

OXYGEN AND THE ECOLOGY OF *ARMILLARIELLA ELEGANS* HEIM

By A. M. SMITH*† and D. M. GRIFFIN*‡

[Manuscript received August 18, 1970]

Abstract

Oxygen is important in the ecology of *A. elegans* because it affects both the rate of growth and form of rhizomorphs. Maximum growth is dependent on high rates of oxygen diffusion within the central canals of rhizomorphs but partial pressures of oxygen in excess of 0.04 atm in contact with the outside surfaces of rhizomorphs are inhibitory.

In long rhizomorphs, the rate of diffusion is maintained because of repeated connections that are made between the central canals and substrate-air interfaces. In the absence of such interfaces, rhizomorphs grow for about 20 cm when the rate of diffusion of oxygen to the apices becomes limiting. Then the rhizomorphs flatten and become plaque-like and their growth slows. Such rhizomorphs are well adapted for absorbing nutrients from a host but not for spreading from host to host.

Partial pressures of oxygen in excess of about 0.04 atm in contact with the outsides of rhizomorphs stimulate the activity of *p*-diphenol oxidase. This enzyme catalyses the formation of a brown pigment in the rhizomorphs and circumstantial evidence indicates that when this pigment forms in the apices of rhizomorphs it inhibits their growth. This pigment overlays the walls of the cells and probably prevents either the uptake of nutrients or the disposal of waste products by the cells.

In soil, oxygen partial pressures are usually above 0.04 atm but rhizomorphs continue to grow if water films form around their apices. Oxygen diffuses slowly in these films and the rhizomorphs reduce the partial pressure of oxygen at their surface to below 0.04 atm. The formation of such water films depends on the moisture content, texture, and bulk density of the soil. By altering these properties and preventing the pathogen spreading from host to host, the disease caused by *A. elegans* may be controlled.

Carbon dioxide had little effect on the initiation and growth of rhizomorphs except that partial pressures between about 0.01 and 0.07 atm were slightly stimulatory. Because this is the range of partial pressures normally found in soil and hosts, carbon dioxide is unlikely to significantly affect *A. elegans*.

I. GENERAL INTRODUCTION

Armillariella elegans Heim and the closely related species *A. mellea* (Vahl in Fl. Dan. ex Fr.) Karst. and *A. tabescens* (Scop. ex Fr.) Sing. are important soil-borne plant pathogens with a wide host range especially among woody perennials (Rhoads 1956; Raabe 1962; Dadant 1963). Both the generic and specific status of these fungi are confused. Singer (1962) placed these fungi in the genus *Armillariella* (Karst.)

* Department of Agricultural Botany, University of Sydney, Sydney, N.S.W. 2006.

† Present address: Biological and Chemical Research Institute, New South Wales Department of Agriculture, Private Mail Bag No. 10, Rydalmere, N.S.W. 2116.

‡ Present address: Burgmann College, P.O. Box 1345, Canberra City, A.C.T. 2601.

Karst. and, in the present study, this classification is followed. Morquer, Lacoste, and Blaha (1967) consider *A. mellea* as the only species of *Armillariella* with *A. elegans* and *A. tabescens* merely as biological races. Heim (1963, 1967), however, considers the species to be distinct and claims that *A. elegans* is the typical pathogenic *Armillariella* sp. of tropical regions with *A. mellea* restricted to temperate regions.

The influence of oxygen on soil organisms has been discussed recently (Griffin 1968; Stolzy and Van Gundy 1968). From these reviews it is clear that oxygen can be a limiting factor for growth of fungi either generally in a soil mass or more commonly within scattered, localized sites. Carbon dioxide also may affect fungi but its importance in soil is unresolved (Griffin 1969). Because of its great solubility and reaction to form ions, partial pressures of carbon dioxide either in soil or at the surface of respiring organs in soil rarely exceed 0.02 atm (D. J. Greenwood, personal communication). The growth of most fungi is not restricted by carbon dioxide partial pressures less than 0.10 atm and thus this gas is unlikely to limit the activity of fungi in soil (Griffin 1963a; Stolzy and Van Gundy 1968; Tabak and Cooke 1968). Indeed, slight increases in the partial pressure of carbon dioxide may even be stimulatory (Thacker and Good 1952; Gundersen 1961).

In the present study, emphasis was placed on determining the role of oxygen in the ecology of *A. elegans*. Carbon dioxide was also studied even though it is unlikely to be limiting except in woody host plants where high partial pressures may occur (Chase 1934; Thacker and Good 1952; Gundersen 1961). Rhizomorphs have an essential function in the life cycle of *A. elegans* (Garrett 1951, 1960; Jacques-Felix 1967) and the roles of both oxygen and carbon dioxide in their development were examined.

II. QUALITATIVE STUDY OF THE EFFECT OF OXYGEN ON THE INITIATION AND GROWTH OF RHIZOMORPHS

(a) Introduction

Evidence establishing the role of oxygen in the initiation and growth of rhizomorphs of *Armillariella* spp. is confused. Reitsma (1932) and Snider (1957, 1959) claimed that partial pressures of oxygen approaching 0.21 atm were required for the initiation of rhizomorphs of *A. mellea*. However, these studies were made on submerged mycelium and no attempts were made to determine whether submerging affects initiation of rhizomorphs independently of the partial pressure of oxygen. Jacques-Felix (1968) found that rhizomorphs did not form if *Armillariella* spp. were grown in closed tubes containing only a limited quantity of oxygen or in evacuated tubes free of oxygen. Under these conditions, increased levels of carbon dioxide and direct effects of reductions in atmospheric pressure may have inhibited the initiation of rhizomorphs independently of the effects of oxygen.

Evidence is available that oxygen diffuses through the central canals of rhizomorphs to their growing apices enabling *Armillariella* spp. to grow into oxygen-deficient situations (Reitsma 1932). Also, rhizomorphs may be able to establish new, direct connections between their central canals and the atmosphere whenever they encounter a substrate-air interface (Snider 1959) and this may explain why rhizomorphs longer than 30 ft occur (Ellis 1929; Findlay 1951).

Snider (1959) suggested that a requirement for a high concentration of oxygen for the initiation of rhizomorphs would be an adaptive mechanism because it would ensure that the oxygen concentration in the central canals of resulting rhizomorphs would be sufficient to maintain their growth. If this hypothesis is correct, it may explain, at least in part, why *Armillariella* spp. cause disease in specific areas or specific seasons or both. Furthermore, in problem areas it may be possible to prevent the initiation of rhizomorphs by controlling oxygen levels in the soil and so limit the disease.

Experiments were designed to confirm qualitatively that oxygen is involved in the initiation and growth of rhizomorphs of *A. elegans*. The ability of rhizomorphs to establish direct connections between the atmosphere and their central canals at substrate-air interfaces was also studied.

(b) *Materials and Methods*

The isolate of *A. elegans* used in these studies was obtained from the stipe of a sporophore which developed on the base of a diseased tree (*Dizygotheca* sp.) growing in the Royal Botanic Gardens, Sydney. Isolations were made from a sporophore stipe to ensure that the genotype of the cultures was identical with that of the wild type. The sporophores were identified as *A. elegans* Heim by Dr. D. Reid, Royal Botanic Gardens, Kew, England. Specimens are lodged in the herbarium of the New South Wales Department of Agriculture, Rydalmere (DAR 16415). Because *Armillariella* spp. are extremely variable in culture (Gibson 1961; Raabe 1966, 1967) most experiments were repeated using a second isolate of *A. elegans* obtained from diseased geraniums (*Pelargonium × hortorum* Bailey). The two isolates, however, behaved so similarly (Smith 1969) that only the results obtained with the isolate from *Dizygotheca* are reported.

Stock cultures of *A. elegans* were maintained on malt Vegemite agar (MVA) (50 g malt extract, 5 g Vegemite, 20 g agar per litre). Inoculum was grown in Petri dishes on distilled water agar (20 g agar per litre distilled water) and, under these conditions, mycelium free from rhizomorphs and microsclerotia was produced (Garrett 1953). Uniform disks of inoculum, cut from the edge of colonies 3–4 weeks old with a No. 1 cork-borer (4 mm diam.), were used throughout these studies.

Several hardwood fence posts, naturally colonized by *A. elegans*, were obtained during the course of these studies. In one experiment, wooden blocks cut from these posts were used as a food base for the production of rhizomorphs. *A. elegans* was identified from sporophores which developed on these posts. Rhizomorphs and xylostroma were present on the underground parts of the posts and mycelial sheets were found in the wood.

(c) *Experimental*

(i) *Growth of Still, Submerged Colonies*

Test tubes containing 15 ml of either potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 20 g agar per litre) or MVA were inoculated with *A. elegans*. Oxygen diffusion barriers were obtained by immediately covering the inoculum with 0.5-, 1.0-, or 2.0-cm layers of either sterilized distilled water, sterilized paraffin oil, or sterilized soft agar (MVA containing 0.5% agar). The liquids were placed on a mechanical shaker for 12 hr before use to equilibrate the partial pressure of oxygen with that of the atmosphere at the time of inoculation. Each combination of treatments was replicated five times. Inoculated tubes without a liquid covering were used as a control. The experiment was conducted at 25°C.

Mycelium grew in all tubes within 3 days and microsclerotia appeared in the control tubes 5–7 days after inoculation. In tubes treated with distilled water or soft

agar, the development of microsclerotia was delayed until the mycelium reached the air-liquid interface. Then, hydrophobic hyphae developed from which microsclerotia formed. Hydrophobic hyphae did not develop in tubes treated with paraffin oil and microsclerotia were not formed even when the mycelium reached the air-oil interface.

(ii) *Growth of Aerated, Submerged Colonies*

Boiling tubes, 3.0 cm in diameter, were partially filled with either PDA or MVA and inoculated with *A. elegans*. The inoculum was covered with a layer of sterilized distilled water 4.0 cm deep and the tubes incubated at 25°C. An air stream (0.5 litres/min) was passed through the water layer to remove the oxygen diffusion barrier. This air stream was first passed through concentrated sulphuric acid and sterilized distilled water to remove spores of bacteria and fungi.

Under these steady-state conditions, mycelium continued to grow in all the tubes but rhizomorphs were not initiated.

(iii) *Role of Oxygen Diffusion in the Growth of Rhizomorphs*

The design of this experiment was similar to that of experiment (i). Inoculated tubes were incubated at 25°C for 12 days and when rhizomorphs 4.0–5.0 cm long had formed the liquid layers were applied. Each combination of treatments was replicated five times. Tubes containing rhizomorphs but without a liquid layer were used as a control. Rhizomorphs stopped growing immediately in all treated tubes irrespective of the type or depth of the liquid layer. Active growth continued in the control tubes until the media were thoroughly permeated.

(iv) *Role of Oxygen in the Development of Rhizomorphs from Naturally Colonized Wooden Blocks*

Gard (1927) found that when root fragments infected with *A. mellea* were half-submerged in water rhizomorphs were consistently produced. This method was used in the present study to determine the effect of oxygen on the development of rhizomorphs from wooden blocks naturally colonized by *A. elegans*.

Blocks, 5.0 by 1.0 by 1.0 cm, with xylem either parallel or at right angles to the long axis, were cut from the naturally colonized fence posts. Forty blocks of each type were placed in test tubes containing about 2.5 cm of water and a further 40 of each type were submerged completely in water. Plastic caps through which air could pass readily were placed on all the tubes to maintain a high relative humidity. The experiment was conducted at 25°C.

With the half-submerged blocks, microsclerotia were initiated on the exposed surfaces within 10 days but did not complete their development. Rhizomorphs, however, developed from the submerged surfaces of almost all these blocks within 14–50 days. On blocks with xylem parallel to the long axis, most rhizomorphs grew from the basal ends of the blocks and only rarely from the sides whereas on blocks with xylem at right angles to the long axis, rhizomorphs developed only from immediately below the air-water interface. Initiation of rhizomorphs was inhibited on all completely submerged blocks.

(v) *Effects of a Substrate-Air Interface on the Growth of Rhizomorphs*

Growth tubes, 40 by 1.5 cm, were half-filled with either PDA or MVA, sloped, inoculated with *A. elegans*, and incubated at 25°C. Rhizomorphs were initiated and grew through the media with the apices often growing into the air above the surface of the medium for up to 1.0 cm. When this occurred, the rhizomorphs became dark brown and stopped growing until branches developed beneath the agar surface forming new growing tips. This growth pattern was repeated irrespective of the medium used until the rhizomorphs reached the ends of the tubes.

Whenever the tips grew into the air, small lateral tufts of cinnamon-coloured hyphae developed from about 0.5 cm behind the apices. Sections were made through these structures and showed that the tufts of hyphae were associated with aborted side branches that had burst through the rind of the main rhizomorph but whose development was incomplete. The apices of these branches were composed of a mass of intertwining hyphae instead of normal rind cells (Figs. 1 and 2). There was no

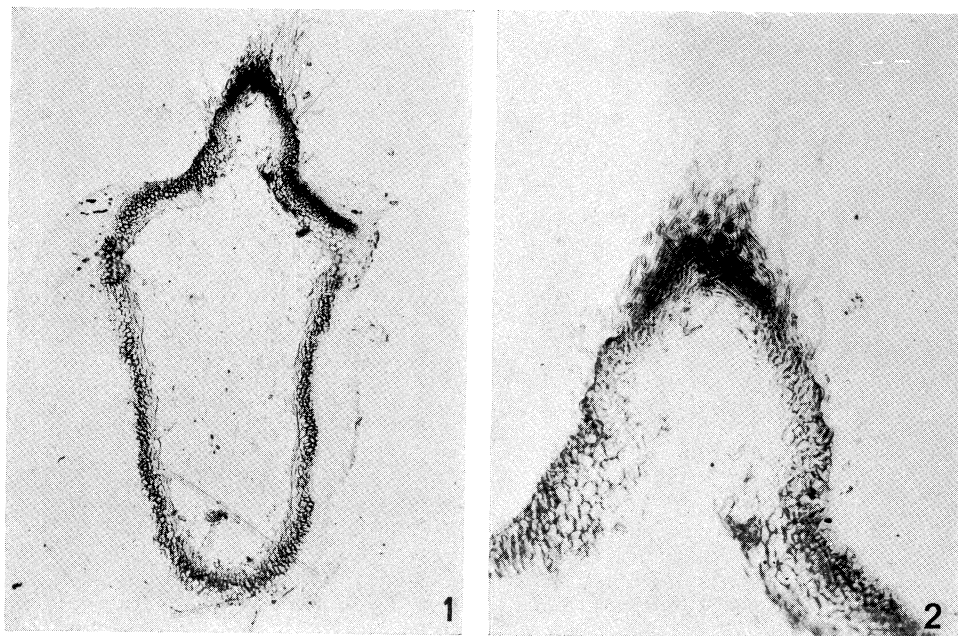


Fig. 1.—Transverse section through a rhizomorph showing a “breathing pore” formed on an aborted side branch ($\times 60$).

Fig. 2.—Section through a “breathing pore” in a rhizomorph. At the apex, the rind and meristem are replaced by interwoven hyphae ($\times 170$).

evidence of an organized meristem such as that found in the main apex of rhizomorphs (Motta 1969). The central canal of the rhizomorph and the aborted branch were directly connected. Oxygen probably diffused through the intertwining hyphae at the tip of the branch and then into the central canal of the rhizomorph.

(d) Discussion

Our results indicate that rhizomorphs need high partial pressures of oxygen in contact with their central canals but lower partial pressures in contact with their outside surfaces if they are to develop and grow. High partial pressures of oxygen in contact with the outside surfaces of rhizomorphs appear to inhibit growth. The inhibition of growth coincides with the formation of a brown pigment at the apex of the rhizomorph.

Submerging the mycelium of *A. elegans* inhibited the initiation of rhizomorphs independently of the diffusion or partial pressure of oxygen in the supernatant liquid. Fungi produce spherical colonies in submerged culture and such colonies soon reach a critical size because of the limitations imposed by the diffusion of oxygen through the water-saturated mass of respiring hyphae. These critical radii are small, varying from 0.01 to 0.04 cm for the fungi investigated (Griffin 1968). Once these critical radii are exceeded, the centres of the spherical colonies become anaerobic.

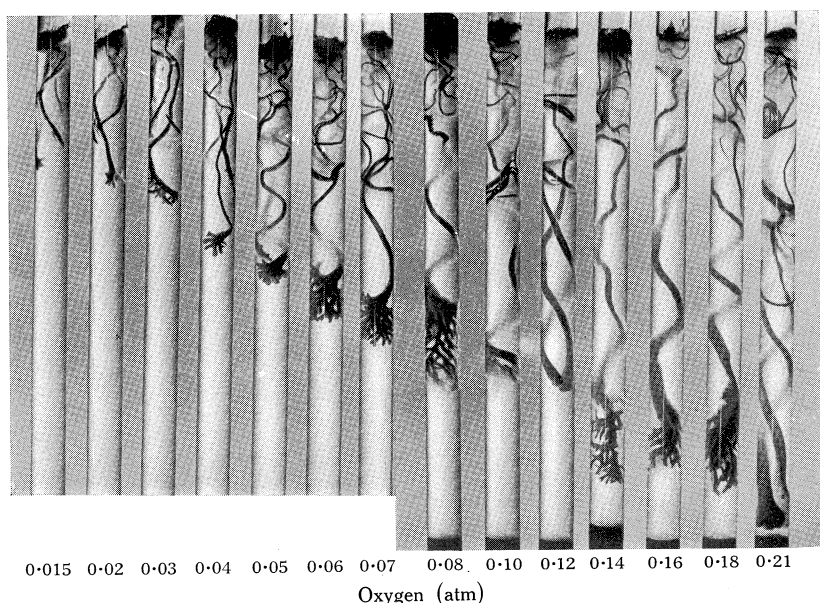


Fig. 3.—Growth in agar of rhizomorphs originating in oxygen partial pressures between 0.015 and 0.21 atm. The rhizomorphs flatten at progressively shorter lengths as the partial pressure of oxygen at their origins is lowered. All tubes were inoculated on the same day ($\times \frac{1}{4}$).

Such a consideration suggests two hypotheses concerning determinants for rhizomorph initiation. First, the formation of microsclerotia in *A. elegans* may be inhibited in submerged culture by limitation in the diffusion of oxygen within the early stages of the microsclerotium. If a mass of hydrophobic hyphae forms at a liquid-gas interface, however, the interhyphal volume will be gas-filled and the diffusion rate of oxygen will be rapid. Thus, secondly, the formation of microsclerotia may well be limited to sites where these hydrophobic hyphae form.

These two hypotheses would explain also why rhizomorphs grew from different positions on the partially submerged wooden blocks depending on whether the blocks

were cut parallel to, or at right angles to, the xylem. The blocks were impregnated, particularly along the xylem, with sheets of hydrophobic hyphae which prevented water entering the continuous vessels. Microsclerotia were able to develop and the rhizomorphs continued to elongate because oxygen diffused to their apices firstly through the xylem and then through the central canals.

We showed that rhizomorphs can make direct connections between their central canals and the atmosphere at substrate-air interfaces. Thus, cylindrical rhizomorphs can grow for great distances through soil because they can replenish their supply of oxygen by repeatedly making direct connections between their central canals and air-filled pores in the soil. Snider (1959) suggested that rhizomorphs had this ability but we have shown the structure of these "breathing pores" which develop on modified side branches and form when the rhizomorph, or part of it, is in contact with a gaseous atmosphere.

III. QUANTITATIVE EFFECTS OF OXYGEN AND CARBON DIOXIDE ON THE INITIATION, GROWTH, AND MORPHOLOGY OF RHIZOMORPHS

(a) *Introduction*

The results in Section II indicate that oxygen is important in the initiation and growth of rhizomorphs of *A. elegans*, but quantitative information is lacking. We now report quantitative data from a series of experiments and also describe and test the validity of a model system designed to simulate a rhizomorph.

The effect of carbon dioxide on the initiation and growth of rhizomorphs was also studied because, at least in woody host plants, concentrations sufficiently high to restrict the growth of fungi may occur (Chase 1934; Thacker and Good 1952; Gundersen 1961).

(b) *Materials and Methods*

A brass diffusion column was constructed, identical in principle to that of Griffin *et al.* (1967). Controlling atmospheres in the displacement chambers were provided by the appropriate humidified gases flowing at 0.5 litres/min. Gases used were air and commercially supplied nitrogen, oxygen, and carbon dioxide. The nitrogen contained about 0.002 atm oxygen and this was not removed. Gas mixtures were obtained by mixing the component gases in the laboratory, the flow of the components being controlled by needle valves and flow metres. The gases in the column were allowed to reach a steady state over a period of 48 hr before the start of each experiment.

Glass tubes 30 cm long and 1.25 cm diameter, sealed at one end with a rubber bung, were attached to the side-arms of the diffusion column by short rubber tubes. PDA was used as a medium, agar content being 1%, to reduce cracking. Tubes were filled with 20 ml medium, autoclaved, cooled in an atmosphere of nitrogen, inoculated, and attached to the column. All experiments were conducted at 25°C and with four replicates at each gas concentration.

Tubes were examined daily for rhizomorph initials and from the tenth day the linear growth of rhizomorphs was measured every second day. There was a tendency for rhizomorphs to grow down the tubes in a spiral fashion but it was impracticable to measure the true lengths of such rhizomorphs while the tubes were attached to the column. The recorded length was the length of tube colonized.

(c) *Experimental*

(i) *Oxygen*

A linear oxygen gradient (0.002–0.21 atm) was produced in the diffusion column by passing commercial nitrogen and air through the displacement chambers.

Four days after the start of the experiment, rhizomorph initials were present in all tubes between 0.03 and 0.21 atm oxygen. Initials developed within 7 days at 0.01–0.02 atm oxygen and within 11 days at 0.005 atm oxygen. At 0.002 atm oxygen, mycelium did not appear for at least 16 days and initials were not apparent until 20 days after inoculation.

Rhizomorphs grew through the agar (Fig. 3) and their lengths at different partial pressures of oxygen and times are shown in Figure 4(a).

Rhizomorphs gradually became less cylindrical and more ribbon-like as they grew down the tubes. Eventually the apices were flat and lobed and the distance grown before flattening occurred was related to partial pressure of oxygen [Figs. 3 and 4(b)]. Flat rhizomorphs grew 0.1–0.2 cm per day compared with a maximum growth rate of *c.* 0.75 cm per day for cylindrical rhizomorphs.

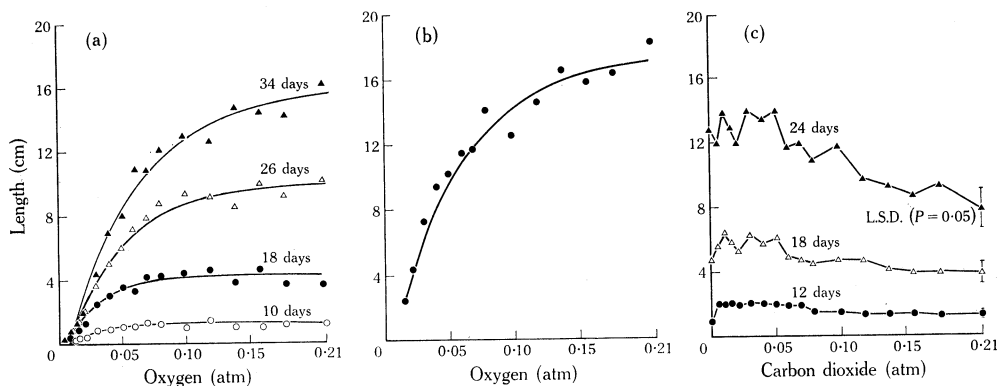


Fig. 4.—Relationship between the partial pressure of oxygen at the origin of rhizomorphs and (a) their length at various times, (b) the length they attained before flattening. (c) Relationship between the partial pressure of carbon dioxide at the origin of rhizomorphs and their length at various times.

(ii) Carbon Dioxide

A linear carbon dioxide gradient at constant oxygen partial pressure was produced by passing air through the displacement chamber at one end of the diffusion column and a mixture of 0.21 atm oxygen, 0.21 atm carbon dioxide, and 0.58 atm nitrogen through the other chamber.

Rhizomorphs were initiated simultaneously under all conditions and the relationship between rhizomorph length and partial pressure of carbon dioxide at various times is shown in Figure 4(c). Rhizomorph apices flattened when the rhizomorphs attained a length of 20 cm regardless of partial pressure of carbon dioxide.

(iii) Oxygen : Carbon Dioxide Ratios

Inverse gradients of oxygen and carbon dioxide were produced by passing air and a mixture of 0.21 atm carbon dioxide and 0.79 atm nitrogen through the displacement chambers of the diffusion column.

Rhizomorph initials appeared 4 days after inoculation in all gas mixtures with oxygen : carbon dioxide partial pressures between 0.03 : 0.18 and 0.21 : 0.0003 atm

and after 7 days in mixtures with partial pressures between 0.01 : 0.2 and 0.03 : 0.18 atm. No other initials were formed during the experiment.

The relationship between rhizomorph length and partial pressures of gases is shown in Figure 5(a) whereas the relationship between gas composition and the length rhizomorphs attained before their apices flattened is shown in Figure 5(b).

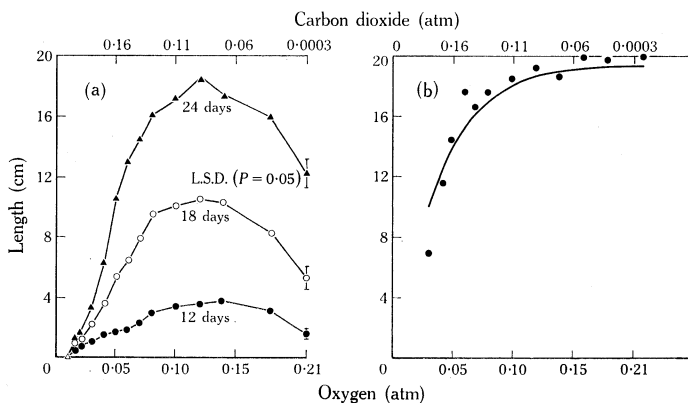


Fig. 5.—Relationship between the partial pressures of oxygen-carbon dioxide mixtures at the origin of rhizomorphs and (a) their length at various times, (b) the length they attained before flattening.

(d) Theoretical Model

(i) Derivation of the Model

To construct a model of a rhizomorph and its oxygen relations which permits subsequent analysis, the rhizomorph may be simplified as in Figure 6. Following the

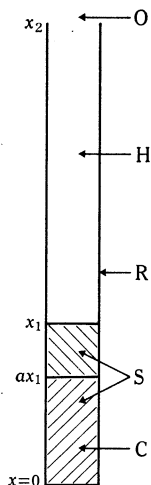


Fig. 6.—Model of a rhizomorph used in analysis of oxygen relationships:

- O, origin—opening to atmosphere;
- H, hollow central canal in which coefficient of diffusion of oxygen is D_e ;
- R, rhizomorph wall;
- S, solid apical cylinder in which coefficient of diffusion of oxygen is D_i ;
- C, cell division; elongation.

Other symbols as defined in text.

descriptions of De Bary (1887) and Motta (1969), the apex may be considered as a cylindrical mass of rather compact cells, of length x_1 . The terminal portion of this

solid cylinder is the zone of cell division and elongation. The proximal portion is the zone of established cells derived from the meristematic zone. Proximal to the compact apex, the rhizomorph may be considered as a hollow cylinder, the walls of which are composed of fungal cells of negligible metabolic activity compared with those of the apex. Such a model permits an analysis of the role of oxygen analogous to those given by Griffin (1968). There, many arguments were presented relevant to the present model, in detail and at length: here only an abbreviated version will be given. Let

- a = proportion of apical tissue which is undergoing cell division or rapid increase in cell size;
- C = concentration of oxygen in the gas phase within a rhizomorph;
- c, c_i = concentration of oxygen within apical tissue of rhizomorph, and particularly at site of terminal oxidase (g cm^{-3});
- C_x, c_x = concentration at distance x ;
- C'_x, c'_x = concentration at x when concentration at $x = 0$ is just zero;
- d = time from commencement of experiment (day);
- D_e = diffusion coefficient of oxygen in air ($\text{cm}^2 \text{sec}^{-1}$);
- D_i = diffusion coefficient of oxygen within the apical cylinder of the rhizomorph;
- P = partial pressure of oxygen (atm);
- P'_x = partial pressure of oxygen at x when concentration at $x = 0$ is just zero;
- q = maximum rate of oxygen uptake by unit volume of apical cylinder ($\text{g cm}^{-3} \text{sec}^{-1}$);
- q_m = measured rate of oxygen uptake under specific conditions;
- v = rate of elongation of rhizomorph (cm day^{-1});
- V_d = rate of elongation of rhizomorph on day d when oxygen is not limiting;
- x = length (cm);
- x_1 = length of apical cylinder;
- x_2 = length of rhizomorph;
- x_i = length within apical cylinder ($x_i \leq x_1$).

Assume that throughout the apical cylinder the diffusion coefficient of oxygen and the maximum rates of oxygen consumption are constant. Further assume that diffusion is entirely parallel with the axis of the rhizomorph and that the apex is entirely supplied by diffusion within the cylindrical canal delimited by the walls, i.e. diffusion has linear co-ordinates. (These assumptions cannot be justified now with rigour but if they are false, the model is unlikely to account for the data.) Then the equation governing the rate of utilization of oxygen within the apical cylinder, under steady-state conditions, is:

$$D_i(d^2c/dx^2) = \sum_{p=1}^n [q_p c / (K_{m_p} + c)], \quad (1)$$

where q_p is maximum rate of oxygen uptake by the p th terminal oxidase, and K_{m_p} is the Michaelis constant of the p th terminal oxidase and n = number of terminal oxidases.

As the cytochrome system may be presumed to be pre-eminent as a sink for oxygen, and as the Michaelis constant for the reaction of cytochrome oxidase with oxygen is negligible compared with atmospheric oxygen concentration (Griffin 1968) equation (1) may be simplified to

$$D_i(d^2c/dx^2) = q \quad (\text{for } c_i > 0). \quad (2)$$

Upon integration, equation (2) becomes

$$dc/dx = qx/D_i + A, \quad (3)$$

and subsequently

$$c_{x_i} = qx_i^2/2D_i + Ax_i + B, \quad (4)$$

where A and B are constants of integration and will depend upon particular boundary conditions. With boundary conditions taken as

$$dc/dx = 0 \quad \text{and} \quad c_{x_i} = 0 \quad \text{at } x_i = 0, \quad (5)$$

then $A = 0$ and $B = 0$, so

$$c_{x_i} = qx_i^2/2D_i, \quad (6)$$

and

$$c'_{x_1} = qx_1^2/2D_i, \quad (7)$$

(that is, the minimum oxygen concentration at the proximal boundary of the apical cylinder that permits maximum respiration by that whole cylinder is $qx_1^2/2D_i$).

But the rate of consumption of oxygen by the apical cylinder must, under steady-state conditions, be balanced by diffusion along the rhizomorph canal. So, by Fick's Law,

$$qx_1 = D_e(C'_{x_2} - C'_{x_1})/(x_2 - x_1). \quad (8)$$

Therefore

$$C'_{x_2} = C'_{x_1} + qx_1(x_2 - x_1)/D_e, \quad (9)$$

and

$$P'_{x_2} = P'_{x_1} + k(x_2 - x_1). \quad (10)$$

From our own observations, and those of Motta (1969), the length of the apical cylinder of the rhizomorph (x_1) is of the order of 0.01 cm. In all our data it is thus negligible compared with x_2 and so equation (10) may be simplified to

$$P'_{x_2} \doteq P'_{x_1} + kx_2. \quad (11)$$

Equation (11) is of value for it is used later in testing the validity of the model. Further tests can be applied if the analysis is developed further as follows.

Instead of the boundary conditions given in (5), let $c_i = 0$ and $dc/dx = 0$ at $x_i = x_b$ where

$$0 < x_b < x_1 \quad (12)$$

(that is, oxygen does not diffuse to the end of the apical cylinder but only as far as a plane x_b from the distal end). From (3), (4), and (12),

$$2c_i D_i / q = (x_i - x_b)^2, \quad (13)$$

so

$$2c_{x_1} D_i / q = (x_1 - x_b)^2. \quad (14)$$

Now

$$q_m x_1 = q(x_1 - x_b), \quad (15)$$

so

$$x_1 - x_b = q_m x_1 / q, \quad (16)$$

$$2c_{x_1} D_i / q x_1^2 = (q_m / q)^2 \quad (17)$$

$$= c_{x_1} / c'_{x_1} \quad [\text{from (7)}] \quad (18)$$

$$= C_{x_1} / C'_{x_1}. \quad (19)$$

But

$$x_1 q_m = D_e (C_{x_2} - C_{x_1}) / (x_2 - x_1). \quad (20)$$

Therefore

$$C_{x_2} = C_{x_1} + q_m x_1 (x_2 - x_1) / D_e \quad (21)$$

From (12), (19), and (21), when $C_{x_2} \leq C'_{x_2}$,

$$C_{x_2} = C'_{x_1} (q_m / q)^2 + q_m x_1 (x_2 - x_1) / D_e. \quad (22)$$

Unfortunately q_m and q are unknown and the technical difficulties of evaluating them appear insuperable at the moment. Let us assume, however, that the rate of elongation on any given day (v) is proportional to the total rate of respiration of the elongating zone (length ax_1). Let the rate of elongation on day d be V_d when oxygen is not limiting. Thus

$$v / V_d = (ax_1 - x_b) / ax_1, \quad (23)$$

and

$$x_b = ax_1 (1 - v / V_d). \quad (24)$$

But, from (16),

$$x_b = x_1 - q_m x_1 / q. \quad (25)$$

Therefore

$$q_m / q = 1 - a + av / V_d. \quad (26)$$

From (22) and (26)

$$C_{x_2} = C'_{x_1} (1 - a + av / V_d)^2 + q x_1 (x_2 - x_1) (1 - a + av / V_d) / D_e, \quad (27)$$

and

$$P_{x_2} = P'_{x_1} (1 - a + av / V_d)^2 + k x_2 (1 - a + av / V_d). \quad (28)$$

(ii) *Test of Validity of Model*

It is a consequence of the model adopted, and particularly of the assumptions made in simplifying equation (1) to equation (2), that the rate of respiration, and thus of growth, will be independent of oxygen concentration if $C_{x_2} \geq C'_{x_2}$. The model thus predicts on a given day an increase in length of rhizomorph with increase in oxygen concentration at the origin until a certain critical oxygen partial pressure (P'_{x_2}) is reached but thereafter length of rhizomorph will be constant. The first step in testing the validity of the model is to see if this expectation is supported. An inspection of the data, given in part in Figure 4(a), strongly supports such an expectation but it is not possible by inspection to say precisely what are the values of P'_{x_2} for each day and thus for each rhizomorph length.

A statistical estimate of P'_{x_2} can be obtained as follows. Inspection of the data relating x_2 and P_{x_2} [Fig. 4(a)] suggests that the data might be divided into two portions, the first with a curvilinear exponential relationship of the form

$$x_2 = \alpha + \beta\mu^s \quad (\text{when } s \leq \delta), \quad (29)$$

the second with a linear relationship of the form

$$x_2 = \alpha + \beta\mu^\delta = \text{constant} \quad (\text{when } s > \delta), \quad (30)$$

where $s = P_{x_2}$ and α , β , and μ are constants.

[It must be emphasized that equations (29) and (30) are used solely in the evaluation of P'_{x_2} and form no part of the model.] By computer analysis, it is possible to vary δ and so to find a value for δ such that the sum of squares of the deviations of the observed from the fitted values is minimized. The values of δ so found are the estimates of P'_{x_2} , for each value of x_2 . This analysis was performed on the data for 10 days between days 10 and 34. The analysis showed values of P'_{x_2} between 0.07 and 0.1 atm oxygen for days 10–26 but for days 28–34, P'_{x_2} was greater than 0.21 atm and so was not determined.

In the equation

$$P'_{x_2} \doteq P'_{x_1} + kx_2. \quad (11)$$

P'_{x_1} and k are constants so that if P'_{x_2} is plotted against x_2 , the relationship should be linear. The relationships between the values of P'_{x_2} and x_2 derived from the statistical analysis outlined above are shown in Figure 7. A regression analysis shows that $P'_{x_2} = 0.0641 + 0.0029x_2$ ($r = 0.9432$, probability $< 1\%$). The main feature of equation (11) is thus in accord with the data.

It remains to apply the further check provided by equation (28), which may be re-expressed as:

$$v/V_d = (1/a)\{a - 1 - [kx_2 - (k^2x_2^2 + 4P'_{x_1}P'_{x_2})/2P'_{x_1}]\}. \quad (29)$$

In equation (29), P'_{x_1} and k are known, being the intercept and slope in equation (11), and the experimental data provide paired values of x_2 and P_{x_2} .

It would therefore be possible to calculate and so predict values of v/V_d , and to compare these with the experimentally determined values, if a were known. Unfortunately the value of a is not known with any degree of accuracy, although

microscopic evidence suggests it might be about 0.5. It is, however, possible by computer to fit values of a , such that $0 < a < 1$, into equation (29) and thus obtain v/V_d . Such a procedure has shown that the best agreement between experimental and predicted values of v/V_d occurs if $a = 0.566$ and this value has been adopted. Table 1 contains values of x_2 , V_d , and v/V_d (experimental and predicted) for six values of P_{x_2} and seven values of d . (Note that values are only given when $P_{x_2} < P'_{x_2}$ because the theory indicates that both x_2 and v are independent of P_{x_2} if $P_{x_2} \geq P'_{x_2}$.)

It is now possible to apply a comprehensive test of the validity of the model. Let the experimental and predicted values of v/V_d be X and Y . Then, ideally, the relationship between X and Y is expressed by $Y = 0.1 + 1.00X$.

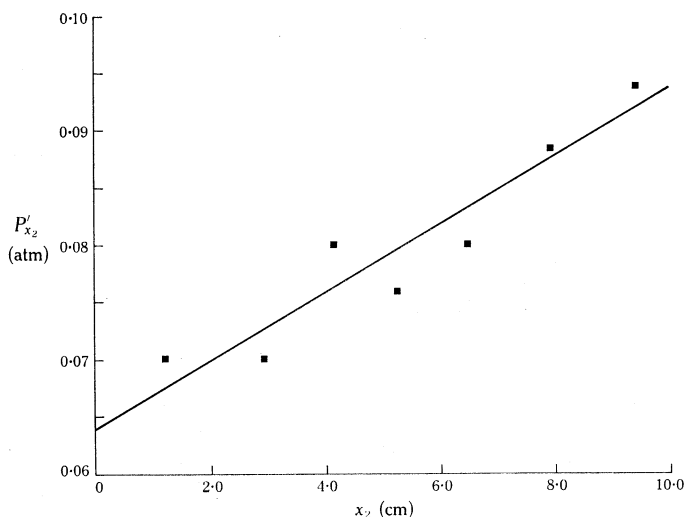


Fig. 7.—Relationship between length of a rhizomorph (x_2) and the minimum partial pressure of oxygen at the origin of the rhizomorph that permits maximum rate of growth (P'_{x_2}).

A regression analysis of the values in Table 1 shows that $Y = 0.051 + 0.94X$ ($r = 0.8943$, probability $\ll 0.1\%$), where neither the intercept on the Y axis nor the slope differ significantly from the ideal values. Analysis thus reveals that the relative growth of a rhizomorph predicted by the model, under any relevant conditions of growth, is very close to the experimentally determined rate. It may be argued that this close correlation between predicted and experimental values is because of the deliberate selection of that value for a which maximizes the correlation. Such an argument, however, fails to allow for the fact that the correlation, though maximized, would still be poor if a did not represent a physical entity of relatively constant value, of a nature similar to that in the model. We thus conclude that the model, as a whole, is in excellent accord with actuality.

(e) Discussion

The initiation of rhizomorphs of *A. elegans* was unaffected by partial pressures of oxygen between 0.03 and 0.21 atm and was only slightly delayed between 0.01 and 0.02 atm. Thus, in nature, differences in oxygen concentrations are unlikely to affect the ecology of this fungus by limiting or delaying the initiation of rhizomorphs. The hypothesis that proposes that the initiation of rhizomorphs has a high

oxygen requirement as an adaptive mechanism to ensure that the oxygen supply to the subsequent rhizomorphs would be continuous with the atmosphere (Snider 1959) is now untenable.

TABLE 1

RELATIONSHIP BETWEEN PARTIAL PRESSURE OF OXYGEN AT ORIGIN OF RHIZOMORPH, LENGTH, MAXIMUM GROWTH RATE, AND EXPERIMENTAL AND PREDICTED RELATIVE GROWTH RATES OF RHIZOMORPHS OF VARIOUS AGES

| Age of Rhizomorphs (days) | | Partial Pressure of Oxygen (atm) | | | | | | | | Max. Growth Rate (cm/day) |
|---------------------------|------------------|----------------------------------|------|------|------|------|------|------|------|---------------------------|
| | | 0.015 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | |
| 10 | Length (cm) | 0.40 | 0.45 | 0.85 | 0.95 | 1.05 | 1.10 | | | 0.18 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0.14 | 0.28 | 0.56 | 0.70 | 0.84 | 0.84 | | | |
| | Predicted | 0.07 | 0.20 | 0.41 | 0.59 | 0.75 | 0.90 | | | |
| 15 | Length (cm) | 0.70 | 0.90 | 1.90 | 2.20 | 2.60 | 2.70 | | | 0.37 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0.23 | 0.29 | 0.63 | 0.73 | 0.87 | 0.79 | | | |
| | Predicted | 0.06 | 0.18 | 0.37 | 0.54 | 0.69 | 0.84 | | | |
| 18 | Length (cm) | 1.00 | 1.30 | 2.50 | 3.10 | 3.60 | 3.40 | 4.10 | | 0.47 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0.17 | 0.26 | 0.38 | 0.60 | 0.81 | 0.64 | 0.94 | | |
| | Predicted | 0.05 | 0.17 | 0.35 | 0.51 | 0.66 | 0.81 | 0.92 | | |
| 20 | Length (cm) | 1.10 | 1.50 | 2.80 | 3.60 | 4.50 | 4.20 | 5.30 | | 0.57 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0.04 | 0.18 | 0.26 | 0.31 | 0.57 | 0.70 | 0.91 | | |
| | Predicted | 0.04 | 0.16 | 0.33 | 0.49 | 0.62 | 0.78 | 0.88 | | |
| 22 | Length (cm) | 1.10 | 1.70 | 3.10 | 3.80 | 4.90 | 5.00 | 6.20 | | 0.67 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0.08 | 0.15 | 0.23 | 0.41 | 0.41 | 0.75 | 0.60 | | |
| | Predicted | 0.04 | 0.15 | 0.32 | 0.48 | 0.61 | 0.75 | 0.85 | | |
| 24 | Length (cm) | 1.30 | 1.90 | 3.40 | 4.70 | 5.60 | 6.20 | 6.90 | 7.70 | 0.70 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0.07 | 0.07 | 0.22 | 0.43 | 0.40 | 0.75 | 0.58 | 0.72 | |
| | Predicted | 0.04 | 0.15 | 0.31 | 0.45 | 0.58 | 0.71 | 0.82 | 0.92 | |
| 26 | Length (cm) | 1.30 | 1.90 | 3.70 | 5.00 | 6.00 | 7.10 | 7.80 | 8.70 | 0.75 |
| | Rel. growth rate | | | | | | | | | |
| | Experimental | 0 | 0 | 0.13 | 0.33 | 0.30 | 0.60 | 0.57 | 0.77 | |
| | Predicted | 0.04 | 0.15 | 0.30 | 0.44 | 0.57 | 0.68 | 0.79 | 0.89 | |

Partial pressure of oxygen at the rhizomorph origin, however, markedly affected their growth. Thus, the critical partial pressure required to keep a cylindrical rhizomorph growing at maximal rate increased as the length of the rhizomorph increased (Figs. 4(a) and 7). A theoretical model was constructed and tested which verified

the relationship between partial pressure of oxygen and growth of rhizomorphs. This model showed clearly that the growth of rhizomorphs between days 10 and 26 is largely explicable by purely physical considerations of oxygen diffusion and utilization. After 26 days the model fails, possibly because the apex of the rhizomorph starts to flatten and changes the size and shape of the air canal. The model provides no explanation, however, for the increase of V_d (maximum growth rate on a given day) with time (Table 1). The simplest hypothesis is probably to suppose that the nutrients available to the apex increase with time as a few of the rhizomorphs assume dominance and as the potential area for absorption increases. Were this to be so, however, q should increase with time and the data would no longer conform to equation (11). The agreement of the data with expectation based on diffusion analysis thus indicates that q is fairly steady and that perhaps it is the energy yield of respiration that varies. In the absence of further data, however, it is profitless to pursue the supposition. Garrett (1956) showed that the maximum growth rate of rhizomorphs on a given day (V_d) decreased with time. However, his experimental design differed so much from ours that a comparison of results cannot be made.

These experiments also showed that oxygen, but not carbon dioxide, can affect the morphology of rhizomorphs. When submerged in agar, rhizomorphs flattened and became plaque-like when the diffusion rate of oxygen down their central canals became limiting for the needs of the meristem. The mechanisms involved, however, remain to be elucidated and are probably complex because nutrition and pressure have already been implicated in flattening (Campbell 1934; Hamada 1940; Rhoads 1945; Townsend 1954; Jacques-Felix 1967). Our theoretical model fails to account for any changes in morphology of the rhizomorph associated with flattening.

When sufficient oxygen was present, partial pressures of carbon dioxide between 0.0003 and 0.21 atm did not affect the initiation of rhizomorphs of *A. elegans*. This result is similar to that obtained by Raabe and Gold (1967) for the initiation of rhizomorphs by *A. mellea*.

Carbon dioxide also affected the growth of rhizomorphs but much less than oxygen. Carbon dioxide partial pressures of about 0.01–0.07 atm stimulated the growth of rhizomorphs slightly whereas partial pressures above about 0.14 atm were inhibitory, especially in long rhizomorphs. These results are similar to those obtained with other wood-rotting fungi (Thacker and Good 1952; Gundersen 1961).

In gas mixtures of oxygen and carbon dioxide, rhizomorphs made optimum growth in mixtures varying between 0.08 : 0.13 and 0.18 : 0.03 atm oxygen : carbon dioxide. The oxygen : carbon dioxide partial pressures recorded in some woody plants correspond to these optimum gas compositions (Chase 1934; Thacker and Good 1952). Thus, *A. elegans* is well adapted to the gas environment within woody host plants.

IV. AN INTERRELATIONSHIP BETWEEN THE BROWNING OF RHIZOMORPHS, THE PARTIAL PRESSURE OF OXYGEN AT THEIR SURFACES, AND THEIR RATE OF GROWTH

(a) Introduction

Rhizomorphs of *Armillariella* spp. are normally dark brown when in contact with air and our results in Section II indicated that the formation of this pigment may be associated with the inhibition of the growth of rhizomorphs.

Melanins can inhibit enzymes in model systems (Kuo and Alexander 1967; Bull and Alexander 1969) but there is scant evidence that this occurs in intact organs. In cultural studies, Snider (1957) found that rhizomorphs submerged in agar grew faster than those growing along the surface. The submerged rhizomorphs were white but those at the surface were pigmented thus suggesting a relationship between growth and pigmentation. Another characteristic of *Armillariella* spp. in culture is that their rhizomorphs usually do not grow more than about 1.0 cm out of the surface of agar (Rhoads 1945; Dadant 1963). These aerial rhizomorphs rapidly become pigmented even at the apices thus again suggesting a relationship between browning and the inhibition of growth. Aerial rhizomorphs, however, may be subjected to desiccation and different light intensity and these factors can inhibit their growth (Townsend 1954; Snider 1957; Raabe 1958). Nutrition is not involved because rhizomorphs grow for long distances through non-nutrient media provided they are attached to a food base (Findlay 1951; Snider 1957).

In our study, experiments were made to determine whether browning of an aerial rhizomorph, particularly its apex, inhibited the growth of the rhizomorph. Attempts were made to separate the effects of browning, light, and desiccation. The role of oxygen in the formation of the brown pigment, and its location within the tissues of the rhizomorph were also studied.

(b) *Experimental*

(i) *Some Effects of the Environment on Browning and Growth of Rhizomorphs*

An experiment, designed as a 2³ factorial, was made to determine the effect of browning on the growth of rhizomorphs. The variables were media, light conditions, and relative humidities.

Flasks containing 50 ml of either PDA or MVA were autoclaved and inoculated with *A. elegans*. Because aerial rhizomorphs are normally exposed to higher light intensities than those submerged in media, half the inoculated flasks were wrapped in black paper to eliminate light. Similarly, to ensure that desiccation of the aerial rhizomorphs did not restrict growth, half the inoculated flasks were incubated in a saturated atmosphere, and the remainder in relative humidities which fluctuated between 70 and 90%. To obtain a saturated atmosphere, flasks were sealed in plastic bags and the insides of the bags were sprayed regularly with water. The experiment was conducted at 25°C and replicated four times. Sample flasks were examined weekly for 4 weeks.

In all flasks the aerial rhizomorphs rapidly became dark brown. None grew more than 1.0 cm out from the surface of the agar. Cultures grown in the flasks from which light was excluded developed a more luxuriant mycelium which was more darkly pigmented than that in the other flasks. In MVA only the aerial rhizomorphs were brown but in PDA rhizomorphs submerged in the top 0.5 cm of the agar were also pigmented. However, the tips of these latter rhizomorphs remained white and continued to grow when submerged in the agar.

(ii) *Effect of Oxygen on the Browning of Rhizomorphs*

(1) White, cylindrical rhizomorphs 10–12 cm long were obtained by growing *A. elegans* in tubes almost filled with potato dextrose broth. These rhizomorphs

were washed thoroughly in distilled water after removal from the tubes and then placed in two McIntosh and Fildes anaerobic culture jars. A nitrogen atmosphere was made in one jar by passing humidified, oxygen-free nitrogen through the jar for 15 min before sealing. In the second jar, humidified air was used to obtain an oxygen partial pressure of 0.21 atm. The jars were kept at room temperature and the rhizomorphs examined daily for browning.

Within 24 hr the rhizomorphs in air started to brown and after 48 hr were dark reddish brown. In the nitrogen atmosphere, the rhizomorphs remained white for the whole of the experimental period.

(2) A microelectrode, designed by Charlton (1961), was used to measure the partial pressure of oxygen at the surface of brown and white rhizomorphs in an attempt to correlate the colour of a rhizomorph with the oxygen tension at its surface.

TABLE 2
PARTIAL PRESSURES OF OXYGEN AT THE SURFACE OF BROWN AND WHITE RHIZOMORPHS

| Rhizo- morph No. | Rep- licate No. | Rhizomorphs Grown in Potato Dextrose Agar* | | | | Rhizomorphs Grown in Malt Vegemite Agar† | | | |
|------------------------|-----------------------|---|------------------------------------|------------------------------|------------------------------------|---|------------------------------------|------------------------------|------------------------------------|
| | | Oxygen at Apex‡ | | Oxygen at Base§ | | Oxygen at Apex‡ | | Oxygen at Base‡ | |
| | | Partial Pressure (atm) | 10 ⁵ × Concn. (M) | Partial Pressure (atm) | 10 ⁵ × Concn. (M) | Partial Pressure (atm) | 10 ⁵ × Concn. (M) | Partial Pressure (atm) | 10 ⁵ × Concn. (M) |
| 1 | i | 0.022 | 2.7 | 0.028 | 3.4 | 0.024 | 3.0 | 0.003 | 0.5 |
| | ii | 0.023 | 2.8 | 0.070 | 8.6 | 0.030 | 3.7 | 0.003 | 0.5 |
| 2 | i | 0.030 | 3.7 | 0.060 | 6.4 | 0.006 | 0.7 | 0.015 | 1.8 |
| | ii | 0.028 | 3.4 | 0.090 | 11.1 | 0.015 | 1.8 | 0.009 | 1.1 |
| 3 | i | 0.025 | 3.1 | 0.084 | 10.3 | 0.013 | 1.6 | 0.013 | 1.6 |
| | ii | 0.033 | 4.1 | 0.040 | 4.9 | 0.013 | 1.6 | 0.013 | 1.6 |
| 4 | i | 0.029 | 3.6 | 0.070 | 8.6 | 0.003 | 0.5 | 0.020 | 2.5 |
| | ii | 0.026 | 3.2 | 0.075 | 9.2 | 0.003 | 0.5 | 0.013 | 1.6 |
| 5 | i | 0.028 | 3.4 | 0.080 | 9.8 | 0.010 | 1.2 | 0.013 | 1.6 |
| | ii | 0.040 | 4.9 | 0.070 | 8.6 | 0.027 | 3.3 | 0.020 | 2.5 |

* Partial pressure of oxygen in agar = 0.21 atm.

† Partial pressure of oxygen in agar = 0.13 atm.

‡ Rhizomorphs white.

§ Rhizomorphs brown.

Brown and white rhizomorphs were obtained by growing *A. elegans* in Petri dishes containing either PDA or MVA. The agar content of the media was reduced to 0.5% to prevent cracking when the electrode was introduced. After 9 days at 25°C, rhizomorphs 2.0–5.0 cm long had developed in both media. Four readings of oxygen levels were made on five different rhizomorphs growing in each medium. Two readings were made within 0.5 cm of the tip where, in both media, the rhizomorphs were white and fringing mycelium was absent. The other two readings were made about 4.0 cm from the tip where the rhizomorphs were white if the medium was MVA and brown if PDA. Fringing mycelium was present on rhizomorphs in both media although it appeared to be deteriorating on rhizomorphs grown in PDA. Finally, the electrode was used to measure the partial pressure of oxygen in each medium.

The results of these measurements are given in Table 2. The equivalent molar concentrations are included so that these readings can be compared more easily with the results of some later experiments.

(iii) *Identification of the Brown Pigment*

The brown pigment from rhizomorphs was extracted using a method described by Potgieter and Alexander (1966). The rhizomorphs were hydrolysed in 72% sulphuric acid for 48 hr and the black precipitate was filtered, washed, dried, and crushed into a fine black powder. Brown rhizomorphs of *A. elegans* obtained from culture and from infected peach roots in the field were similarly treated.

The extracted pigments were examined to determine whether they were melanins. They were insoluble in hot and cold water, and in methanol, ethanol, xylol, chloroform, acetone, and ether. They were, however, decolourized within 12 hr by 5% sodium hypochlorite and 30% hydrogen peroxide. Neither material reduced ammoniacal silver nitrate. The extracted pigments were dissolved separately in hot 0.5N sodium hydroxide and the optical densities of these solutions were measured at wavelengths between 400 and 600 nm using a Bausch & Lomb Spectronic 20 spectrophotometer. When the logarithm of absorbancy was plotted against the wavelength of light for each sample, straight lines, each with a slope of -0.0037 , were obtained. Alkaline solutions of the extracted pigments were saturated with ammonium sulphate and a brown flocculent precipitate was formed by the extracts from both kinds of rhizomorphs.

(iv) *Ability of Brown Pigment to Precipitate Protein*

A method described by Gustavson (1956) was used to test whether either extracted brown pigment or intact brown rhizomorphs could combine with protein.

Extracted pigment (0.02 g) was added to 1.0-ml samples of a 1% solution of gelatin in 10% sodium chloride. Five tubes were prepared and examined over 5 hr for precipitation of the gelatin.

In another test, intact brown rhizomorphs about 5.0 cm long were suspended in 5-ml samples of a 1% solution of gelatin in 10% sodium chloride. Five tubes were prepared and examined over 5 hr for precipitation of the gelatin.

Neither extracted nor intact brown pigment from rhizomorphs caused precipitation of gelatin.

(v) *Location of the Brown Pigment within the Rhizomorphs*

Studies of transverse sections through brown rhizomorphs with the light microscope (Section II) suggested that the pigment was restricted to the cell walls of the rind. To determine more precisely where the brown pigment is located in the cell walls, sections were prepared and examined with an electron microscope.

Brown and white rhizomorphs were obtained from nutrient broth culture, washed carefully, split longitudinally, and fixed in either 1% potassium permanganate in veronal acetate buffer at pH 7.6 for 45 min at 4°C, or in 6.5% glutaraldehyde in cacodylate buffer at pH 7.6 for 4 hr at 4°C. After fixing, the specimens were treated in 1% osmium tetroxide in veronal acetate buffer at pH 7.6 for 2 hr at 4°C. Then the specimens were washed, dehydrated in an ethanol series, and infiltrated with a mixture of propylene oxide and Araldite followed by Araldite before ultra-

thin sections were cut with a LKB Ultratome. The specimens were stained in lead citrate for 10 min before being examined with a Siemens Elmiskop I electron microscope. The specimens were prepared by Dr. N. G. Nair, Department of Agricultural Botany, University of Sydney, who also operated the electron microscope.

Both potassium permanganate and glutaraldehyde gave good fixation of white rhizomorphs. Brown rhizomorphs were more difficult to fix although potassium permanganate was reasonably successful.

Characteristically, the rind of rhizomorphs is composed of cells with thin walls surrounded by large intercellular spaces (Fig. 8). In brown rhizomorphs the pigment is localized in these intercellular spaces (Fig. 9).

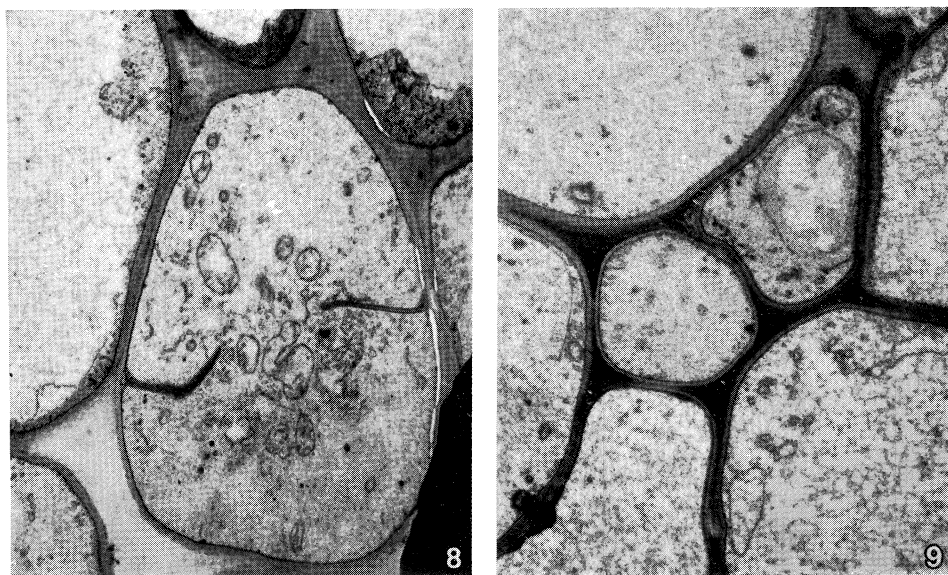


Fig. 8.—Electron micrograph of rind cells of a white rhizomorph showing the large intercellular spaces ($\times 5300$).

Fig. 9.—Electron micrograph of rind cells of a brown rhizomorph showing the pigment localized in the intercellular spaces ($\times 5750$).

(c) Discussion

These studies confirmed that the aerial growth of rhizomorphs is restricted coincidentally with the browning of their apices by a melanin-like pigment (Thomas 1955; Thomson 1962, 1965). Browning occurs only when the partial pressure of oxygen at the surface of the rhizomorph exceeds 0.04 atm. No attempts were made to test directly the ability of the extracted brown pigment to inhibit the growth of rhizomorphs because any such treatment would probably have altered the chemistry of the pigment (Mason 1948).

Townsend (1954) claimed that the rind of rhizomorphs of *A. mellea* is composed of cells that are irregular in both size and shape, closely packed so that there are no intercellular spaces, and with dark brown walls that are thickened and fused. Our

electron micrographs show that the rind of rhizomorphs of *A. elegans* is formed from thin-walled cells that are loosely packed to produce large intercellular spaces. In brown rhizomorphs the pigment is not in the cell walls but restricted to the intercellular spaces. The brown pigment appears unable to precipitate protein and so how it interferes with the growth of rhizomorphs remains unresolved. It seems likely, however, that the heavy deposit of pigment around the cells could interfere with the uptake of nutrients or the excretion of waste products by the cells or both (Chet 1969).

It is now clear that not all the oxygen uptake by the rhizomorphs is through the central canal as assumed in the model in Section III because reduced oxygen partial pressures were recorded at the surface of submerged rhizomorphs. Hence, it may be that rhizomorphs flatten when diffusion of oxygen down their central canals becomes limiting, an increase in the surface to volume ratio ensuring better uptake of oxygen present in the surrounding medium.

V. STUDIES ON THE OXIDASE ENZYME CAUSING BROWNING OF RHIZOMORPHS

(a) *Introduction*

The oxidation of phenols causes tissues to become brown (Mason 1955) and so pigment formation on the rhizomorphs of *A. elegans* is likely to be catalysed by either *o*-diphenol oxidase (*o*-diphenol : oxygen oxidoreductase, E.C. 1.10.3.1), *p*-diphenol oxidase (*p*-diphenol : oxygen oxidoreductase, E.C. 1.10.3.2), or peroxidase (diphenol : hydrogen peroxide oxidoreductase, E.C. 1.11.1.7). Our results in Section IV, however, showed that rhizomorphs browned only when oxygen was present. This indicated that either *o*- or *p*-diphenol oxidase is responsible because peroxidase is active in the absence of oxygen (Theorell 1951).

o- and *p*-Diphenol oxidase have not been identified in *A. elegans* although both are formed by the mycelium of *A. mellea* (Boidin 1951; Käärik 1965; Jacques-Felix 1968). Lindeberg and Holm (1952) showed that these enzymes may be produced by different tissues of the one fungus. Therefore, to identify the diphenol oxidase that causes rhizomorphs of *A. elegans* to brown, the enzymes must be shown to exist in the rhizomorphs and not simply in the mycelium. Also, rhizomorphs from different sources should be studied because nutrition may influence the production of these enzymes (Jacques-Felix and Legrand 1954).

The method described by Demoulin (1967) to identify diphenol oxidases from other fungi was modified in our experiments. Although peroxidase is unlikely to cause browning, extracts of rhizomorphs were examined for this enzyme. Studies with enzyme inhibitors were also made on intact rhizomorphs to help establish a causal relationship between specific diphenol oxidases and pigmentation.

(b) *Experimental*

(i) *Examination of Rhizomorphs for Diphenol Oxidases and Peroxidase*

Rhizomorphs were grown in 30 by 1.25 cm glass tubes filled with nutrient broth and were freed of undifferentiated mycelium by removing the top 1.0 cm from each tube and washing thoroughly in distilled water. Samples, each of 5 g (wet weight), were then ground in the cold with a mortar and pestle in a small quantity

of acid-washed sand and 10 ml of MacIlvaine's buffer (0.2M disodium hydrogen orthophosphate and 0.1M citric acid). The ground material was made up to 25 ml with cold buffer and centrifuged at 31,000 *g* for 20 min at 2°C. The clear, pale yellow supernatant was tested for diphenol oxidases and peroxidase using a modification of the method described by Demoulin (1967). Two substrates were used: either (1) 1% guaiacol for *p*-diphenol oxidase and peroxidase; or (2) a saturated L-tyrosine for *o*-diphenol oxidase. Cuvettes containing 0.5 ml of substrate, 4.0 ml of MacIlvaine's buffer, and 0.5 ml of enzyme extract were incubated at 25°C in a water-bath. To test for peroxidase, 0.5 ml of buffer were replaced by an equal quantity of 0.2 volume hydrogen peroxide. The presence of the various enzymes was determined by measuring changes in the optical density of the solutions in the cuvettes with a Bausch & Lomb Spectronic 20 spectrophotometer at a wavelength of 490 nm at predetermined times after incubation.

In the present study, enzyme extracts were prepared at pH 3.0, 4.0, 5.0, 6.0, and 7.0 in MacIlvaine's buffer and tested for activity over this same range. Rhizomorphs that had developed 12, 20, 28, and 84 days after inoculation were examined. In these studies, malt Vegemite broth (MVB) was used as the standard medium. The activity of these enzymes was also examined on duplicate samples of extracts from rhizomorphs grown for 28 days in potato dextrose broth (PDB) and in a semi-synthetic broth (Garrett 1953) to ensure that the enzymes were not induced by the MVB.

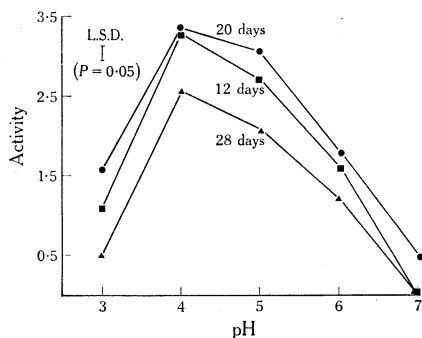


Fig. 10.—Effect of pH on the activity of *p*-diphenol oxidase extracted from rhizomorphs of different ages.

The extracts of enzymes from rhizomorphs were also checked for catalase (hydrogen peroxide : hydrogen peroxide oxidoreductase, E.C. 1.11.1.6) which catalyses the breakdown of hydrogen peroxide, and, in these experiments, may mask peroxidase. Duplicate samples of each enzyme extract at each pH were examined by adding two drops of 30 volume hydrogen peroxide to each tube. Violent bubbling occurred immediately when catalase was active.

Dry weight determinations were made on 10 samples of rhizomorphs grown in MVB for 12, 20, 28, and 84 days after inoculation so that the interaction between the activity of the enzyme, the age of the rhizomorphs, and pH could be studied.

p-Diphenol oxidase and catalase were identified in extracts from rhizomorphs of all ages irrespective of the medium in which they were grown. Catalase was active only at pH 6.0 and 7.0 whereas *p*-diphenol oxidase was active over a wide range of pH (Fig. 10; Table 3). In a few instances, activity of *p*-diphenol oxidase was not

strictly linear with time but this is to be expected when crude extracts are tested over such a range of conditions. Peroxidase and *o*-diphenol oxidase were not identified in any of the extracts even though the reaction tubes were incubated for 24 hr.

TABLE 3

ACTIVITY OF *p*-DIPHENOL OXIDASE IN EXTRACTS FROM RHIZOMORPHS GROWN FOR 84 DAYS IN MALT VEGEMITE BROTH, FOR 28 DAYS IN POTATO DEXTROSE BROTH, OR FOR 28 DAYS IN A SEMISYNTHETIC BROTH

Activity measured as change in optical density of solution at 490 nm

| pH | Replicate No. | Activity | | | | |
|-----------------------|---------------|----------|--------|--------|--------|-------|
| | | 5 min | 10 min | 15 min | 20 min | 3 hr* |
| Malt Vegemite broth | | | | | | |
| 3 | 1 | 0 | 0 | 0 | 0 | 0.045 |
| | 2 | 0 | 0 | 0 | 0 | 0.045 |
| 4 | 1 | 0.045 | 0.115 | 0.185 | 0.260 | —† |
| | 2 | 0.045 | 0.110 | 0.220 | 0.325 | —† |
| 5 | 1 | 0.015 | 0.055 | 0.110 | 0.175 | —† |
| | 2 | 0.015 | 0.060 | 0.120 | 0.190 | —† |
| 6 | 1 | 0.005 | 0.020 | 0.040 | 0.070 | —† |
| | 2 | 0.010 | 0.025 | 0.055 | 0.090 | —† |
| 7 | 1 | 0 | 0 | 0 | 0 | 0.030 |
| | 2 | 0 | 0 | 0 | 0 | 0.030 |
| Potato dextrose broth | | | | | | |
| 3 | 1 | 0 | 0.015 | 0.020 | 0.035 | — |
| | 2 | 0 | 0.015 | 0.020 | 0.025 | — |
| 4 | 1 | 0.140 | 0.280 | 0.450 | 0.580 | — |
| | 2 | 0.140 | 0.280 | 0.440 | 0.580 | — |
| 5 | 1 | 0.055 | 0.175 | 0.290 | 0.400 | — |
| | 2 | 0.060 | 0.180 | 0.295 | 0.410 | — |
| 6 | 1 | 0.015 | 0.030 | 0.050 | 0.070 | — |
| | 2 | 0.010 | 0.030 | 0.050 | 0.070 | — |
| 7 | 1 | 0 | 0 | 0 | 0 | 0.030 |
| | 2 | 0 | 0 | 0 | 0 | 0.025 |
| Semisynthetic broth | | | | | | |
| 3 | 1 | 0.070 | 0.190 | 0.300 | 0.400 | — |
| | 2 | 0.070 | 0.170 | 0.290 | 0.400 | — |
| 4 | 1 | 0.520 | 0.900 | —† | — | — |
| | 2 | 0.560 | 0.940 | —† | — | — |
| 5 | 1 | 0.160 | 0.390 | 0.570 | 0.750 | — |
| | 2 | 0.150 | 0.370 | 0.560 | 0.740 | — |
| 6 | 1 | 0.040 | 0.060 | 0.110 | 0.160 | — |
| | 2 | 0.035 | 0.065 | 0.110 | 0.150 | — |
| 7 | 1 | 0 | 0 | 0 | 0 | 0.060 |
| | 2 | 0 | 0 | 0 | 0 | 0.060 |

* Reaction time 5 hr for potato dextrose broth solution.

† Off scale.

Dark-brown, cylindrical rhizomorphs of *A. elegans* collected from roots of a diseased peach tree were also examined for *o*- and *p*-diphenol oxidases, peroxidase,

and catalase. Because few rhizomorphs were collected, enzyme extracts were tested for activity only at pH 4.0 and 6.5. Catalase immediately gave a positive reaction at pH 6.5 and *p*-diphenol oxidase a slight reaction at pH 4.0 after incubation for 24 hr.

(ii) *Effect of Enzyme Inhibitors on Browning*

(1) *Sodium Diethyldithiocarbamate and Phenylthiourea*.—White rhizomorphs grown for 20 days in either MVB, PDB, or Garrett's semisynthetic broth were washed thoroughly in distilled water. Two rhizomorphs from each medium were soaked for 15 min in one of three solutions: 0.001M sodium diethyldithiocarbamate; 0.01M phenylthiourea; or distilled water. The rhizomorphs from each treatment were placed in a McIntosh and Fildes anaerobic jar in a humid atmosphere of air. Rhizomorphs treated in phenylthiourea or distilled water were dark brown after 48 hr while those treated in sodium diethyldithiocarbamate were still white after 2 weeks.

(2) *Carbon Monoxide*.—White rhizomorphs grown for 20 days in either MVB, PDB, or Garrett's semisynthetic broth were washed in distilled water. Two rhizomorphs from each medium were placed in four McIntosh and Fildes anaerobic jars. Two of the jars contained a humidified atmosphere of air and two a humidified gas mixture (4:1 v/v) of carbon monoxide and oxygen. Within 24 hr all the rhizomorphs were brown irrespective of the treatment and no differences were evident in the speed or intensity of the colour change.

(3) *4-Chlororesorcinol*.—Twenty Petri dishes containing either PDA, or PDA plus 0.001M 4-chlororesorcinol were inoculated with *A. elegans* and incubated at 25°C. The latter medium was prepared by adding the 4-chlororesorcinol through a Millipore filter to autoclaved PDA. After 10 days rhizomorphs had developed well in all Petri dishes, and, regardless of the media used, submerged rhizomorphs rapidly browned except at their apices.

(iii) *Michaelis-Menten Constant for the Reaction of p-Diphenol Oxidase with Oxygen*

The *p*-diphenol oxidase for this determination was prepared from rhizomorphs cultured for 20 days in MVB. The enzyme was prepared at pH 4.0, as described in experiment (i), and its activity was also measured at pH 4.0. These conditions are optimum for the activity of *p*-diphenol oxidase (Fig. 10).

The Michaelis-Menten constant (K_m) was determined by measuring the initial velocity of the oxidation of guaiacol by *p*-diphenol oxidase at oxygen partial pressures of 1.00, 0.21, 0.12, 0.08, 0.04, and 0 atm, obtained by using mixtures of oxygen and oxygen-free nitrogen.

A series of cuvettes containing 4.0 ml of MacIlvaine's buffer at pH 4.0 and 0.5 ml of a 1% solution of guaiacol were placed in a water-bath at 25°C. A humidified gas mixture at the partial pressure of oxygen being studied was bubbled through each cuvette for 2 min at the rate of 200 ml min⁻¹. After 0.5 ml of *p*-diphenol oxidase was added, the gas mixture was bubbled through the cuvette for 5 min. Then the cuvette was placed in a Bausch & Lomb Spectronic 20 spectrophotometer and the initial velocity of the oxidation reaction read directly from the change in the optical

density of the solution at a wavelength of 490 nm. Values obtained are given in the following tabulation:

| Partial Pressure of Oxygen (atm) | Replicate 1 | Replicate 2 |
|--|-------------|-------------|
| 1.00 | 0.850 | 0.900 |
| 0.21 | 0.720 | 0.720 |
| 0.12 | 0.650 | 0.650 |
| 0.08 | 0.630 | 0.580 |
| 0.04 | 0.450 | 0.420 |
| 0 | 0.035 | 0.025 |

The reaction was not stopped before the readings were made because the commonly used inhibitors perchloric acid (3%), trichloroacetic acid (3%), azide (1 mM), or heat all caused marked colour change.

The K_m value for *p*-diphenol oxidase was determined by calculating the linear regression of the reciprocals of the initial velocity (v) on the partial pressure of oxygen (S) when the value of $1/v$ in 1 atm oxygen was made unity and the other values were scaled accordingly. Thus:

$$1/v = 0.042(1/S) + 1.008.$$

When $1/v = 0$, from the Lineweaver and Burk plot $1/S = -1/K_m$ and so $K_m = 0.042$ atm oxygen, i.e. 5.19×10^{-5} M oxygen. From the regression line, the 95% confidence interval for the K_m value of *p*-diphenol oxidase was $(5.19 \pm 0.42) \times 10^{-5}$ M oxygen.

(c) Discussion

p-Diphenol oxidase causes rhizomorphs of *A. elegans* to brown. This was concluded partly from enzyme analyses which showed that rhizomorphs produce this enzyme and partly from enzyme-inhibitor studies that established a causal relationship between this enzyme and browning. Studies with enzyme inhibitors must be interpreted carefully but if used in combination with other methods, such as enzyme analyses of extracts, valuable results can be obtained (Hill and Hartree 1953; James 1953). Sodium diethyldithiocarbamate inhibited the browning of rhizomorphs, indicating that either *o*- or *p*-diphenol oxidase is responsible (Dubois and Erdway 1946; James and Garton 1952), whereas the failure of both carbon monoxide and 4-chlororesorcinol to inhibit browning indicates that the reaction is catalysed by *p*-diphenol oxidase (Dawson and Tarpley 1951; Kull, Bonorden, and Mayer 1954; Bonner 1957; Hampton and Fulton 1961).

o-Diphenol oxidase and peroxidase were not found in rhizomorphs of *A. elegans*. However, Dawson and Tarpley (1951) claimed that active *o*-diphenol oxidase is sometimes difficult to demonstrate in enzyme extracts because it has a long induction period which is increased in the presence of *p*-diphenol oxidase. In our experiments the enzyme extracts were examined for *o*-diphenol oxidase activity over a period of 24 hr and hence it is unlikely that its presence was not detected. Catalase was present in the rhizomorphs and may have masked peroxidase. Catalase, however, was active only at pH 6.0 and 7.0 whereas peroxidase from fungi usually has an optimum between pH 4.0 and 6.0 (Lyr 1956; Demoulin 1967). Therefore, it should have

been possible to identify peroxidase in the extracts from rhizomorphs even in the presence of catalase. Catalase may be active over a wider pH range in extracts from other fungi and it is suggested that Demoulin's (1967) method for identifying peroxidase in fungi be modified to include a test for catalase.

p-Diphenol oxidase was most active at pH 4.0 which agrees with results found for the same enzyme obtained from other fungi including *A. mellea* (Demoulin 1967; Jacques-Felix 1968). Jacques-Felix and Legrand (1955) claimed that *p*-diphenol oxidase was not produced by cultures of *A. mellea* older than 40–60 days. Rhizomorphs of *A. elegans* still produced this enzyme after 84 days although activity was reduced in cultures older than 20 days. This may explain why the enzyme was difficult to demonstrate in rhizomorphs collected in the field.

The K_m value of oxygen for *p*-diphenol oxidase is of the same order as that obtained by Goddard and Bonner (1960) and Frieden, Osaki, and Kobayashi (1965) for *o*-diphenol oxidase. This is an expected result because of the close relationship between the two enzymes (Bonner 1957). The K_m value is in accord with the results obtained in Section IV where the pigmentation of rhizomorphs occurred only when the partial pressure of oxygen at their surface exceeded 0.04 atm.

Finally, these results showed that the brown pigment on the surface of the rhizomorphs is not a melanin because its formation is catalysed by *p*- and not by *o*-diphenol oxidase (Thomas 1955; Thomson 1962, 1965).

VI. EFFECT OF SOIL MOISTURE ON THE GROWTH OF RHIZOMORPHS

(a) Introduction

Our results show that rhizomorphs, for optimum growth, require low partial pressures of oxygen in contact with the external surfaces of their apices and high partial pressures in their central canals. Thus, soil moisture through its effect on oxygen content of the soil should influence the growth of rhizomorphs. Similarly, a correlation should exist between soil moisture and incidence of disease caused by *A. elegans* because rhizomorphs are vital to the spread of *Armillariella* spp. in soil. Information regarding any such relationship is confused (Dadant 1963).

Experiments were done to determine the indirect effects that soil moisture has on the growth of rhizomorphs of *A. elegans*. Sandy soils were used because they do not shrink appreciably on drying and so maintain a precise inverse relationship between their moisture content and their air-filled porosity. Thus, any changes in the growth of rhizomorphs at different matric suctions can be correlated with changes in the oxygen content of the soil. Another advantage of using sandy soils is that small changes in matric suction cause large changes in their oxygen content with the result that change in the potential of the water will not complicate the results. Moisture characteristics for each soil were determined so that the air-filled porosity of the soil at the different suctions was known. Also, a mechanical analysis of each soil was made to assess whether changes in texture modified the growth of rhizomorphs.

(b) Experimental

(i) Moisture Characteristics

The moisture characteristics of three sandy soils were obtained using the technique described by Griffin (1963*b*). The soils were passed through a 25-mesh

sieve and the moisture characteristics of each determined on a 20-g sample of oven-dried soil (Fig. 11).

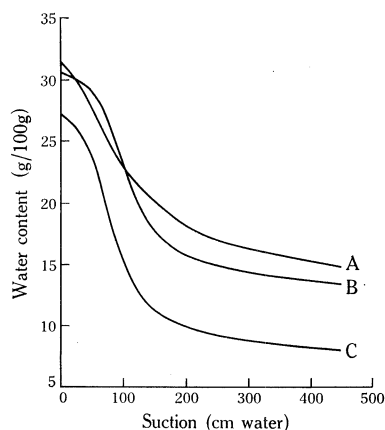


Fig. 11.—Drying boundary curves of the moisture characteristics of the three soils used to study the effect of changes in soil moisture on the growth of rhizomorphs.

(ii) Mechanical Analyses

A mechanical analysis was made on sieved samples of each soil (Table 4). The method of Bouyoucos (see Bayer 1956) was used.

TABLE 4
MECHANICAL ANALYSES OF SOILS A, B, AND C
Values given as grams per 100 g sample

| Particle Size (mm) | Soil A | Soil B | Soil C |
|-----------------------|--------|--------|--------|
| 1—0.2 | 44.50 | 56.67 | 64.83 |
| 0.2—0.02 | 18.17 | 24.00 | 20.83 |
| 0.02—0.002 | 3.80 | 6.93 | 3.77 |
| <0.002 | 28.27 | 6.53 | 3.20 |

(iii) Soil Moisture and the Growth of Rhizomorphs

Balsa wood blocks, 2.5 by 0.6 by 0.6 cm, impregnated with MVB, were used to provide a food base for the rhizomorphs. The blocks were autoclaved in the nutrient broth, soaked for 24 hr, and then transferred aseptically to 500-ml Erlenmeyer flasks in which *A. elegans* had been growing for 4 weeks on MVA. The cultures were then incubated for 4 weeks at 25°C.

A series of 7.5-cm diameter sintered-glass Buchner funnels (porosity grade 5) was prepared, as described by Griffin (1963*b*). About 1.0 cm of sieved soil was placed in each funnel and saturated with water. The funnels were tapped vigorously to consolidate the soil and to remove air bubbles. Four balsa wood blocks colonized by *A. elegans* were placed in each funnel. Soil was slowly added until 4.0 cm deep.

All funnels were flooded with water and care taken to consolidate the soil and exclude air bubbles. The growth of rhizomorphs was studied in the three soils (A, B, C) at eight different matric suctions. The experiment was made at 25°C and the funnels covered with plastic to minimize water loss.

After 4 weeks the soil was carefully washed from each funnel and rhizomorphs attached to each block collected and their dry weight recorded. The growth of rhizomorphs in each soil at the different matric suctions was analysed by a one-way analysis of variance and the mean dry weights compared by the method of the least significant difference ($P = 0.05$). Significant results were obtained with soils A and B [Figs. 12(a) and 12(b)].

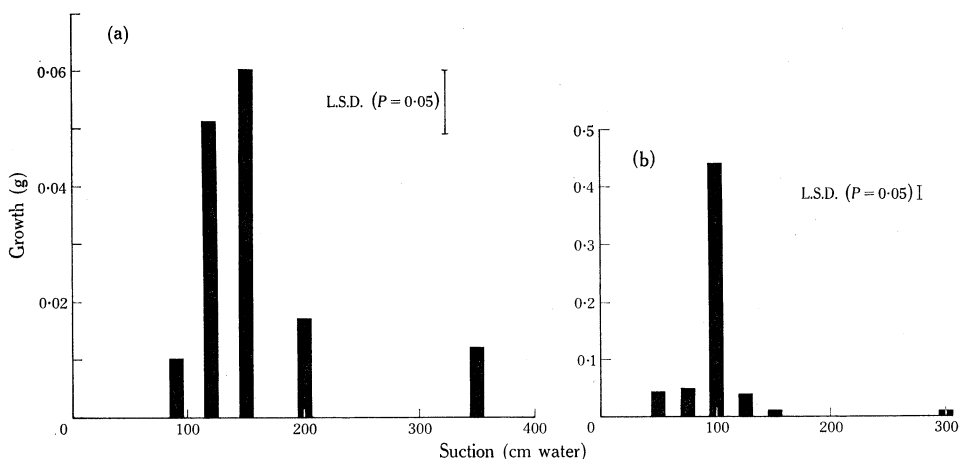


Fig. 12.—Growth of rhizomorphs in soil A (a) at suctions of 30, 50, 70, 90, 120, 150, 200, and 350 cm water and in soil B (b) at suctions of 20, 50, 75, 100, 125, 150, and 300 cm water.

(c) Discussion

These studies showed that rhizomorphs of *A. elegans* were able to grow from buried inocula for extended distances through soil only under specific moisture conditions. Only two of the soils gave significant results but the third soil (C) was so sandy that even minor differences in packing, either within or between funnels, may have changed significantly the moisture content at a given suction. The variation within replicates recorded from soil C supported this conclusion.

It seems likely that rhizomorphs were not formed at small matric suctions because the soils were then almost water-saturated. Under these conditions, continuity of the gas phase between the atmosphere and the surface of the inoculum would be extremely tenuous and growth of rhizomorphs would be negligible. In the field the inoculum is not always completely buried but, as in a tree stump, may be in contact with the atmosphere. Under these conditions rhizomorphs may grow into a saturated soil [cf. Section II(c)(iv)].

At high matric suctions it is likely that growth was inhibited because the water film covering the apex of the rhizomorph was removed. The resulting high concentration of oxygen in contact with the apex would be inhibitory. At a given suction it would be easier to retain a water film over the apex of a rhizomorph in heavier, rather than lighter, soils. The increased bulk density of the soil adjacent

to a penetrating organ (Farrell and Greacen 1966) may also be important here. Growth of rhizomorphs across small air-filled voids or for a short distance above an agar surface may be facilitated by the barrier to oxygen diffusion presented by the mucilaginous sheath surrounding the apices (Motta 1969).

These studies indicated that rhizomorphs will not form in light-textured, well-drained soils of low bulk density. Thus, it may be possible to control disease caused by *A. elegans* by altering the properties of a soil to produce such conditions. Field control of soil texture and moisture are difficult but regular cultivation reduces the bulk density in the top layers of the soil. Most rhizomorphs are found in the top few inches of soil (Day 1927; Boullard 1961; Redfern 1968) and, therefore, this treatment may be sufficient to control their growth. Also, the rhizomorphs in the surface soil are the most important because they are the ones most likely to initiate a fatal infection by directly attacking the collar of a plant (Bliss 1946; Larue *et al.* 1962). Knowing the specific conditions that favour the growth of rhizomorphs also makes it possible to predict the location of problem areas in an orchard or plantation. Thus, preventative measures may be adopted early enough to either prevent infection of hosts or limit the spread of the disease.

VII. CONCLUSION

Our studies showed that oxygen has a key role in the ecology of *A. elegans* because it controls the rate of growth and morphology of the rhizomorphs.

In soil, rhizomorphs are cylindrical and grow rapidly for extended distances from a single apical meristem when attached to a food base. Because of the concentration of tissue in rhizomorphs and their rapid growth, apices have a high requirement for oxygen. This is satisfied by the specially adapted central canals and by the ability of rhizomorphs to make repeated connections between their central canals and the air-filled pores of the soil. When formed under the bark of a host, rhizomorphs no longer have a single apical meristem but become flat and plaque-like and their growth slows rapidly. Butler and Jones (1949) and Jacques-Felix (1967) claimed that these flat rhizomorphs are caused by pressure. We have no information on the role of pressure but our results suggest that flattening of rhizomorphs under bark may occur, at least in part, because of limitations placed on the diffusion rate of oxygen down their central canals. This occurs because rhizomorphs are unable to form functional "breathing pores" under intact bark where substrate-air interfaces do not occur. Cylindrical rhizomorphs are important in the ecology of *A. elegans* because they enable this pathogen to spread rapidly from host to host and also increases its infection potential (Garrett 1951). Flat rhizomorphs, on the other hand, with their slow rate of growth and increased surface to volume ratio, are more efficient for absorbing nutrients from the host.

p-Diphenol oxidase causes rhizomorphs of *A. elegans* to brown and there is strong circumstantial evidence that rapid browning of the apices of rhizomorphs inhibits their growth. Thus, the effect of oxygen on the kinetics of *p*-diphenol oxidase may have a practical application because it may be used to control disease. By manipulating the soil, the concentration of oxygen at the surface of the apices of rhizomorphs may be increased sufficiently to stimulate the activity of *p*-diphenol oxidase and prevent rhizomorphs spreading from host to host.

There is evidence that these brown pigments protect fungal organs from microbial lysis in soil (Potgieter and Alexander 1966; Bloomfield and Alexander 1967; Kuo and Alexander 1967; Bull and Alexander 1969; Hurst and Wagner 1969). In both higher plants and fungi, however, the role of diphenol oxidases and the brown pigments whose formation they catalyse are often not well understood (Thomas 1955). Clearly they are important in the ecology of *A. elegans*.

VIII. ACKNOWLEDGMENTS

The authors wish to thank Dr. D. G. Pederson, Biometry Section, Department of Agricultural Botany, University of Sydney, for the computer analyses. The investigation was financed by a Rural Credits Development Fund Grant from the Reserve Bank of Australia.

IX. REFERENCES

- BAVER, L. D. (1956).—"Soil Physics." (John Wiley & Sons: New York.)
- BLISS, D. E. (1946).—The relation of soil temperature to the development of *Armillaria* root-rot. *Phytopathology* **36**, 302-18.
- BLOOMFIELD, B. J., and ALEXANDER, M. (1967).—Melanins and resistance of fungi to lysis. *J. Bact.* **93**, 1276-80.
- BOIDIN, J. (1951).—Recherche de la tyrosinase et de la laccase chez les Basidiomycetes en culture pure. Milieux différentiels-intérêt systématique. *Revue Mycol.* **16**, 173-97.
- BONNER, W. D. (1957).—Soluble oxidases and their function. *A. Rev. Pl. Physiol.* **8**, 427-52.
- BOULLARD, B. (1961).—Étude d'une attaque de "*l'Armillariella mellea*" (Vahl) Qué. sur l'épicéa de Sitka (*Picea sitchensis*); biologie du parasite, moyens de lutte. *Revue for. fr.* **13**, 16-24.
- BULL, A. T., and ALEXANDER, M. (1969).—Inhibition of enzyme polysaccharases by melanin and its significance in mycolysis. *Bact. Proc.* **69**, 36-7.
- BUTLER, E. J., and JONES, S. G. (1949).—"Plant Pathology." (Macmillan: London.)
- CAMPBELL, A. H. (1934).—Zone lines in plant tissue. II. Re black lines formed by *Armillaria mellea* (Vahl) Qué. *Ann. appl. Biol.* **21**, 1-22.
- CHARLTON, G. (1961).—A microelectrode for determination of dissolved oxygen in tissue. *J. appl. Physiol.* **16**, 729-33.
- CHASE, W. (1934).—The composition, quantity, and physiological significance of gases in tree stems. Tech. Bull. Minn. agric. Exp. Stn No. 99.
- CHET, I. (1969).—The role of sclerotial rind in the germinability of sclerotia of *Sclerotium rolfsii*. *Can. J. Bot.* **47**, 593-5.
- DADANT, R. (1963).—Contribution à l'étude du pourridié du Caféier causé par le *Clitocybe elegans* Heim à Madagascar. *Revue Mycol.* **28**, 95-168.
- DAWSON, C. R., and TARPLEY, W. B. (1951).—Copper oxidases. In "The Enzymes". (Eds. J. Sumner and K. Myrbäck.) Vol. 2. (Academic Press: New York.)
- DAY, W. R. (1927).—The parasitism of *Armillaria mellea* in relation to conifers. *Qd J. For.* **21**, 9-21.
- DE BARY, A. (1887).—"Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria." (Clarendon Press: Oxford.)
- DEMOULIN, V. (1967).—Proposition d'un processus de détection des phénoloxydases et peroxydases chez les Macromycetes. Application à quelques Gasteromycètes. *Planta* **76**, 129-37.
- DUBOIS, K. P., and ERDWAY, W. F. (1946).—Studies on the mechanisms of action of thiourea and related compounds. II. Inhibition of oxidative enzymes and oxidations catalysed by copper. *J. biol. Chem.* **165**, 711-21.
- ELLIS, E. H. (1929).—*Armillaria mellea* in a mine-working. *Trans. Br. mycol. Soc.* **14**, 305-7.
- FARRELL, D. A., and GREACEN, E. L. (1966).—Resistance to penetration of fine probes in compressible soil. *Aust. J. Soil Res.* **4**, 1-17.
- FINDLAY, W. P. K. (1951).—The development of *Armillaria mellea* rhizomorphs in a water tunnel. *Trans. Br. mycol. Soc.* **34**, 146.

- FRIEDEN, E., OSAKI, S., and KOBAYASHI, H. (1965).—Copper proteins and oxygen. In "Oxygen". Proceedings of symposium sponsored by the New York Heart Association. (J. & A. Churchill Ltd.: London.)
- GARD, M. (1927).—À propos des "Mourios" de l'Aveyron. *Revue Path. vég. Ent. agric. Fr.* **14**, 24–6.
- GARRETT, S. D. (1951).—Ecological groups of soil fungi: a survey of substrate relationships. *New Phytol.* **50**, 149–66.
- GARRETT, S. D. (1953).—Rhizomorph behaviour in *Armillaria mellea* (Vahl) Quél. I. Factors controlling rhizomorph initiation by *A. mellea* in pure culture. *Ann. Bot.* **17**, 63–79.
- GARRETT, S. D. (1956).—Rhizomorph behaviour in *Armillaria mellea* (Vahl) Quél. II. Logistics of infection. *Ann. Bot.* **20**, 193–209.
- GARRETT, S. D. (1960).—Inoculum potential. In "Plant Pathology". (Eds. J. Horsfall and A. Dimond.) Vol. 3. (Academic Press: New York.)
- GIBSON, I. A. S. (1961).—A note on variation between isolates of *Armillaria mellea* (Vahl ex Fr.) Kummer. *Trans. Br. mycol. Soc.* **44**, 123–8.
- GODDARD, D., and BONNER, W. D. (1960).—Cellular respiration. In "Plant Physiology 1A". (Ed. F. C. Steward.) (Academic Press: New York.)
- GRIFFIN, D. M. (1963a).—Soil moisture and the ecology of soil fungi. *Biol. Rev.* **38**, 141–66.
- GRIFFIN, D. M. (1963b).—Soil physical factors and the ecology of fungi. I. Behaviour of *Curvularia ramosa* at small soil water suctions. *Trans. Br. mycol. Soc.* **46**, 273–80.
- GRIFFIN, D. M. (1968).—A theoretical study relating the concentration and diffusion of oxygen to the biology of organisms in soil. *New Phytol.* **67**, 561–77.
- GRIFFIN, D. M. (1969).—Soil water in the ecology of fungi. *A. Rev. Phytopath.* **7**, 289–310.
- GRIFFIN, D. M., NAIR, N. G., BAXTER, R. I., and SMILES, D. E. (1967).—Control of gaseous environment of organisms using a diffusion column technique. *J. exp. Bot.* **18**, 518–25.
- GUNDERSEN, K. (1961).—Growth of *Fomes annosus* under reduced oxygen pressure and the effect of carbon dioxide. *Nature, Lond.* **190**, 649.
- GUSTAVSON, K. H. (1956).—"Chemistry of Tanning Processes." (Academic Press: New York.)
- HAMADA, M. (1940).—Physiologisch- morphologische Studien über *Armillaria mellea* (Vahl) Quél., mit besonderer Rücksicht auf die Oxalsäurebildung. *Jap. J. Bot.* **10**, 387–464.
- HAMPTON, R., and FULTON, R. (1961).—The relation of polyphenol oxidase to instability *in vitro* of prune dwarf and sour cherry necrotic ringspot virus. *Virology* **13**, 44–52.
- HEIM, R. (1963).—L'*Armillariella elegans* Heim. *Revue Mycol.* **28**, 90–4.
- HEIM, R. (1967).—Notes sur la flore mycologique des Terres du Pacifique Sud. VI. Note complémentaire sur l'*Armillariella elegans* Heim. *Revue Mycol.* **32**, 1–15.
- HILL, R., and HARTREE, E. (1953).—Hematin compounds in plants. *A. Rev. Pl. Physiol.* **4**, 115–50.
- HURST, H. M., and WAGNER, G. H. (1969).—Decomposition of ¹⁴C-labelled cell wall and cytoplasmic fractions from hyaline and melanin fungi. *Proc. Soil Sci. Soc. Am.* **33**, 707–11.
- JACQUES-FELIX, M. (1967).—Recherches morphologiques, anatomiques, morphogenetiques et physiologiques sur des rhizomorphes de champignons superieurs et sur le déterminisme de leur formation. *Bull. trimest. Soc. mycol. Fr.* **83**, 5–103.
- JACQUES-FELIX, M. (1968).—Recherches morphologiques, anatomiques, morphogenetiques et physiologiques sur des rhizomorphes de champignons superieurs et sur le déterminisme de leur formation. Deuxieme partie. *Bull. trimest. Soc. mycol. Fr.* **84**, 166–307.
- JACQUES-FELIX, M., and LEGRAND, G. (1954).—Influence du milieu de culture sur l'activité oxydasique du mycélium et des rhizomorphes d'*Armillariella mellea*. *C.r. hebd. Séanc. Acad. Sci., Paris* **239**, 1404–6.
- JACQUES-FELIX, M., and LEGRAND, G. (1955).—Présence, chez *Armillariella mellea*, d'un système oxydant les phénols en milieu acide. *C.r. hebd. Séanc. Acad. Sci., Paris* **240**, 2257–9.
- JAMES, W. O. (1953).—The use of respiratory inhibitors. *A. Rev. Pl. Physiol.* **4**, 59–90.
- JAMES, W. O., and GARTON, N. (1952).—The use of sodium diethyldithiocarbamate as a respiratory inhibitor. *J. exp. Bot.* **3**, 310–18.
- KÄÄRIK, A. (1965).—The identification of the mycelia of wood-decay fungi by their oxidation reactions with phenolic compounds. *Studia forestal. suec.* **31**, 1–80.
- KULL, F., BONORDEN, R., and MAYER, R. (1954).—Inhibition of melanin formation *in vivo* by 4-chlororesorcinol. *Proc. Soc. exp. Biol. Med.* **87**, 538–40.

- KUO, M. J., and ALEXANDER, M. (1967).—Inhibition of the lysis of fungi by melanins. *J. Bact.* **94**, 624–9.
- LARUE, J., PAULUS, A., WILBUR, W., O'REILLY, H., and DARLEY, E. (1962).—*Armillaria* root rot fungus controlled with methyl bromide soil fumigation. *Calif. Agric.* **16**, 8–9.
- LINDBERG, G., and HOLM, G. (1952).—Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some Hymenomycetes. *Physiologia Pl.* **5**, 100–14.
- LYR, H. (1956).—Untersuchungen über die Peroxydasen höherer Pilze. *Planta* **48**, 239–65.
- MASON, H. S. (1948).—A classification of melanins. Spec. Publ. N.Y. Acad. Sci. No. 4, pp. 399–404.
- MASON, H. S. (1955).—Comparative biochemistry of the phenolase complex. *Adv. Enzymol.* **16**, 105–84.
- MORQUER, R., LACOSTE, L., and BLAHA, G. (1967).—Contribution à l'étude pathogénique de *Clitocybe mellea* (Vahl) Ricken. *Bull. trimest. Soc. mycol. Fr.* **83**, 124–97.
- MOTTA, J. J. (1969).—Cytology and morphogenesis in the rhizomorph of *Armillaria mellea*. *Am. J. Bot.* **56**, 610–19.
- POTGIETER, H. J., and ALEXANDER, M. (1966).—Susceptibility and resistance of several fungi to microbial lysis. *J. Bact.* **91**, 1526–32.
- RAABE, R. D. (1958).—The effect of light upon growth of *Armillaria mellea* in culture. *Phytopathology* **48**, 397.
- RAABE, R. D. (1962).—Host lists of the root-rot fungus, *Armillaria mellea*. *Hilgardia* **33**, 25–88.
- RAABE, R. D. (1966).—Variation of *Armillaria mellea* in culture. *Phytopathology* **56**, 1241–4.
- RAABE, R. D. (1967).—Variation in pathogenicity and virulence in single spore isolates of *Armillaria mellea*. *Phytopathology* **57**, 826.
- RAABE, R., and GOLD, A. H. (1967).—Effects of different levels of carbon dioxide on the growth of *Armillaria mellea* in culture. *Phytopathology* **57**, 101.
- REDFERN, D. B. (1968).—The ecology of *Armillaria mellea* in Britain: biological control. *Ann. Bot.* **32**, 293–300.
- REITSMA, J. (1932).—Studien über *Armillaria mellea* (Vahl) Qué. *Phytopath. Z.* **4**, 461–522.
- RHOADS, A. S. (1945).—A comparative study of two closely related root-rot fungi, *Clitocybe tabescens* and *Armillaria mellea*. *Mycologia* **37**, 741–66.
- RHOADS, A. S. (1956).—The occurrence and destructiveness of *Clitocybe* root rot of woody plants in Florida. *Lloydia* **19**, 193–240.
- SINGER, R. (1962).—“The Agaricales in Modern Taxonomy.” (J. Cramer: Weinheim.)
- SMITH, A. M. (1969).—Oxygen and the ecology of *Armillariella elegans* Heim. Ph.D. Thesis, University of Sydney.
- SNIDER, P. J. (1957).—Rhizomorph development in *Armillaria mellea* (Vahl ex Fr.) Qué. Ph.D. Thesis, Harvard University.
- SNIDER, P. J. (1959).—Stages of development in rhizomorphic thalli of *Armillaria mellea*. *Mycologia* **51**, 693–707.
- STOLZY, L. H., and VAN GUNDY, S. D. (1968).—The soil as an environment for microflora and microfauna. *Phytopathology* **58**, 889–99.
- TABAK, H. H., and COOKE, W. B. (1968).—The effects of gaseous environments on the growth and metabolism of fungi. *Bot. Rev.* **34**, 126–252.
- THACKER, D. G., and GOOD, H. M. (1952).—The composition of air in trunks of sugar maple in relation to decay. *Can. J. Bot.* **30**, 475–85.
- THEORELL, H. (1951).—The iron-containing enzymes. B. Catalases and peroxidases. In “The Enzymes”. (Eds. J. Summer and K. Myrbäck.) Vol. 2. (Academic Press: New York.)
- THOMAS, M. (1955).—Melanins. In “Modern Methods of Plant Analysis”. (Ed. K. Paech and M. V. Tracey.) (Springer-Verlag: Berlin.)
- THOMSON, R. H. (1962).—Melanins. In “Comparative Biochemistry”. (Eds. M. Florkin and H. S. Mason.) Vol. 3. (Academic Press: New York.)
- THOMSON, R. H. (1965).—Miscellaneous pigments. In “Chemistry and Biochemistry of Plant Pigments”. (Ed. T. W. Goodwin.) (Academic Press: London.)
- TOWNSEND, B. B. (1954).—Morphology and development of fungal rhizomorphs. *Trans. Br. mycol. Soc.* **37**, 222–33.