased. Polyphenol oxidase appeared to be insensitive to the presence or absence of alcohol in the growth medium; no difference in activity was noticed between treatments over 72 hr incubation period.

The author discusses mechanisms which could help explain the stimulatory effect of ethanol on rhizomorph development and growth in
light of his data. Ethanol and/or its metabolites cause suppression of toxic phenol accumulation and peroxidase activity was reduced. This suppression may occur through the effect of ethanol and/or its metabolites on primary metabolic pathways. The author also attempts to discuss how the results presented in this thesis may give alternative explanations of studies involving growth of *A. mellea*. 
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