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RHIZOMORPH EXUDATE OF ARMILLARIA MELLEA

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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.....

submitted by .....Kenneth Ian Mallett.....

in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor

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Date August 7, 1981

ABSTRACT

Exudate has been associated with a number of different fungal structures. Droplets of exudate were observed on rhizomorphs of Armillaria mellea, and subsequent study showed that rhizomorph exudate was produced in great quantities on carrot agar medium. Light microscopy and scanning electron microscopy studies were done on rhizomorphs with exudate droplets and showed that the droplets were initially hyaline but darken with age and that a membranous material is left after freeze-drying. Some physical and biochemical properties of the exudate were determined. Inorganic elements, protein, and free amino acids were detected. Oxalic acid which has been found in other fungal exudates was not found in A. mellea exudate. Proteins from exudate and from rhizomorph extracts were separated by the isoelectric focusing technique. B-glucosidase, peroxidase, polyphenoloxidase, acid and alkaline protease activities were also detected.

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CHAPTER I

INTRODUCTION

Armillaria mellea (Vahl. ex Fr.) Kummer, (=Armillariella mellea Karst.), is a root rotting basidiomycete belonging to the order Agaricales. It is commonly found in forest soils throughout the world, and has an extremely wide host range, principally forest trees but also including many horticultural crops (Raabe 1962). A. mellea causes the disease in plants known as 'shoestring rot', 'crown rot', or 'mushroom root rot'. White fans of mycelium and black cord-like rhizomorphs of A. mellea can be found between the bark and the wood in the root collar and roots of infected trees. The rhizomorphs may be attached to root surfaces and/or growing in the soil surrounding the plant roots. Rhizomorphs are the principal means of infection and spread of the fungus (Rishbeth 1970), as they can grow through soil and attack healthy plant roots.

Armillaria mellea has been the subject of considerable study because of its' wide host range and destructiveness of certain economically

important plants (Foster and Foster 1951, Marsh 1952). Hartig (1873), was one of the first workers to examine A. mellea from a taxonomic point of view. He showed that Rhizomorpha substerannea and Rhizomorpha subcorticalis were the same fungus, A. mellea. Since then, much effort has been carried out in the study of A. mellea's biology, cultural characteristics, rhizomorph behavior, genetics, etiology, and control.

Hartig (1873) originally thought that A. mellea was a 'wound' parasite and that it was unable to enter actively growing tissue, but later reported that A. mellea was neither a parasite of healthy nor wounded roots. However, Thomas (1934) concluded, from a long and detailed study, that penetration of the root by a branch of the rhizomorph was direct and through intact periderm. Penetration involved two processes, mechanical force from the rhizomorph, and by chemical means as there appeared to be destruction of the suberized cell walls. Further observations revealed that death of the plant cells always occurred well in advance of the penetrating rhizomorphs, thus indicating the presence of fungal toxins and/or enzyme systems.

Ward (1888) pointed out the possibility that fungi might produce toxic substances which were related to pathogenesis. De Bary (1887) and Brown (1915) found that cultural filtrates of fungi had the ability to destroy host tissue. It is now a commonly held view that fungi excrete extracellular enzymes in order to break down foodstuffs.

Since the time of de Bary (1887), liquid droplets have been reported by many investigators on fungi from almost every taxonomic class. In the basidiomycetes, many authors have described liquid droplets being associated with different parts of the basidiocarp.

Reports of droplets on the upper portion of the stipe have been made for Agaricus polyporus (Tulasne and Tulasne 1931), Paneaeolus solidipes (Hard 1908, McIlvaine 1912, Gussow and Odell 1927), Tricholoma grande (McIlvaine 1912), Marasmius elongatipes, Hygrophorus eburnes, Panoeolus campanulatus (Kauffman 1918), Boletus subaurens, Boletus punclipes (Coker and Beers 1943), Suillus plirans, Limacium poitarum, Limacium pudorinum and Russula foetans (Pilat 1961). McIlvaine (1912), Kauffman (1918), Coker and Beer (1943), and Pilat (1961)

noted that the droplets were colored and that when dried left colored spots on Lepoita lenticularis, Suillus plorans, Marasmius elongatipes, Boletus subaureus, Boletus punctipes. Droplets on gills or tubes have been reported for Hypholoma lachrymabundum (Hard 1908, Atkinson 1911, Kauffman 1918), Boletus subaureus (McIlvaine 1912), Poliota albocrenulata, Hebeloma fastible, Hebeloma crustuliniforme (Kauffman 1918), Boletus granulatus, Polyporus hipidus, Polyporus stiptiais, Psilobybe areolata, Russula luteo-tacla (Rea 1922), Russula delica (Rea 1922, Gussow and Odell 1927), Suillus plorans (Pilat 1961), Russula foetans (Groves 1962), Polyporus berkeleyi, Polyporus schweinitzii (Krieger 1967). A chestnut colored watery liquid exuded by spores of Gyroden proximus was reported by Singer (1977). Buller (1958) described, for a number of basidiomycetes, droplets of liquid that formed on basidiospores just before their ejection. Exuded droplets on Hymenophores have been noted for Tricholoma grande (McIlvaine 1912), Polyporus hispidus (Massee), Merulius lachrymans (Rea 1922), Polyporus resinosus (Groves 1962), Suillus subaureus and Pulveroboletus certisii (Snell and Dick 1970).

Several early twentieth century plant physiologists, Pfeffer (1900), Lepeschkin (1906), and Knoll (1912) studied liquids exuded from fungi to understand guttation in higher plants. All of these workers found that the droplets contained other substances besides water. Pfeffer (1900) observed crystalline material after letting liquid droplets from the sporangiophores of Pilobus dry on a coverslip. He was not sure however, as to how the droplets were formed. Lepeschkin (1906) analyzed droplets from Pilobus longipes and found them to contain several inorganic mineral salts but no organic substances. Small droplets of liquid were observed by Knoll (1912) on the ends of cystidia of Coprinus ephemerus. The ends of the cystidia were considered, by him, to be acting as hydathodes. He also noted that as the droplets dried their surfaces wrinkled and became irregular in shape, which led him to believe that a colloidal substance was present in the droplets. Pilat (1961) described a reddish-brown liquid which was exuded from the cystidia on the stipe of Suillus plorans. Watting (1977) noted that cystidia of Bolbitaceae exuded droplets of liquid. Droplets of liquid can be found

on the cystidia and basidiospores of Ophiobolus canarii (Corner 1948). In Corners' opinion, these droplets were made up of cytoplasmic and vacuolar sap, and were exuded through an excessively permeable cystidial wall.

Exudate droplet formation, in some fungi, appears to be associated with periods of active growth (Fenner 1932, Macdonald 1934, Cooke 1971, Colotelo 1973, McPhee and Colotelo 1977). Exudate droplets from developing sclerotia of Sclerotinia sclerotiorum have been shown to exhibit non-enzymatic (Cooke 1969, Jones 1970, Colotelo et al. 1971a, 1971b, 1973) and enzymatic characteristics (Jones 1970, Colotelo et al. 1971a, 1971b, 1973). The presence of hydrolytic enzymes, fatty acids, free amino acids, cations, oxalic acid, and ammonia in exudate from Sclerotinia sclerotiorum have been determined by Colotelo et al. (1971a, 1971b). Christias (1980) noted the free amino acid composition, total protein and carbohydrate, electrical conductivity and the effect of sclerotial exudate on growth of selected soil microorganisms for sclerotial exudate Sclerotium rolfsii. Exudate droplets from a wide variety of fungi have also been

shown to contain hydrolytic enzymes (Colotelo 1978). McPhee (1978) showed that hyphal exudate from Fusarium culmorum contained hydrolytic enzymes and also had the ability to degrade plant tissue. Droplet formation on the mycelium of Serpula lacrimans was described by Coggins et al. (1980) and related to pressure driven flow along the hyphae.

Droplets of exudate have been observed on the mycelium of Armillaria mellea (Hamada 1940) and on the rhizomorphs growing above the cultural media (Raabe 1967). Hamada (1940) referred to the exudate as guttation droplets and observed the droplets to grow larger with time, eventually coalescing into several large drops. The color of the exudate was noted to change from colorless to black with time. Hamada also found the quantity of guttation droplets to vary as the C:N ratio of the media was changed. The greatest amount of exudate was produced when the C:N ration was 1:1. No one has reported on the chemical composition of the exudate droplets of A. mellea rhizomorphs; however, Lanphere (1934) did examine extracts made from rhizomorphs for the presence of various hydrolytic enzymes.

The purpose of this study was to deter-

mine some of the physical and biochemical characteristics of Armillaria mellea rhizomorphal exudate.

CHAPTER II

OBSERVATIONS OF RHIZOMORPHAL EXUDATE IN CULTURE

Objective

In order to study exudate droplets produced on rhizomorphs of A. mellea, it was necessary to determine when the droplets appeared, what their physical appearance was like, and which media or medium produced the greatest amount of exudate.

Materials and Methods

A culture of Armillaria mellea (Vahl. ex Fries) Kummer, isolate C49, was obtained from the Canadian Forestry Service, Pacific Forest Research Center, Victoria, British Columbia. The isolate was grown on the following media:

(a) carrot agar

(b) potato dextrose agar

(c) synthetic

(a) Carrot Agar

The carrot agar was prepared by grinding 300 g. of washed carrots with 400 ml deionized distilled water in a Waring blendor for 45 seconds.

In a separate flask, 16g. of difco agar was added to 300 ml of deionized distilled water. The two flasks were then autoclaved for 40 minutes at 121°C and 16 psi. The agar was added to the homogenized carrots aseptically before dispensing into 140mm (i. diam.) x 20 mm Petri plates, 125 mls per Petri plate.

(b) Potato Dextrose Agar (PDA) Medium

PDA was made by autoclaving 200 g. of peeled potatoes in one liter of deionized distilled water for 20 minutes at 121°C and 16 psi. The liquid portion of this was filtered through cheesecloth into a flask containing 20 g. dextrose and 15 g. agar, then brought up to one liter with deionized distilled water. This mixture was autoclaved for 20 minutes at 121°C and 16 psi before being dispensed into 140 mm (i. diam.) x 20 mm Petri plates, 125 mls per Petri plate.

(c) Synthetic Medium

The synthetic medium was composed of the following:

5 g	glucose
2 g	l-asparagine
1.75 g	KH_2PO_4
0.55 g	$MgSO_4 \cdot 7\ H_2O$

2.0 ml of a solution containing

0.15 g/l KCl

0.00365 g/l $FeSO_4 \cdot 7\ H_2O$

0.00365 g/l $ZnSO_4 \cdot 7\ H_2O$

0.00314 g/l $MnCl_2 \cdot 4\ H_2O$

100 g thiamine

20 g difco agar

These chemicals were dissolved in one liter of deionized distilled water and autoclaved for 20 minutes at 121°C and 16 psi. The medium was dispensed into 140 mm (i. diam.) x 20 mm Petri plates, 125 mls per Petri plate.

Petri plates containing media were inoculated with agar plugs, cut with a number five cork-borer from the margin of a five-day-old culture grown on the same medium as the inoculum plug was to be placed upon. Care was taken to insure that no rhizomorphs were included in the inoculum plug for these experiments. Petri plates were sealed with masking tape to prevent the cultures from drying out. All cultures were grown in an incubator at 25°C. Observations were made daily for four weeks.

Microscopic examinations were done using a Carl Zeiss low power binocular microscope

equipped with a photographic apparatus. Material used for scanning electron microscope (SEM) purposes came either from carrot agar or synthetic media cultures. Rhizomorphs were removed from the culture with a scalpel and immediately frozen in liquid Freon 12 followed by liquid Nitrogen. The samples were placed in an Edwards Pearse tissue drier (EPD_2) and dried for 10 hours at $-70^{o}C$. Samples were sputtercoated with gold (approximately 200Å thick) in an Edwards Vacuum Coating unit (E12E) and examined using a Cambridge S150 Stereoscan Scanning electron microscope.

Results

On carrot agar medium, A. mellea grew from the inoculum plug onto the agar surface within two days of inoculation. The mycelium was white, sparse in growth, and appressed to the agar surface. Six to seven days after inoculation, the diameter of the colony was approximately 4 cm. During this time, the mycelium around the plug grew upwards by several millimeters and had turned from white to brown in color. The agar immediately surrounding the colony was a much darker color than the agar near the edge of the Petri plate. Nine

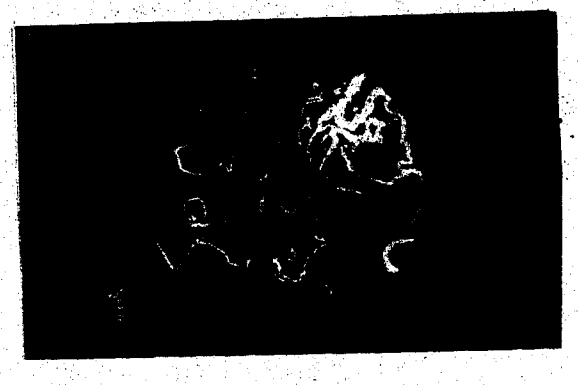
to ten days after inoculation, small white tufts of mycelium could be seen growing randomly out of the surface mycelium around the agar plug; the number of tufts varied from plate to plate. Twelve to fourteen days after inoculation, the tips of round white rhizomorphs could be seen growing down and outward in the medium. At first, there were only five or six of these round white rhizomorphs, but several days later profuse branching had occurred and the rhizomorphs had become flattened or ribbon shaped. These rhizomorphs could be seen growing between the media and the glass of the bottom half of the Petri plate, Fig. 1. There was no directionality to the growth of the rhizomorphs. Sixteen to eighteen days after inoculation, rhizomorphs emerged through the agar surface, Fig. 2. These rhizomorphs were at first white and had a mucilaginous-like coating, but developed peripheral hyphae, some more so than others, and took on a fuzzy appearance within 24 hours. Small clear droplets that turned brown over time were scattered over the rhizomorph surface. These droplets, and others which developed later, grew larger and eventually coalesced into several large droplets,

FIGURE 1

A colony of Armillaria mellea grown on carrot agar illustrating the submerged white rhizomorphs growing between the media and the bottom half of the Petri plate.

FIGURE 2

A colony of Armillaria mellea grown on carrot agar, illustrating aerial rhizomorphs, mature brown rhizomorphs, and (arrow) immature white rhizomorphs.



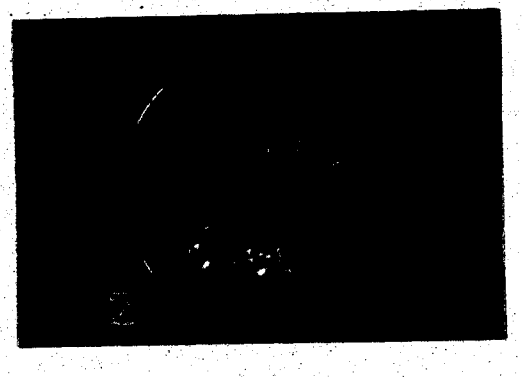


Fig 3. These exudate drops were observed on all parts of the rhizomorph, tip to base, Fig. 4. Droplet formation was random over the rhizomorph surface and did not correlate with the age of the rhizomorph part. Small clear droplets could also be seen on the surfaces of the peripheral hyphae, Fig. 5. When exudate droplets were observed for a length of time under the microscope, the droplets' surfaces seemed to shrivel and wrinkle as if water was being lost from the droplets, either through evaporation or reabsorption, and thus indicating that the surface of the droplets was membranous. Shrivelled and hardened blobs, droplets from which the water had evaporated or reabsorbed, were found on surface rhizomorphs growing from three to four-week-old cultures.

Surface rhizomorphs varied in height, from several millimeters to one centimeter. The tips of the rhizomorphs were either cylindrical or forked, the forked-tipped rhizomorphs were usually flat. All surface rhizomorphs, regardless of shape, had exudate droplets on them. Three to four days after the rhizomorph emergence, surface mycelium grew from the base of the rhizomorphs onto the agar surface. This mycelium resembled the mycelium that

FIGURE 3a

An aerial rhizomorph of Armillaria mellea with clear exudate droplets.

FIGURE 3b

A scanning electron micrograph of an Armillaria mellea aerial rhizomorph with droplets of exudate. (50x)


3a

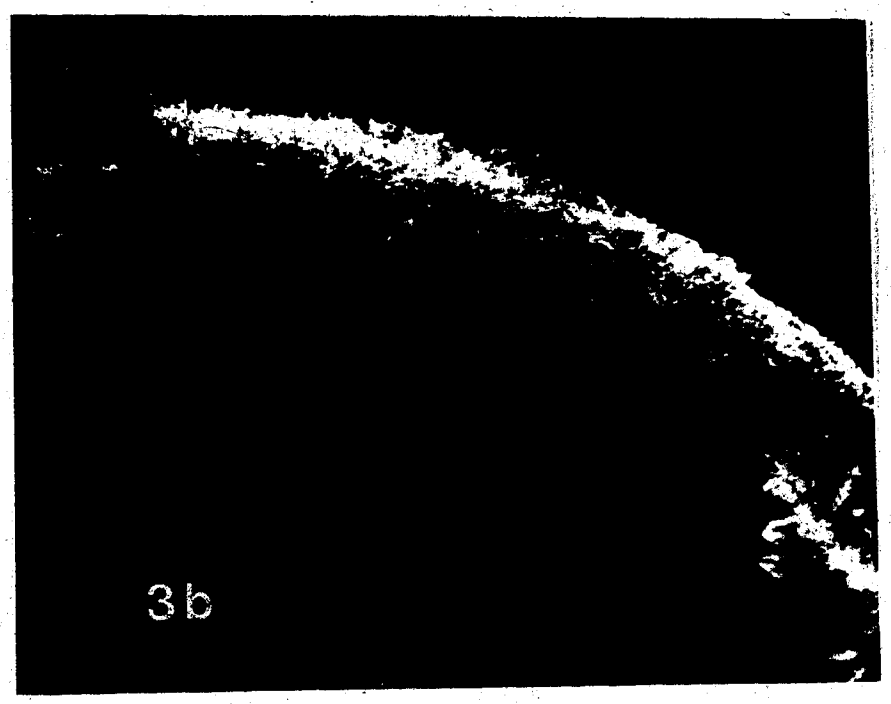

3b

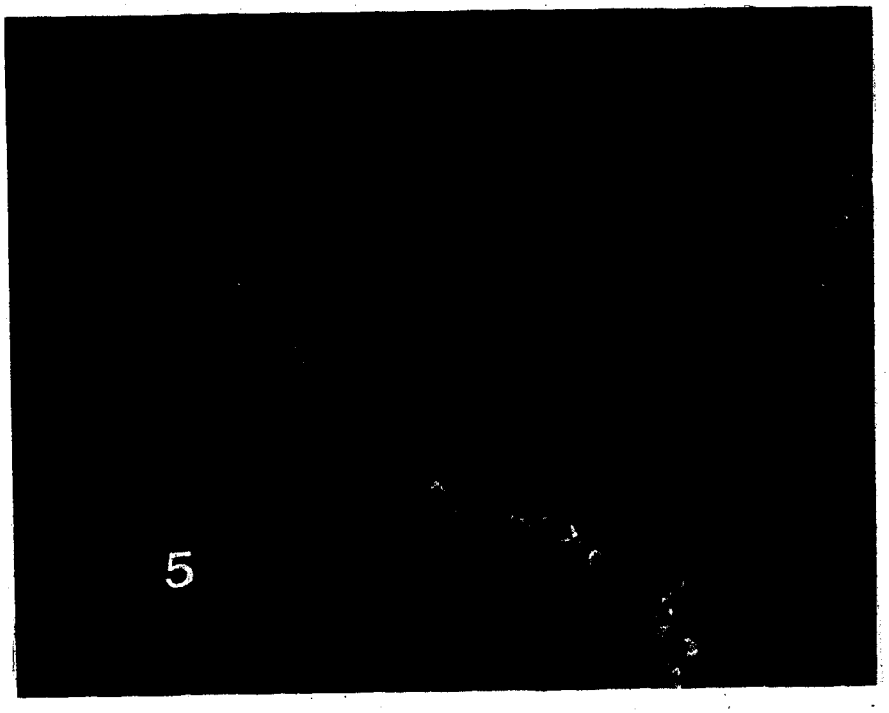
FIGURE 4

An aerial rhizomorph of Armillaria mellea with droplets of exudate, illustrating that exudate droplets can be found from the base to the tip of the rhizomorph. Clear droplets (arrow) and dark brown-black droplets can be seen.

FIGURE 5

A scanning electron micrograph of Armillaria mellea aerial rhizomorph peripheral hyphae with exudate droplets. (4,000x)


4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grew from the inoculum plug and followed the same developmental stages except that rhizomorphal initials were not formed. The mycelia or rhizomorphs growing in close proximity to each other eventually coalesced.

Isolate C49 did not grow well on PDA. A small amount of mycelium formed around the inoculum plug but very few cultures produced rhizomorphs that grew beyond this margin of the colony. The isolate did grow on synthetic medium and produced rhizomorphs. On the synthetic medium the fungus grew in a manner similar to that on the carrot agar except that the rhizomorphs produced in the agar were black in color and tubular in shape resembling rhizomorphs growing under natural conditions, Fig. 6.

The black rhizomorphs had white apical tips and numerous branches along their lengths. Surface rhizomorphs were also black with white tips. Some of these rhizomorphs grew over the agar surface. None of these colonies covered the entire Petri plate nor did any of the rhizomorphs have as much peripheral hyphae as the carrot agar surface rhizomorphs. Exudate droplets were observed on the surface rhizomorphs, but not in as great a quanti-

FIGURE 6

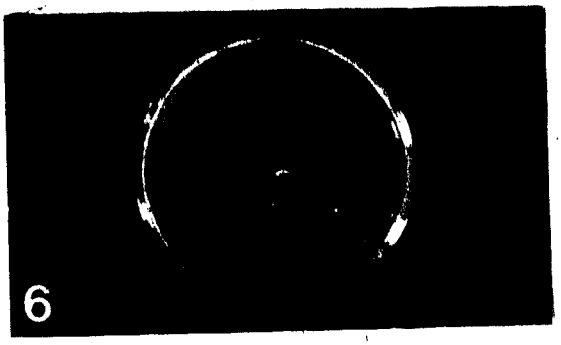
A colony of Armillaria mellea grown on synthetic medium illustrating the black surface rhizomorphs with white apical tips and numerous branches. These rhizomorphs resemble rhizomorphs of Armillaria mellea found naturally in the soil.

FIGURE 7a

A surface rhizomorph of Armillaria mellea grown on synthetic agar. A droplet of exudate is indicated by the arrow.

FIGURE 7b

A scanning electron micrograph of a surface rhizomorph of Armillaria mellea grown on synthetic medium. (60x)


6


7a


7b

ty as that found on carrot agar-grown rhizomorphs, Fig. 7.

Discussion

The cytology and morphogenesis of A. mellea rhizomorphs have been studied in detail by Townsend (1954), Motta (1969), Schmid and Liese (1970), and Wolkinger et al. (1975). The various cell layers of a rhizomorph can be seen in Fig. 8. (Motta 1969).

Snider (1959) described five stages of development for the growth of A. mellea in culture. These stages are as follows:

Stage 1. Mycelium only is present, no rhizomorphs have emerged although rhizomorphal initiation occurs (microscopically) at the end of Stage 1.

Stage 2. The emergent phase, microsclerotia form and the apices of the rhizomorphs emerge.

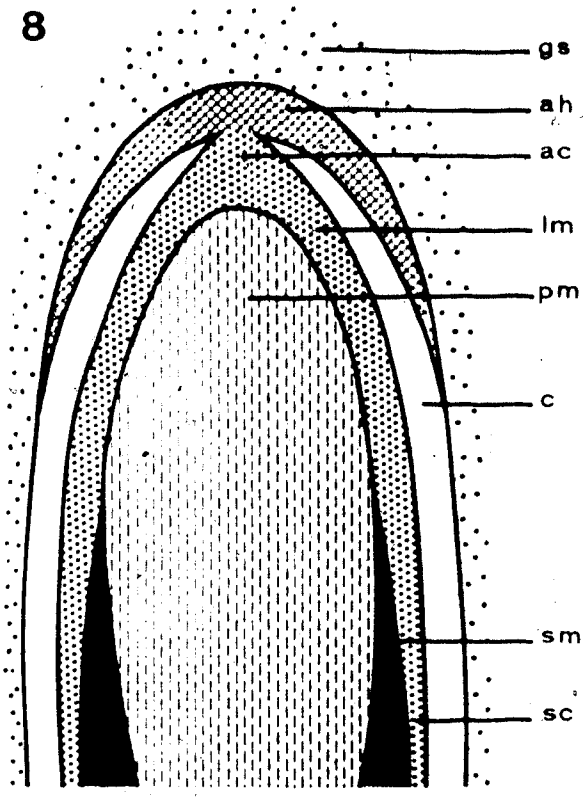
Stage 3. The lag phase, the rhizomorphs have not yet grown past the mycelial margin.

Stage 4. The linear phase, rhizomorph apical growth rate is maximal and lateral branching begins.

FIGURE 8

A diagram of an apex of an Armillaria mellea rhizomorph illustrating the distribution of tissue and their origins: ah, apical hyphae; ac, apical center; lm, lateral meristem; pm, primary medulla; c, cortex; gs, gelatinous sheath; sm, secondary medulla; sc, subcortex; taken from Motta (1969).

8



Stage 5. The terminal phase, radical growth stops or is retarded; lateral mycelium and lateral branches extend laterally from rhizomorphs, pseudosclerotia may form in the media. (Pseudosclerotia may be defined as a thin layer of bulbous cells often found on the surface or within media or wood.)

Isolate C49, when grown on carrot agar, followed Snider's five stages of development, the isolate did not exhibit all of the characteristics of each stage when grown on synthetic medium. For instance, lateral mycelia was not formed in Stage 5. It is known that different isolates of A. mellea differ in their development on certain media (Benton and Ehrlich 1941, Gibson 1961, Raabe 1966). It is also known that, in nature, two types of rhizomorphs of A. mellea are formed, the R. subterranea, tubular black rhizomorphs found in the soil, and R. subcorticalis, flattened white rhizomorphs found growing between the bark and the wood in roots of infected trees. De Bary (1884) regarded the differences in form as due to a nutritional factor.

Hamada (1940) found that R. subterranea type was formed in yeast agar and R. subcorticalis type was formed on soya sauce agar. The experiments carried out on carrot agar and synthetic medium also showed the importance of a nutritional factor(s) in the morphological type of rhizomorph formed in the media. The rhizomorphs of C49 found in synthetic medium resembled R. subterranea and the rhizomorphs of the same isolate (C49) found in the carrot agar were like the R. subcorticalis.

The appearance of surface rhizomorphs occurred during what Snider (1959) termed the linear phase of growth, the period when rhizomorphal tissue grows most rapidly. During the latter stages of this phase the rhizomorphs had ramified throughout most of the carrot agar medium and the majority of the surface rhizomorphs that would form were formed. It was also during this stage of development that one could collect the most exudate. Carrot agar was the best medium to use in order to obtain an appreciable volume of exudate for analyses.

The difference between the amount of liquid exuded by rhizomorphs of the same isoalte grown on different media can be accounted for by the follow-

ing explanation based on nutritional differences of the two media. The carrot agar is rich in organic substances and perhaps unidentified growth stimulants, whereas the synthetic media can be chemically defined and may be considered to be a minimal medium. Many investigators have noted the stimulatory effect of certain organic compounds on rhizomorph formation of A. mellea (Weinhold 1963, Garraway and Weinhold 1970, Garraway 1970, Moody and Weinhold 1972a, 1972b, Sortkjaer and Allerman 1972, Garraway 1975, Guillauman and Leprince 1979).

Droplets seen on peripheral hyphae did not appear to be associated with the brown exudate droplets. This was because none of these droplets was ever observed to grow exceptionally large in size nor change color and brown exudate droplets were observed at the tips of rhizomorphs where there were no peripheral hyphae.

Exudation on rhizomorphs grown on carrot agar and synthetic medium was noted to occur during the linear phase of development, i.e. a stage of rapid growth. This observation, that exudation is associated with periods of active growth has been made for many other fungi (Fenner 1932, Macdonald 1934, Cooke 1971, Colotelo et al. 1971b, Colotelo

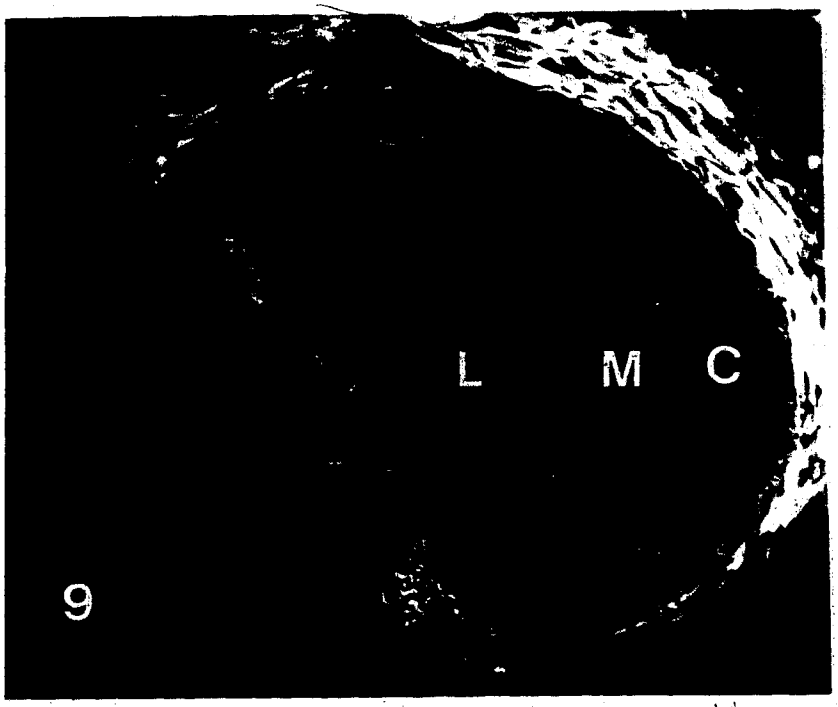
1973, McPhee and Colotelo 1977). Isolate C49 produces more exudate on carrot agar grown rhizomorphs than on synthetic medium rhizomorphs because of the richer nutritional status of the carrot agar allowing the rhizomorphs to grow more quickly and abundantly. Two theories have been put forward to explain the exudation phenomena of actively growing tissues. Cooke (1971) has described exudation in developing sclerotia as an effort to maintain an internal physiological balance by exuding water that contains carbon compounds. This maintenance is necessary because the fungus is rapidly moving soluble carbohydrates into growing tissues and cannot convert the carbohydrates to storage and structural compounds fast enough. Colotelo et al. (1971b), also describes exudation as being a function of rapidly growing tissues. Exudation is the result of cell wall growth. When polymerization of short and long chain polymers occurs, water and related compounds are expelled; a form of syneresis as well as cell rupture. Exudation as a result of cell rupture could be caused by the rupture of rind cells but also by the collapse of medullary cells to form the lumen in the rhizomorph, Figs. 9 and 10. Motta

FIGURE 9

A scanning electron micrograph of a cross section through a surface rhizomorph of Armillaria mellea grown on synthetic medium, illustrating the distribution of tissue: L, lumen; M, medullary cells; C, cortical cells (rind). (420x)

FIGURE 10

A scanning electron micrograph of a cross section through an aerial rhizomorph of Armillaria mellea grown on carrot agar medium, illustrating the distribution of tissue: L, lumen; M, medullary cells; C, cortical cells (rind). (300x)


L
M
C
9

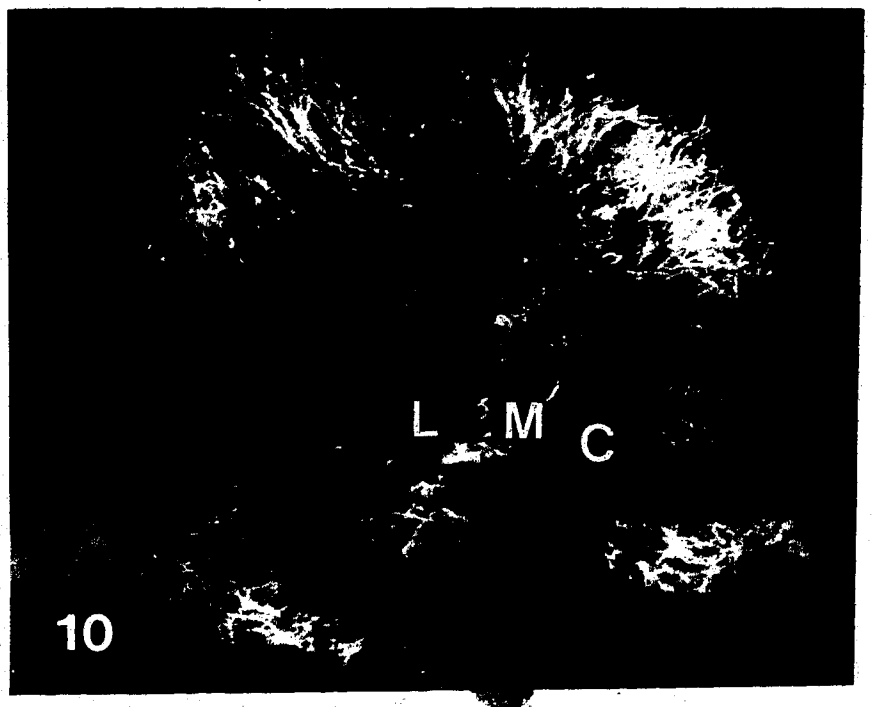
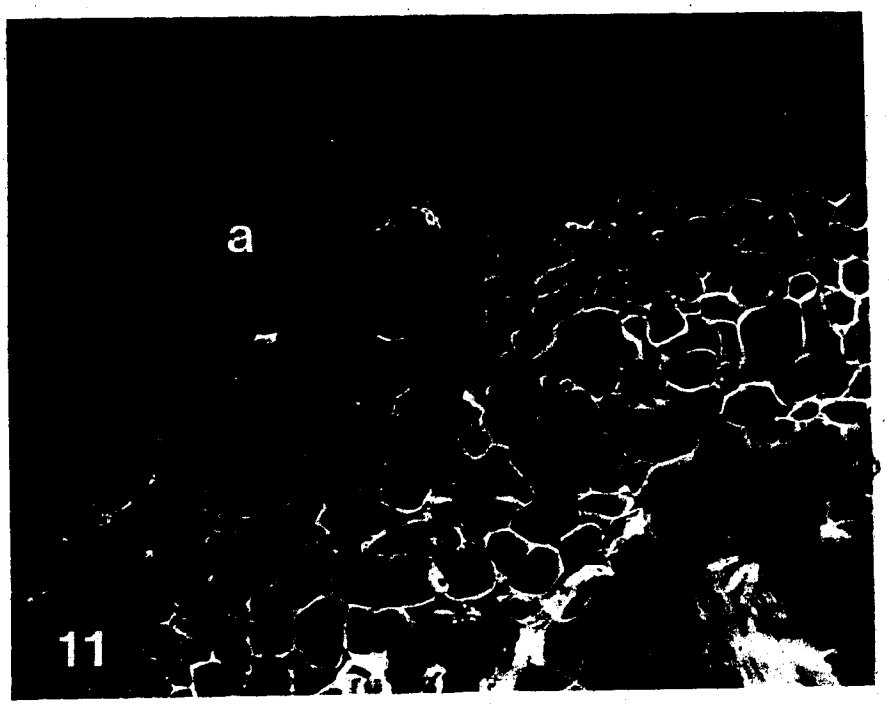

L
M
C
10

FIGURE 11

A scanning electron micrograph of a cross section through an aerial rhizomorph of Armillaria mellea grown on carrot agar medium, illustrating collapsed medullary cells (arrow) and an a, exudate droplet. (1,200x)


a
11

(1969) detailed the morphogenesis of A. mellea rhizomorphs and indicated that there was stretching and tearing of the medullary cell walls during elongation. He also suggested that there may be autolysis in the inner medulla during medullary differentiation, Fig. 11. The contents of these lysed cells may well be exuded from the rhizomorph in the form of exudate droplets.

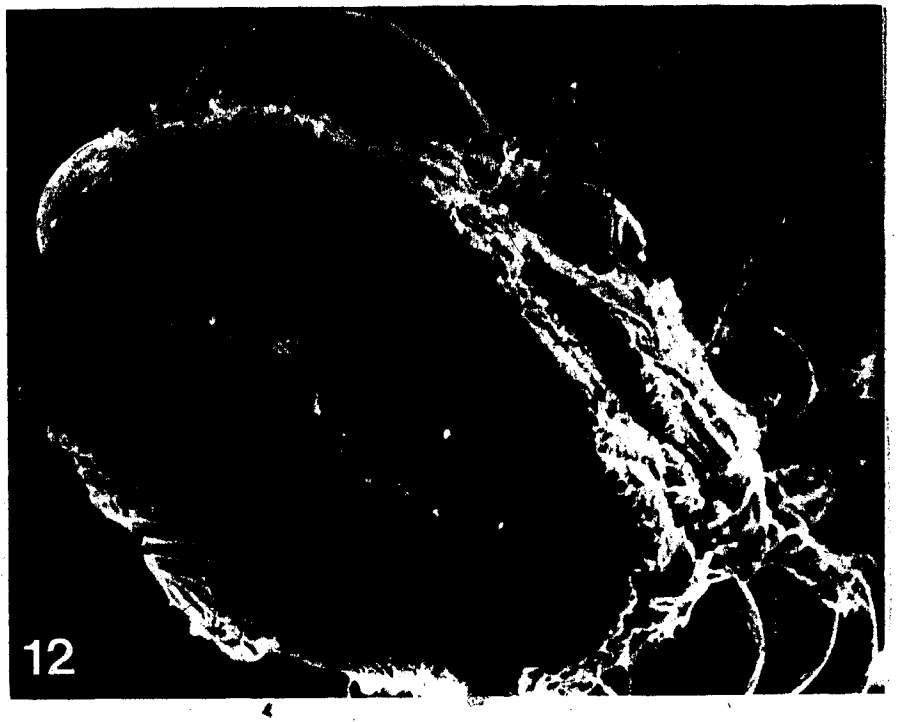
Membranous material surrounding exudate droplets has been reported by Colotelo (1971b, 1973, 1978) for Sclerotinia sclerotiorum, Phycomyces blakesleeanus, Rhizopus nigracans, Thanatephorus cucumeris, Claviceps purpurea, and Nectria inventa. Upon fixation with osmium tetroxide and examination with the scanning electron microscope, the inside of these freeze-dried droplets were shown to contain a network of membranous material (Colotelo 1973, 1978). Similarly, A. mellea exudate droplets also exhibit a membranous coat surrounding the droplet as well as the membranous material inside the freeze-dried droplets. This was evident from living cultures and from SEM micrographs, Figs. 12 and 13. The droplets on the peripheral hyphae also show sac-like membranes. Colotelo (1971b) stated that these sacs may form from the associa-

FIGURE 12

A scanning electron micrograph of a cross section through an aerial rhizomorph of Armillaria mellea grown on carrot agar medium, illustrating exudate droplets with a membranous coat on the outer surface of the rhizomorph. A cross section through an exudate droplet showing membranous micelles is indicated by the arrow. (465x)

FIGURE 13

A scanning electron micrograph of exudate droplets found on the surface of an aerial rhizomorph of Armillaria mellea grown in carrot agar medium, illustrating the membranous surface of the exudate droplets. (2100x)


12


13

tion of proteins and lipids found in the exudate. However, he could not get reformation of the membranous material after removal of the original membranous material by filtration through a 0.45 μm millipore filter. Colotelo (1973) concluded that shrinkage of droplets in Sclerotinia sclerotiorum was due to evaporation of water and reabsorption of droplet constituents.

CHAPTER III

SOME PHYSICAL AND BIOCHEMICAL CHARACTERISTICS OF RHIZOMORPHAL EXUDATE

Objective

Upon observing rhizomorphal exudation, it was noted that the exudate droplets became pigmented with time. When the droplets dried, a membranous sac could be seen. This observation would suggest that the droplets contained constituents other than water. Therefore, a series of experiments were designed to determine some of the physical and biochemical characteristics of exudate droplets.

Materials and Methods

(i) Cultures

Armillaria mellea, isolate C49, was used throughout the experiments. Carrot agar was used as the culture medium since the isolate grew quickly and produced an appreciable volume of exudate when grown on it. The preparation of the

medium and growth conditions are described in Chapter II. Inoculum plugs were cut with a number five cork-borer from 10- to 15-day-old cultures and purposely contained rhizomorphs. This was done so that rhizomorphs would be initiated immediately and thus reduce the time necessary for the production of exudate droplets. Using this method, aerial rhizomorphs with exudate droplets could be found in all regions of the Petri plate after 20 days incubation. All exudate used in the experiments was harvested from 20-day-old cultures.

(ii) Collection of Exudate

Exudate was collected with a pasteur pipette before being passed through a 0.45 μ millipore filter and into a clean test tube. The filtered exudate was kept at 5^{o}C until used.

(iii) pH Measurements

The pH of filtered exudate was determined with a Radiometer pH meter, model 22, using a combined glass-calomel electrode. For comparative purposes the pH of a ground aerial rhizomorph extract was also taken. Aerial rhizomorphs from a 20-day-old culture were ground in a mortar using acid washed sand with a minimal amount

of distilled water. The pH of the slurry was determined.

(iv) Viscosity

Viscosity measurements of filtered exudate were performed at 30°C using a Cannon-Manning 150A610 viscometer.

(v) Dry Weight

The dry weight of 0.45 μmillipore filtered exudate was determined by adding 1 ml of filtered exudate to a weighing pan of known weight and then air drying the exudate at 60°C for 16 hours in a Thelco circulating air oven. The residue and pan were subsequently weighed.

(vi) Inorganic Elements

Exudate was collected and filtered through a 0.45 μmillipore filter using acid washed glassware. Two milliliters of exudate were added to a crucible and air dried at 100°C for 16 hours. The sample was ashed in a muffle furnace at 300°C for one hour and 550°C for six hours. Ten milliliters of 2N Instra-analyzed HCl (Baker) was added to the residue. The control consisted of ten milliliters of 2N Instra-analyzed HCl (Baker). The

sample was analyzed in an Applied Research Lab Inductive Coupling argon plasma emission spectrophotometer, model 13700, with a torch temperature of 9000°K. Analysis was carried out at the Alberta Agriculture Soil and Feed Testing Laboratory by Mr. Lloyd Hodgins.

(vii) Oxalic Acid

The quantity of oxalic acid in the exudate was ascertained using the method of Bateman and Beer (1965) and by x-ray diffraction. Several collections of exudate were combined to obtain 5 ml necessary for analysis; the exudate was kept frozen until used. The sample for x-ray diffraction was prepared by adding 4 ml of $CaCl_2$ in acetate buffer to the exudate and letting it stand for 16 hours. The sample was centrifuged at 3000 x g for 10 minutes and the pellet was air dried at 60°C for several hours. The pellet was then crushed to a fine powder in a mortar before being mounted on a glass slide. A Phillips x-ray diffraction unit with a monochromatic attachment, operated at 40 kV, 10 mA, was used for analysis, located in the Department of Soil Science, University of Alberta, and performed by Dr. M. Nudas.

(viii) Total Proteins

Two methods for determining total protein were used, the method of Lowry et al. (1951) and the procedure developed by Bradford (1976). Exudate that was to be used in the Lowry test was dialyzed for 24 hours at 5°C against 0.02M phosphate pH 7. Protein complexed with copper in an alkaline solution reduces the folin reagent to produce a blue color. The absorption of this color was read on a Hitachi Perkin Elmer, model 139, spectrophotometer at 630 nm. An autoclaved sample of exudate plus substrate was used as a control. The concentration of protein was quantitatively determined using Bovine serum albumin protein standard. Exudate to be used for the Bradford method was dialyzed against 0.02M phosphate buffer pH 7 for 24 hours. In the Bradford method, Coomassie brilliant blue G-250 complexes with protein to give a blue color. The absorption of this color was determined on the above spectrophotometer at 595 nm. An autoclaved sample of exudate plus substrate was used as a control. The concentration of protein was quantitatively determined using a Bovine serum albumin protein standard.

(ix) Qualitative Determination and Separation of Exudate Protein by Disc Gel Electrophoresis

Ammonium sulphate, 0.662 g/ml, (this amount gives a saturated solution at 5°C), was added to a known volume of exudate to precipitate protein. The exudate - $(NH_4)_2SO_4$ solution was kept at 5°C over a 24 hour period and then centrifuged at 32,000 x g for 15 minutes in a Sorvall superspeed automatic refrigerated centrifuge, model RC2-B. The pellet was resuspended in deionized distilled water and dialyzed against deionized distilled water for six hours and 0.02M phosphate buffer pH 7 for 18 hours. The separation and detection of exudate proteins in the above purified sample was attempted using the disc gel electrophoresis method of Davis (1964). Separated protein band(s) in the gel were stained with 0.2% Coomassie brilliant blue G-250 in 3.5% perchloric acid (Reisner et al. 1975).

(x) Qualitative Determination and Separation of Exudate Protein and Rhizomorph Extract Protein by Isoelectricfocusing

(a) Exudate Proteins

Filtered exudate was dia-

lyzed against 0.02M phosphate buffer pH 7 for 24 hours and concentrated in a Minicon B15 concentrator to 10x original concentration. Exudate proteins were separated and detected using thin layer isoelectric focusing technique of Vesterberg (1973). A 6% polyacrylamide gel containing Servalyt pH 4-9 ampholine solution was prepared using 0.004% riboflavin as the catalyst. A Dessaga thin-layer electrophoresis apparatus, model 121221, operated at 5°C was used to separate the proteins. A strip of heavy filter paper, (1 x 10cm), soaked in a 1M NaOH was used at the cathode and another similar strip of heavy filter paper soaked in 1M phosphoric acid was used at the anode. The distance between these electrodes was 10cm. Small strips of filter paper, (1 x 4mm), were placed 1cm from the cathode electrode and 20µl of sample were applied to each. The current, 3.5mA, was applied and allowed to run for four hours or until the voltage reached 900v. Gels were stained in a solution of 0.25% Coomassie brilliant blue G-250 and 3.5% perchloric acid (Reisner et al. 1975).

(b) Rhizomorph Extracts Proteins

Brown aerial rhizomorphs,

2.2g, and submerged white rhizomorphs, 10 g, were harvested separately from 20-day-old cultures. The rhizomorphs were pressed between paper towels before being ground in a mortar with acid washed sand and 15 ml of 0.02M phosphate buffer, pH 7. The slurry from each was centrifuged at 32,000 x g for 15 minutes before decanting the supernatant. The pellet was washed and centrifuged three more times with 5ml of buffer each time. The 30ml of supernatant had ammonium sulfate added to it, 0.662 g/ml, and the protein was precipitated overnight at 5°C. The protein mixture was centrifuged for 15 minutes at 32,000 x g; the supernatant was discarded and the pellet was redissolved in 3ml of 0.02M phosphate buffer, pH 7. This solution was dialyzed against deionized distilled water for six hours and against 0.02M phosphate buffer for 18 hours to remove the $(NH_4)_2SO_4$. Total protein for each sample was determined by the Bradford method. For isoelectric focusing, the protein was then concentrated in a Minicon B15 concentrator 25x before being applied to strips of filter paper near the cathode. 80µl of white rhizomorph protein was applied to the

filter paper and 40 µl of the black-brown aerial rhizomorph protein was applied to another filter paper.

(xi) Free Amino Acids

(a) Free Amino Acids of Exudate

A 3ml aliquot of filtered exudate was air dried overnight at 60°C in a 50 ml beaker. The dried sample was extracted with 80% ethanol for 30 minutes before the alcohol soluble fraction was centrifuged at 5000 x g for 15 minutes and the pellet discarded. The alcohol was evaporated by drying the sample in a circulating air oven at 60°C. The sample residue was redissolved in 3 mls of 6N constant boiling HCl and autoclaved at 121°C for 16 hours. The free amino acids in the exudate extract and the rhizomorph extract were determined by a Durrum D500 amino acid analyzer, operated in the Department of Biochemistry, University of Alberta, by M. Natriss. Acidic and basic amino acids were separated on a 0.18 x 50cm column packed with Durrum 6A Resin and eluted with sodium citrate buffers; pH 3.25, 4.25 and 7.9 with the column temperature being 51°C and 64°C. The detection reagent was ninhydrin.

(b) Free Amino Acids of the Brown Aerial Rhizomorphs

A sample, 2.2 g, of aerial rhizomorphs was harvested from the surface of a 20-day-old culture and ground in a mortar with acid washed sand and 80% ethanol. The ground rhizomorphs were centrifuged at 10,000 x g for 15 minutes and the supernatant fraction was decanted. The pellet was washed with 80% ethanol and centrifuged twice. The supernatant from the rhizomorph extraction was then treated in a manner identical to the exudate sample.

(xii) Enzyme Assays

(a) B-Glucosidase

B-glucosidase activity was determined using the method of Cohen et al. (1952). Filtered exudate that had been dialyzed against 0.2M phosphate sodium citrate buffer pH 4.5 was incubated with 6 bromo-2-napthol b-B-D-glucopyranoside at 25°C. This resulted in the release of 6 bromo-2-napthol which forms a color complex with tetrazotized diorthoanisidine. The amount of color development was measured at 540 nm. Using a standard curve for 6 bromo-2-napthol the number of μgrams of substrate transformed by the enzyme in the exudate could be determined from the curve and the specific activity of the enzyme could thus be calculated. Autoclaved

filtered exudate plus substrate was used as a control. Specific activity is defined as the number of enzyme units per mg protein. One enzyme unit is defined as the amount of enzyme causing transformation of 1.0 microgram of 6 bromo-2-napthol released per minute at 25°C.

(b) Catalase

A preliminary test for catalase activity was done by mixing filtered exudate with dilute 30% hydrogen peroxide in a test tube and examining it for evolution of oxygen bubbles.

(c) Peroxidase

The peroxidase activity in filtered exudate was determined by the procedure described in Enzymes, Enzyme Reagent (Worthington Biochemical Corporation, Freehold, New Jersey). The exudate was dialyzed against 0.01M phosphate buffer, pH 6, for 24 hours. O-dianisidine was used as the hydrogen donor during the decomposition of hydrogen peroxide. The rate of color development at 25°C was followed at 460 nm. Autoclaved filtered exudate plus substrate was used as a control. Specific activity of the enzyme was determined using the following equation:

$$\frac{\text{change in color development at 460nm/min}}{11.3 \times \text{mg protein/ml reaction mixture}}$$

(d) Polyphenoloxidase

Polyphenoloxidase activity was determined by the method of Hall et al. (1969). Filtered exudate was dialyzed against 0.1M citrate phosphate buffer, pH 4.5. Ferricyanide is released in the presence of catechol when the enzyme was present and an increase in absorbance at 420 nm was measured at 25°C. Autoclaved filtered exudate plus substrate was used as a control. Polyphenoloxidase activity in the exudate was calculated by multiplying the increase in absorbance per minute by 1.45.

(e) Proteases

Acid and alkaline protease activities were determined by the method described by Nagainis (1976). Filtered exudate was divided into equal volumes. Exudate for acid protease was dialyzed against 0.1M citrate buffer, pH 4.5, for 24 hours. Exudate for alkaline protease was dialyzed against 0.1M phosphate buffer, pH 8.0. Bovine serum albumin (fraction v), the substrate, was incubated with either acid protease exudate or alkaline protease exudate dialysates respectively at 25°C. 2,4,6-trinitrobenzene sulfonic acid was

reacted with the released amino acids, the color change was measured at 420 nm. Acid and alkaline protease activities in the exudate were calculated by dividing the change in absorbance at 420 nm by mg/ml total protein.

Results

All experiments were repeated more than once.

(i) Volume of Exudate per Petri plate

The average amount of exudate collected per Petri plate from carrot agar medium was 0.17 ml.

(ii) pH of Exudate and Rhizomorph Extract

The pH of filtered exudate was 7.43, whereas the pH for an aqueous extract of rhizomorphs was 7.64.

(iii) Dry Weight of Filtered Exudate

The dry weight of filtered exudate was 17.25 mg/ml.

(iv) Viscosity of Filtered Exudate

The viscosity of filtered exudate at 30^{o}C was 0.89443 centisokes; 8% greater than the viscosity of deionized distilled water.

(v) Inorganic Elements

The difference between the sample and control values can be seen in Table I.

Table I. Inductively Coupled Plasma Emission Spectroscopic Analysis for some Inorganic Elements in Filtered *Armillaria mellea* Rhizomorphal Exudate in 2N HCl

Element	Concentration (in ppm)
Potassium	1548.14
Sodium	552.10
Phosphorus	535.15
Zinc	43.68
Magnesium	21.66
Boron	7.99
Calcium	4.51
Aluminum	3.22
Iron	0.52
Molybdenum	0.07
Cadmium	0.07
Chromium	0.06
Manganese	0.06
Strontium	0.05
Cobalt	0.05
Arsenic	--
Lead	--
Copper	--
Vanadium	--

1 These values are the average of three separate collections, (two determinations were done per sample), and represent the difference between the sample and the control.

(vi) Oxalic Acid

No oxalic acid was found in the filtered exudate by the method of Bateman and Beer (1967) nor by the x-ray diffraction technique.

(vii) Total Protein

The results of the total protein experiments are given in Table II. The same exudate sample was used for both Lowry and Bradford methods in order to compare and evaluate the two methods. The Bradford method was replicated several more times, the results of this can be seen in Table III. This was done because as Table II indicates, the Bradford method gave the lesser value. The average value for total protein by the Bradford procedure was determined to be 0.31 mg/ml.

(viii) Qualitative Determination and Separation of Exudate Protein by Disc Gel Electrophoresis

A qualitative determination and separation of exudate protein by disc gel electrophoresis was not achieved. Fig. 14 shows a single band of protein at the anode end of the gel. Some of the sample remained at the point of application.

Table II. A Comparison of the Lowry Method vs the Bradford Method for Determining Total Protein of Armillaria mellea Rhizomorphal Exudate

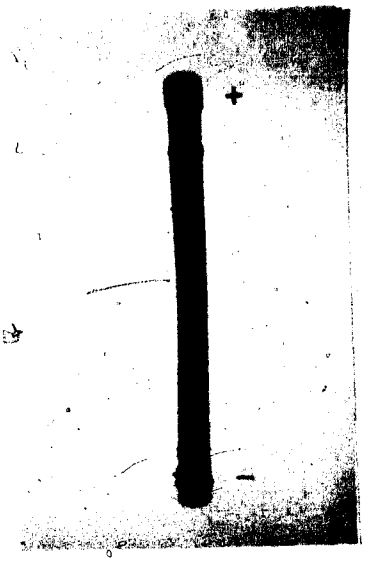
Sample	Total Protein	
	Lowry Method (mg/ml)	Bradford Method (mg/ml)
1	1.55	0.30
2	0.90	0.20
3	2.60	0.36

Table III. Total Protein of Armillaria mellea Rhizomorphal Protein as Determined by the Bradford Method

Replicate	Total Protein (mg/ml)
1	0.30
2	0.20
3	0.36
4	0.20
5	0.27
6	0.34
7	0.33
8	0.38
9	0.38
10	0.38
	$\bar{x}$=0.31

FIGURE 14

A disc gel electrophoresis gel showing a single band of protein from Armillaria mellea exudate at the anode end.



(ix) Qualitative Determination and Separation of Exudate Protein and Black-Brown Rhizomorph and White Rhizomorph Extracted Proteins

Separation of exudate protein and proteins extracted from black aerial rhizomorphs and submerged white rhizomorphs was achieved by the isoelectric focusing technique, Fig. 15.

(x) Free Amino Acids found in Rhizomorphal Exudate and Rhizomorph Extract

The free amino acids found in rhizomorphal exudate and rhizomorph extract are given in Table IV.

(xi) Enzyme Assays

The activities of B-glucosidase, peroxidase, polyphenoloxidase acid and alkaline protease are given in Table V. No catalase activity was found in the pretest.

FIGURE 15a

Protein bands of Armillaria mellea exudate separated by the isoelectric focusing technique.

FIGURE 15b

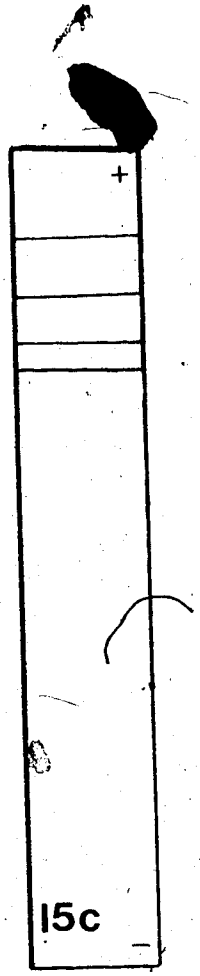
Protein bands of Armillaria mellea brown aerial rhizomorph extract separated by the isoelectric focusing technique.

FIGURE 15c

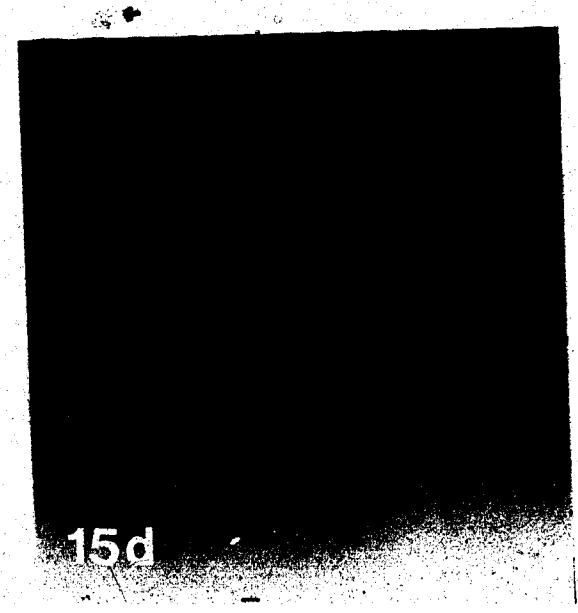
Protein bands of Armillaria mellea submerged white rhizomorphs extract separated by the isoelectric focusing technique.

FIGURE 15d

A sample of Armillaria mellea exudate protein applied near the anode showing migration towards the anode. A control protein also applied at the anode migrated to it's isoelectric point.



15c



15d

Table IV. The Free Amino Acids Found in Rhizomorphal Exudate and in a Brown Rhizomorph Extract of Armillaria mellea

Amino Acid	Exudate (μmoles/ml)		Extract (μmoles/g)	
	Sample 1	Sample 2	Sample 1	Sample 2
Glutamic Acid	1.950	2.100	32.210	38.050
Aspartic Acid	0.863	0.950	18.050	19.840
Alanine	0.497	0.764	6.579	7.789
Serine	0.419	0.411	6.263	6.316
Threonine	0.263	0.278	4.053	5.053
Glycine	0.245	0.260	4.053	3.947
Arginine	0.098	0.083	2.105	1.474
Valine	0.085	0.093	1.105	1.368
Histidine	0.053	0.047	2.210	2.000
Lysine	0.041	0.035	0.821	0.619
Phenylalanine	0.030	0.033	--	--
Isoleucine	--	--	6.000	6.616
Leucine	--	--	4.711	4.430
Proline	--	--	1.315	1.097

Each sample is the average of two runs.

Table V. Specific Activities for B-Glucosidase, Catalase, Peroxidase, Polyphenoloxidase, Acid and Alkaline Protease of Rhizomorphal Exudate from Armillaria mellea.

Enzyme	Specific Activity[2] (units/mg ml^{-1} protein)
B-Glucosidase	88.10
Catalase	--
Peroxidase	1.4×10^{-2}
Polyphenoloxidase	1.6
Acid Protease	7.33×10^{-1}
Alkaline Protease	8.17×10^{-1}

2 Average of 3 experiments

Discussion

The results show that A. mellea rhizomorphal exudate is a complex liquid made up of a variety of substances. From the experiments performed, the true function of exudation is unknown and therefore the discussion of it's purpose is speculative.

The pH of the exudate indicates that it is a weak base but slightly more acidic than the rhizomorph extract. The pH of Sclerotinia sclerotiorum six-day-old sclerotial exudate and sclerotial extract was determined by Colotelo et al. (1971) to be 5.4 and 6.3 respectively. Christias (1980) found sclerotial exudate of Sclerotium rolfsii to have a pH of 4.5. These results would at first suggest that fungal exudates can vary widely with respect to pH between genera. Colotelo (1973) found that the pH of exudate taken from sclerotia of differing ages changes with the age of the sclerotia and becomes more alkaline. This may be caused by the presence of more oxalic acid in young exudate than in old exudate. The high pH in isolate C49's exudate may be due to the lack of oxalic acid. A. mellea exudate was collected from rhizomorphs of differing temporal and physio-

logical age. Moreover, exudate droplets on a single rhizomorph can be of a different temporal and physiological age and so the exudate collected in this study is a composite of exudate droplets that vary in physiological age and therefore physiological characteristics. It is also of interest to note that the pH of the rhizomorphal exudate is very similar to the pH of the rhizomorphal extract which one might expect if the exudate is the result of lysed medullary cells. The viscosity of filtered exudate at 30°C is 0.89443 centistokes, this is 8% greater than the viscosity of deionized distilled water. Colotelo et al. (1971b), found the viscosity for six-day-old sclerotial exudate from S. sclerotiorum to be 0.90016 centistokes, 9% less than that of water, this Colotelo thought to be due to lipid. The greater viscosity for A. mellea rhizomorphal exudate is probably due to the nature of the solute in the exudate.

The ICP results indicate that rhizomorphal exudate contains many inorganic elements, some of which are found in large quantities. These inorganic elements make up 12% of the exudate's dry weight. Large quantities of potassium, sodium, phosphorus, zinc, magnesium and boron were present.

Small quantities of calcium, aluminum and iron as well as trace quantities of chromium, cadmium, manganese, strontium and cobalt were also present. According to Lilly (1965) the essential elements for all fungi include hydrogen, carbon, nitrogen, phosphorus, oxygen, sulphur, potassium, magnesium, iron, zinc, manganese, copper and molybdenum. Some fungi may require calcium. Colotelo et al. (1971b), analyzed exudate from six-day-old cultures of S. sclerotiorum for sodium, potassium, magnesium, iron and calcium and found large quantities of potassium, sodium and magnesium, but did not detect iron or calcium. It would therefore appear that exudate from A. mellea and from S. sclerotiorum contain some similar elements.

Potassium was the element found in the greatest concentration in the exudate. According to Lilly (1965), of all the metals found in spores of mycelia, potassium usually is found in the greatest quantity. In plants, potassium is involved in enzyme activation and acts as a charge-balancing cation during transport of anions (Salisbury and Ross 1978). Sodium was also found

in considerable quantity in the exudate, however, it's role is unknown since it is not considered to be an essential element in fungal or plant nutrition. Phosphorus is involved in many different components of the cell including phospholipid membranes, energy exchange, and nucleic acids. It is difficult to ascribe a function to the phosphorus found in exudate since the form that it was found in is unknown. However, since exudate is associated with actively growing tissue that has high metabolic activity, it is possible that the phosphorus is found in high energy compounds. Zinc is considered to be an essential microelement of fungi necessary for activation and function of some enzymes (Lilly 1965). The presence of zinc in the exudate may well be explained by the finding that enzymes were also found in the liquid.

Magnesium is an essential element necessary for respiratory enzyme activity and is often found chelated to ATP or ADP. The presence of this element can also be accounted for by the presence of enzymes in the exudate.

Boron is not considered to be an essential element for fungi and it's presence in the exudate may be due to a high concentration of

boron in the carrot agar medium which the fungus was using as a food source. Iron is essential for fungal growth and is a constituent of several enzymes such as the cytochromes. Again the presence of this element in the exudate may be explained by exudate enzymes. The elements found in trace quantities such as strontium and manganese may have possible roles but none have been described for other fungi (Lilly 1965). Chromium, cadmium, vanadium, strontium, calcium and aluminum do not have essential roles in fungi and are probably found in the exudate due to their presence in the carrot agar medium. The presence of these elements in the exudate is circumstantial evidence to support the hypothesis that exudate is the result of lysed medullary cells since some of these elements have essential roles within the cell. However, it is also possible that these elements could result from excretion or are lost from the cell as the young or newly-formed cell wall material polymerizes. An important effect of these elements that are found in the exudate is that they could act as a nutrient source for microflora surrounding the rhizomorph in soil.

No oxalic acid was found in the exudate of isolate C49, however, other isolates of A. mellea have been noted to produce oxalic acid in culture (Hamada 1939). The reason for the oxalic acid determinations is that oxalic acid is thought to play an important role in pathogenesis of such fungi as S. sclerotiorum (Debary 1887, Maxwell and Lumsden 1970), Botrytis cinnerea (Smith 1902), Penicillium (Johann et al. 1931), Aspergillis niger (Gibson 1953), and S. rolfsii (Bateman and Beer 1965). However, Foster (1949), thought oxalic acid to be a common metabolite of many fungi. Colotelo (1978) found oxalic acid in the exudate of Claviceps purpurea, Fusarium culmorum, Penicillium notatum, S. sclerotiorum and S. rolfsii.

Total protein in the exudate was determined by two methods for comparative purposes. The results in Table II indicate that the Lowry method gave a greater value for total protein than did the Bradford method. There are some faults in the Lowry method which can give misleading results. Garhardt and Beaver (1968) showed that the amount of sucrose in a sample to be tested can

influence the Lowry method, that is, in samples low in protein, sucrose will give high absorbance readings greater than the true value. Solecka et al. (1968), demonstrated that there are TCA precipitable substances in plant leaf tissue protein that can give misleading results when using the Lowry method. The Bradford method gave a much lower total protein value compared to the Lowry method and so it was used as the method for determining total protein throughout the entire study. The results indicate that the total protein made up 1.82% of the total dry weight of one milliliter of exudate. Colotelo et al. (1971) found the total protein from S. sclerotiorum six-day-old culture sclerotial exudate to be 1.0 mg/ml and that the total protein in sclerotial exudate decreases with age of the culture (Colotelo 1973). This decrease can be dramatic, as exemplified by a decrease by one half of the initial concentration in a day. McPhee (1978) found that there was an increase in the protein content of mycelial exudate from F. culmorum from 24 hours (5.3mg/ml) after the start of exudation, to a maximum at 60 hours (8.7mg/ml) and

then a decrease after 90 hours (6.3 mg/ml). Christias (1980) determined the total protein of S. rolfsii to be 0.498 mg/ml.

When A. mellea exudate proteins were subjected to disc gel electrophoresis, a single brown band formed at the anode end of the gel. However, when the exudate protein was subjected to isoelectric focusing, many brown bands separated out in the gel. A. mellea exudate protein was then applied to the anode end of the gel and gave the result seen in Fig. 15. No protein bands separated out into the gel, as they did in the control, but a brown band was observed at the anode. These results are very similar to what Colotelo (personal communication) found for exudate protein from the sclerotia of Thanatephorus cucumeris. The results would suggest that the proteins were associated with melanin-like substances which can prevent the proteins from migrating to and precipitating at their isoelectric point. In Fig. 15, it can be seen that exudate protein from dark brown aerial rhizomorph extract show similar brown bands in the gel. Submerged white rhizomorph extract protein did not form brown bands in the gel but had to be

dyed with Coomassie blue before the bands could be seen. These bands occurred in similar locations as those from the aerial rhizomorph extract and suggest that these could be the same proteins. Exudate and rhizomorph extracts contain numerous proteins and it would seem that those proteins bound to melanin-like substances can be separated by isoelectric focusing but not by disc gel electrophoresis. Colotelo (1978) showed that proteins in the exudate of a number of fungi can be separated into bands by using disc gel electrophoresis but most of these fungi lack melanin-like substances in their exudate. Exudate from Thanatephorus cucumeris sclerotia, which has melano proteins, also does not separate into bands during disc gel electrophoresis (Colotelo, personal communication). Smith and Griffin (1971) claimed that the pigment extracted from rhizomorphs of A. elegans was not melanin, but melanin-like. Part of their evidence for this was based on their finding that brown pigment formation was catalyzed by p-diphenol oxidase and not by o-diphenol oxidase which is responsible for melanin formation. Rhizomorphs turn brown in color upon exposure to oxygen (Smith and Griffin 1971). There must also

be other causes for rhizomorph browning as the submerged rhizomorphs in the synthetic agar medium also turn brown. Smith and Griffin (1971) found that the extracted brown pigment did not precipitate gelatin. From the results of these experiments, it would appear that the melanin-like pigment ties up protein. A possible function for melanin-like compounds in the rind of the rhizomorph may be similar to that described by Bloomfield and Alexander (1967) for the melanins in the rind of the sclerotia of S. rolfsii. They found that the melanins may serve a protective function, preventing lysis of the fungus by soil microorganisms. This function could be very important to A. mellea rhizomorphs and may account for their longevity in the soil.

Smith and Griffin (1971) have made the observation that the intercellular spaces between rind cells in brown rhizomorphs are filled with melanin-like pigment, but in the white rhizomorphs, intercellular spaces of the rind cells are not. This observation may be explained if one considers the exudate. If the medullary cells are collapsed, then exudate may represent the contents of these

cells that have leaked out of the rhizomorph through the rind cell layer. Since exudate is initially clear but later turns brown like the rind layer, it could be argued that the brown material in the intercellular space between rind cells is exudate. The intercellular spaces between rind cells of white rhizomorphs may also contain non-pigmented exudate.

Eleven free amino acids were detected in the exudate compared to thirteen free amino acids found in the rhizomorphal extract. Cysteine and methionine may well have been present but were not tested for. Glutamine and asparagine were probably detected as glutamic acid and aspartic acid because of hydrolysis. This may be a possible explanation of why glutamic acid and aspartic acid are found in such great quantities. Glutamic and aspartic acids are found in the greatest quantities if the free amino acids from the exudate and the rhizomorphal extract are compared. Relative amounts are the same in both except histidine in the rhizomorphal extract which is found in a greater quantity than valine. No isoleucine, leucine, nor proline were found in the exudate

but were found in the rhizomorph extract. No phenylalanine was found in the rhizomorph extract but was found in the exudate. Colotelo et al. (1971b) found that S. sclerotiorum sclerotial exudate and sclerotial extract from six-day-old cultures contained 17 free amino acids. The only free amino acids that they found that were not found in A. mellea exudate or rhizomorph extract were tyrosine, cysteine and methionine. Chet et al. (1967) did not detect phenylalanine nor tyrosine in hydrolyzates of S. rolfsii sclerotial and hyphal walls. However, Christias (1980), found 14 free amino acids including phenylalanine and tyrosine, in trace quantities, in exudate of S. rolfsii. These free amino acids were identical to those found in A. mellea exudate except that tyrosine was found in trace quantities, leucine and isoleucine were also found. Colotelo et al. (1971b) found it somewhat surprising that Chet et al. (1967) found no tyrosine nor phenylalanine in S. rolfsii sclerotial and hyphal walls, since these amino acids are common constituents of proteins and are also considered to be involved in the formation of melanins. The same is true for

A. mellea rhizomorphal extract. Phenylalanine but not tyrosine was found in the exudate of A. mellea, however, Christias (1980) found small quantities of phenylalanine and tyrosine in the exudate of S. rolfsii. It could be suggested that the extraction methods used on the sclerotia of S. rolfsii and the rhizomorphs of A. mellea did not withdraw these two amino acids from the tissue in a detectable quantity. As with dry weight and total protein, Colotelo (1973) has shown that the concentration of amino acids in S. sclerotiorum sclerotial exudate decreased with the age of the culture. Since the exudate droplets and the rhizomorphs from which they were collected differed in physiological and temporal age, it is most likely that these results presented represent a composite of the free amino acids that are present in exudate and rhizomorphs in a 20 day period of growth. Morrison (1975) extracted 11 free amino acids from A. mellea rhizomorphal apices. His results show the same amino acids to be present as those found in the

extracted rhizomorphs of the experiment except that he did not detect arginine, phenylalanine, proline, threonine, nor serine but did detect tyrosine and methionine.

B-glucosidase activity has been demonstrated in exudate from several fung; S. sclerotiorum (Colotelo et al. 1971), Fusarium culmorum (McPhee 1978), Claviceps purpurea, Nectria inventa, Penicillium flavium, Penicillium notatum, Sclerotium rolfsii and Thanatephorus cucumeris (Colotelo 1978). A. mellea rhizomorphal exudate also posseses B-glucosidase activity. B-glucosidase is a hydrolytic enzyme that may be involved in the autolysis process (McPhee 1978). When cellulose is hydrolyzed, cellobiose is formed, this according to Cochrane (1958) is a substrate for B-glucosidase. It would therefore appear that B-glucosidase is a common constituent of fungal exudates and that it's association with autolysis is particularly interesting if the hypothesis that exudate is a result of medullary cell autolysis is considered. It is also important to note the effect that B-glucosidase in the exudate might have in pathogenesis.

Colotelo (1978) found that the exudate

from F. culmorum, N. inventa, Penicillium claviforme, S. sclerotiorum, and S. rolfsi [illegible] contain alkaline protease activity. S. r[illegible] and S. sclerotiorum exudate were also found to have acid protease activity. A. mellea exudate has both acid and alkaline protease activity, although a greater activity was found for alkaline protease which might be indicative of the pH of the exudate, slightly alkaline. Protease like B-glucosidase is a hydrolytic enzyme.

Peroxidase and polyphenoloxidase activities were found in A. mellea exudate. Lanphere (1934) found peroxidase and catalase activity in A. mellea rhizomorph extract. Smith and Griffen (1971) did not find peroxidase activity in rhizomorphal extract of A. elegans, but did find p-diphenol oxidase activity. This enzyme, they concluded, was responsible for the browning reaction in the rhizomorph. O- and p-diphenol oxidases have been found in the [illegible] of A. mellea (Boiden 1951, [illegible] 1965, Jacque-Felix 1968). McPhee (1978) [illegible] peroxidase and polyphenoloxidase activity in e[illegible]te from F. culmorum. Colotelo (1973) found that peroxidase activity in sclerotial exudate from cultures of S. sclerotiorum to increase

between the fifth and the sixth day and then to remain the same thereafter. Polyphenoloxidase activity increased with age.

It would thus appear that these enzymes are common in many fungal exudates. Colotelo (1978) pointed out that the enzymes B-glucosidase, protease, polyphenoloxidase and peroxidase are characteristic of lysosomal enzymes.

CHAPTER IV

GENERAL DISCUSSION

From this study and from the work of Colotelo et al. (1971b), Colotelo (1973), Colotelo (1978), McPhee (1978) and Christias (1980), it would seem that exudation is a common phenomena in many fungi and is particularly associated with rapidly growing fungal structures. From the work cited and from the experiments carried out, fungal exudate has been shown to be remarkably complex. Many of the exudate characteristics such as free amino acids, some of the inorganic elements, total protein, isoelectric focusing, enzyme content and enzyme activities of different fungi show striking similarities. Colotelo (1973) has demonstrated that exudation is a dynamic event and that exudate constituent concentrations vary with time independently of volume changes in the liquid.

If one were to speculate on the purpose of the exudate of A. mellea, it could conceivably

have several different roles. Smith and Griffin (1971) have demonstrated that the medullary lumen which is formed in rhizomorphs of A. elegans are used to supply oxygen to the growing tip of the rhizomorph. In order for this lumen to form, medullary cells have to collapse; this has been shown by Motta (1969). If these cells collapse, the cause of which could be autolysis or simple pressure from the surrounding cells, the cellular contents would then be freed and it's fate could take several courses. One might be that the material is simply absorbed by surrounding cells. A second fate may be that it is exuded out through the intercellular spaces of the rind layer of cells to the outside of the rhizomorph. The former explanation may account for some of the material but could not handle the full volume of material that would be produced. The data collected in the experiments would tend to support, but does not prove, the latter fate. The pH of the exudate and the rhizomorph are very similar, relative amounts of amino acids and isoelectric focusing patterns are also similar. The presence of enzymes that are characteristic of lysosomal enzymes would also tend

to support this fate.

The exudate would then be a response to growth allowing the growing apex to derive oxygen but also performing several other functions. As the exudate moved through the intercellular spaces some of it would be left behind in the intercellular space. When in contact with oxygen, melanin-like compounds form. This melaninization may have, as noted before, a protective function preventing the rhizomorph from being lysed by soil microflora. Likewise, the inorganic elements and the free amino acids found in the exudate could influence the microflora that surrounds the rhizomorph as roots influence the rhizosphere. Another function which the exudate could play an important role in is pathogenesis. Colotelo et al. (1971), Colotelo (1973),(1978), suggested that this may possibly be a role for exudate of Sclerotinia sclerotiorum and of other fungi. McPhee (1978) demonstrated that exudate from the mycelia of Fusarium culmorum caused plant tissue degradation. Thomas (1934) thought that penetration of host tissue by A. mellea rhizomorphs to be mechanical, but was chemically influenced as he noted that tissue ahead and sur-

rounding the rhizomorph was degraded. Exudate from the rhizomorph should be suspected as being the cause of such degradation. Morrison (1975) showed evidence that rhizomorphs growing through soil are able to absorb and utilize nutrients. Rhizomorphs growing in peat soils had greater dry weights than those not growing in peat soils, Morrison (1972). Since exudate contains hydrolytic enzymes, it might play an important role in breaking down plant material surrounding the rhizomorph which the rhizomorph could use for continued growth.

Summary

Exudation from rhizomorphs of Armillaria mellea is a common phenomena and has been noted by several investigators (Hamada 1940)(Raabe 1967). There is more exudate produced on carrot agar medium grown rhizomorphs than on synthetic agar medium grown rhizomorphs, possibly because synthetic agar is a minimal medium and that carrot agar is nutritionally rich.

Light and scanning electron microscopy revealed that exudate droplets are associated with rapidly growing tissue, that exud[illegible]rkens with

age and that upon freeze-drying of the droplets, membranous material is left. Exudate droplets on peripheral hyphae were observed and did not appear to be related to exudate droplets exuded from the rhizomorph.

Biochemically, the exudate from carrot agar medium grown rhizomorphs is complex. The pH, viscosity, dry weight and oxalic acid content of the exudate were determined. Inorganic elements such as potassium, sodium and phosphorus were found in large quantities as well as trace quantities of other elements. Total protein of the exudate was determined quantitatively by the Bradford method. Qualitatively, proteins in the exudate were separated by the isoelectricfocusing technique. Melanin-like compounds were found to be associated with the proteins. Disc gel electrophoresis separation of the exudate proteins was not successful, a single band was found at the anode end of the gel.

Eleven free amino acids were found in the exudate whereas thirteen free amino acids were found in the rhizomorph extract. B-glucosidase, peroxidase, polyphenoloxidase, acid and alkaline protease activites were found in the exudate.

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