SOME CONTRIBUTIONS TO THE BIOLOGY OF ARMILLARIA MELLEA (VAHL EX FR.) QUEL.

being a thesis presented by

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CONTENTS

SUMMARY

1. Six strains of *A. mellea*, obtained from different sources, were cultured in the laboratory and various aspects of their biology were studied.

2. Formation of rhizomorphs and sclerotia, and dry weight of fungal colonies, were reduced by the incidence of light on both solid and liquid media. The amount and intensity of light affected the form of the colony.

3. Maximum growth of all strains took place at 25°C., but a tropical strain developed better than a temperate one at higher temperatures.

4. Greater rate of linear spread of the mycelium took place at pH 7.

5. Mycelial, rhizomorphic and sclerotial development was affected by the composition of the medium and favoured by those media containing the greatest amount of dissolved nutrients. Glucose and peptone were the most suitable carbon and nitrogen sources, respectively.

6. The inoculum influenced the development of cultures and a rhizomorph-containing portion gave a colony with the maximum formation of rhizomorphs and sclerotia.

7. A colony of one strain of *A. mellea* produced

fructifications on a peptone-glucose-saccharose solution after eight months in daylight.

8. Double culture of A. mellea with various wood-destroying and soil fungi showed that growth of A. mellea was adversely affected in most instances.

9. Spores from field fructifications gave greatest percentage germination on substrates of acid reaction at an optimum temperature of 25°C.

10. Normal growth of mycelium, sclerotium and rhizomorphs occurred in monospore cultures though not all isolates formed colonies.

11. Spores did not act as agents of infection of bean roots.

12. In agar culture of A. mellea and bean seedlings, roots of the latter were penetrated by rhizomorphs and penetration was influenced by the nutrients in the agar.

13. Hyphal invasion of roots took place, and hyphal penetration occurred in advance of attack by rhizomorphs.

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I. INTRODUCTION.

Armillaria mellea (Fr.) Quél. is a Basidiomycete, a member of the order Hymenomycetales, and of the family Agaricaceae which includes the common mushrooms and toadstools.

A. mellea is of world-wide distribution and has been recorded in varying climatic conditions. It has been observed in the continents of Europe, America and Africa, and it is common in many other countries including India and Japan. The host range of A. mellea is great; both hard and soft woods are attacked, also economic and ornamental plants if growing in ground infected with the fungus. Among plants which are severely affected by this organism are the plantation crops of tea, coffee and citrus fruits. Some trees are known to have resistant varieties, The pathogenicity of A. mellea itself is thought to be a variable property, the latter possibly being due to the occurrence of different strains of the fungus, to local environmental conditions, or to the presence of other soil fungi.

Many records of the occurrence of A. mellea are available and a number of investigations have been made. Some detailed studies of the physiology, including cultural characteristics and requirements have been published, but investigations of pathogenicity have often been in the form of field trials, with subsequent detailed

examination of the invaded tissue in some cases.

Thomas (1934) gives a description of the invasion of various plants by A. mellea. The initial penetration of the host takes place by means of rhizomorphic branches which first attach themselves to the roots by a hardening mucilage, and by hyphae growing out from near the rhizomorphic tips. These hyphae only penetrate the dead cork cells and the actual invasion of the root takes place by the entrance of the rhizomorph as a whole.

Butler and Jones (1949) give an account of the Armillaria root rot which is similar to that given by Hiley (1919) for larch. The fungus develops under the bark of the roots and spreads upwards into the trunk, in the form of a rhizomorph network. At a more advanced stage of attack, the wood is also found to be invaded by the fungus which forms xylostromata, and the host tissue finally becomes disintegrated.

The presence of the fungus, as shown by a visible effect on the host, is rarely evident until the decay has reached an advanced stage. The first symptom to be seen is a yellowing of the leaves with subsequent loss of the foliage. Formation of resin appears to be stimulated in some cases, and this oozes out through cracks in the bark. This latter effect is noted in some coniferous hosts, but not in other plants which may also become invaded. Fructifications may be produced in the final stages of the disease, and are usually at ground level (see Plate 1).

The biology, ecology and pathogenicity of A. mellea suggest many lines of work. The author has selected for particular study, various cultural and pathogenic features of the fungus. Scrutiny of the previous investigations made, show that relatively little attention has been paid to the initial penetration of the fungus into the host, except by the agency of rhizomorphs. The particular studies reported here are concerned with possible methods of penetration into the host, and such matters as the effect of fungal invasion on the neighbouring tissues of the host.

From previous investigations and observations recorded, it was seen that although the organism is well known and widespread, no completely effective control measures are available for all areas. It was thought that further information on the pathogenic habit of the fungus, particularly its invasion method and also its interrelationship with other micro-organisms of the soil might contribute to our knowledge of measures of control.

In this study, some interesting data have been obtained. It was not always possible to advance an adequate explanation of the observed phenomena, but where possible this has been attempted.

Plate 1. Fructifications of A. mellea growing from the base of the stem of a young conifer. Note the typical defoliation of the branches due to the disease.

II. REVIEW OF LITERATURE

The majority of records of A. mellea state the host plant, or plants, on which it occurs and occasionally macroscopic features of its appearance are also given. These observations are usually made by pathologists, mycologists or other workers dealing with forests, crops or plantations. In Britain, this fungus is known to occur in most districts, and is well-known to foresters as the cause of a disease of coniferous wood. Many other plants are subject to infection, but in this county the effect on trees is of the greatest economic importance.

Out of the mass of literature available, thepapers which deal in sufficient detail with the physiology and pathology of the organism have been selected for this review. Two sections are presented, each giving the major investigations for the two above-mentioned aspects.

Information about the early work is taken from Hiley (1919), who gave details of his own and other investigations Most accounts of later investigations including those referred to by Reitsma (1932), Garrett (1944) and Butler and Jones (1949), were obtained from the original papers.

a) Literature on physiology of A. mellea

There are not many early records of investigations on A. mellea and the relevant work may be considered as beginning with Hartig in 1874. In this year, Hartig discovered that the forms Rhizomorpha subcorticalis and R. subterranea were the sterile mycelial strands of

A. mellea. He also mentioned that when he grew spores of this fungus on plum decoction they developed into a white mycelial mass and later this developed rhizomorphs. Information was given of the infection method and symptoms of the disease.

The conditions required by A. mellea for growth have received the attention of numerous investigators in more recent times.

The germination of the spores was investigated by Gard in 1923 and he showed that the optimum temperature was 18°- 20°C.; they germinated well on sterilised manure and gave a well-developed mycelium. Germination was inhibited by iron sulphate, which also inhibited the growth of mycelial strands of the organism. Later, in 1928, while investigating the occurrence of A. mellea in alkaline soils he found that the fungus was rarely present in soils containing more than 25 per cent. lime, except where they had an increased water-holding capacity. The spores germinated poorly in 0.13 per cent. lime water; mycelial growth was reduced in nutrient media containing 2 or 1 per 100 parts of precipitated chalk. The occurrence of the fungus was noted, however, in one instance with 55.3 per cent. lime content of the soil, and the previously noted relationship between lime content and the presence of A. mellea was questioned (Jöessel and Bordas, 1931).

Other data have been obtained on the environmental conditions suitable for development, and in Germany it was

reported that damp warm weather favoured saprophytism of the fungus and a dry season, parasitism (Nechleba,1927). Dade (1927) considered humidity to be a determining factor in increasing the tendency to parasitism; this view was supported by Ritchie (1932) who considered that in a wood of open canopy, as one of Scots pine, the conditions are damper and more favourable for infection of the trees, than in a wood of closed canopy. Bliss (1946) stated that soil temperature in South California seemed to prevent the development of A. mellea when it remained at more than 26°C. for some months in the year.

The soil type is also considered to have an effect on the development of the fungus and Reitsma (1932) found that light acid soils allowed its growth. Laurent (1938) stated that heavy wet soils could be associated with the rapid death of hosts due to A. mellea.

Interest has been taken in other aspects of the growth and development of the organism. The well-known luminosity of the mycelium was investigated by Guyot (1927). This feature, most apparent in the vigorously growing parts, was affected by anaesthetics and antiseptics the effect being that of temporary or permanent inhibition respectively; laceration of the mycelium also suppressed luminosity. In cultures, luminosity of the mycelium became apparent after about one month, but not in rhizomorphs formed later on the same media. It was noted that both mycelium and rhizomorphs of this age were

able to produce the disease on trees to which they were experimentally applied.

At about the same time, Bothe (1928) carried out experiments on the effect of various substances on the growth and luminosity of a "mycelium x" and A. mellea. The luminosity varied with the concentration of the materials, which included alkaline compounds and zinc; various media were described which were able to promote luminosity, and for A. mellea the temperature of 18-20 C. stimulated it. The development of rhizomorphs appeared to depend on the medium and the supply of oxygen, the latter also inducing a brown coloration in the mycelium.

In 1928, Guyot continuing his observations on the effect of anaesthetics on A. mellea, observed that the concentrations of various phenols and their derivatives inhibited the growth and luminescence of the organism. Lutz (1931) added further results to this literature; he exposed the luminescent mycelium to various substances known to inhibit oxidation and found that luminescence was suspended for periods varying from 5 to 60 minutes, but that the property could be restored when the material was transferred to an atmosphere with excess oxygen.

The formation of enzymes by fungal mycelium is a well known feature accompanying growth. Investigations to determine which enzymes were formed during the development of A. mellea have been undertaken by several workers, metabolism on certain substrates determining

the presence of the appropriate enzymes.

Hansenohrl and Zellner (1922) conducted a series of experiments to determine the chemical relationships between the higher fungi and their substrates. In some of these organisms including A. mellea, they could not find either cellulose or lignin splitting enzymes. These results were in contradiction to the findings of Lehmann and Scheible (1923), who stated that the utilisation of sugar in certain cultures and that of cellulose in wood, was of approximately the same order for the eight fungi investigated. A. mellea was able to utilise the highest amount of sugar in beerwort cultures (91gms. in three months). In experimental determinations of the optimum water content of wood needed for development of wood-rotting fungi, it was found that A. mellea required 45 per cent. Thakur and Norris in 1929 found that it was capable of decomposing cellulose.

Continuing his experiments on the white rot of wood in 1931, Campbell found that A. mellea and other fungi attacked the cellulose of the wood and also its associated pentosans, while the lignin was not decomposed to the same extent. In subsequent published work (1932), he stated that in white rots of the A. mellea type, the attack on the lignin is delayed. Pearson (1933) aalso found that this fungus attacked lignin in later stages of decay.

A comprehensive study of the enzymes present in

A.mellea was made by Lanphere (1934). Enzymes present were extracted from the rhizomorphs and successful tests were made for diastase, inulase, oxidase, peroxidase, invertase, rennet and catalase. Those stated not to occur were hemicellulase, maltase, lactase and esterases.

Bavendamm and Reichelt (1938) demonstrated the dependence of wood-rotting fungi on the water content of the medium. A. mellea was found to have an optimum requirement of 90.4 to 99 per cent. relative vapour tension on malt agar.

Other conditions for growth have been investigated. Wolpert (1924) discussed the growth of certain wood-destroying fungi in relation to the hydrogen-ion concentration of the medium. He found that the growth of A.mellea was favoured by an acid medium, but that there were inhibiting values. In Richard's solution, growth was inhibited at pH values 2.9 and 7.4, while in his peptone-containing medium the values were 2 and 7.8. The development of the fungus increased the acidity of both Richard's medium and peptone nutrient solution. It appeared to make better growth with an organic source of nitrogen and could utilise peptone as both a carbon and nitrogen source.

Further detailed investigations of cultural aspects of A. mellea were reported by Reitsma (1932). He found that fructifications were formed on the agar media which gave best mycelial and rhizomorph development, and also from a peptone-containing liquid medium standing in diffuse daylight at 18° - 22°C. for about four months.

Development of fructifications was, however, most normal on portions of elm twig. Regular subculturing of the fungus in liquid media suppressed the formation of rhizomorphs, their development being resumed on a solid medium. Optimum growth took place at 25°C. and at a pH of 5. The carbon source giving best development of mycelium was glucose, while peptone and other organic nitrogen sources gave greater growth than inorganic materials. Luminescence, and the effect of some toxic substances on growth were described. Oxygen was found to be essential for growth and was transported in the rhizomorphs.

Campbell (1934) in his account of the black zone lines formed in wood by A. mellea, gave detailed descriptions of their formation and appearance, along with information on the growth of the fungus in culture. The nature of the sclerotium and its significance was also discussed.

An account of investigations on the physiology and morphology of A. mellea, in which nine strains were used, was given by Hamada (1940). The growth of the organism was good on media containing glucose and peptone. The effect of media on rhizomorph and sclerotium forma tion was observed to vary with the nitrogen to carbon (N:C) ratio, the optimum being 1:4. The development of aerial mycelium showed no such dependence, but the secretory reactions had optimum N:C values, that for guttation being

between 1:2 and 1:1; calcium oxalate crystal formation had an optimum of about 1:4. Form and colour of the rhizomorphs were influenced by the substrate. The colour of the guttation liquid and browning of the medium were dependent on the peptone content as also was the form of the calcium oxalate crystals produced. Hamada also showed that the fungus when symbiotic in the orchid Galeola septentrionalis was influenced by the host and developed incompletely.

Benton and Ehrlich (1941) studied several isolations of A. mellea from white pine and found that they showed distinguishing characteristics in culture. They described the cultures and their rhizomorph-forming ability, and determined the degree of saprogenicity of the strains. On malt agar at pH 5, the best growth was obtained in the temperature range 21° - 25°C. At 25°C., the optimum pH values for malt agar were 4.5 - 5.5. The growth rates of the strains were compared by measuring the diametric spread of the mycelium in agar culture. The optimum wood-moisture content for development was 150 per cent. on initial oven dry basis.

A further study of the growth rate of A. mellea was reported by Edgecombe in the same year. He measured diametric spread of the mycelium of six fungi under uniform conditions. They were cultured on a basic one per cent. agar medium with prune extract added, the cultures growing in darkness, shade or full daylight, at 25°C. The observations published were the results for growth in the shade,

though it was stated that the other conditions influenced the appearance of the colonies. A. mellea showed a daily increase of 1.2 mm.

Further investigations of temperature and pH requirements were made by Rhoads (1939). He demonstrated that it grew best at 21.7°and 24.7°C. with only slight development at 35.8°C. Equally good growth occurred from pH 3.9 through pH 6.3, but progressive decrease was noted beyond pH 7.

In L931, Kärcher found that A. mellea survived for 8 days at a temperature of -70°C., and for 13 hours at -192°C, in malt agar culture.

An interesting observation by Weindling (1934) was made while investigating a lethal principle isolated from filtrates of Trichoderma lignorum cultures, and testing its action on Rhizoctonia solani and several other soil fungi. It was noted that T. lignorum was able to parasitise A. mellea. Bliss (1941) found that the growth of the latter in agar culture was suppressed by T. lignorum.

Greathouse and Rigler (1940) assessed the ability of seven fungi, one of which was A. mellea, to tolerate alkaloids, and the ability was found to vary for each fungus. Erythritol was shown by Birkenshaw, Stickings and Tessier (1948), to be produced in liquid cultures of A. mellea.

b) Literature on pathogenicity of A. mellea

Hiley (1919) stated that the infection of trees by rhizomorphs of A. mellea was attempted by Hartig (1894, 1901), Cieslar (1896), and Wagner (1899). The general conclusion reached by these workers was that unwounded trees were not liable to attack by the fungus. Brefeld (1877) showed that pieces of freshly dug root of pine were penetrated by the rhizomorphs both through the cut ends and through the bark. Hiley considered that this was not sufficient evidence to show that the fungus could invade healthy roots, as the cut portions are liable to lose their vitality very quickly. He concluded from his own observations on conifers that the undamaged healthy trees were not susceptible to the attack of A. mellea. He recommended as a measure of suppression, that stumps of trees should be infected with harmless fungi, as the presence of two species of the higher fungi is rare on the same stump, and invasion by A. mellea might be thus discouraged.

Gard (1923) investigated the root rot of walnuts caused by the fungus and stated as a result of his observations that it was able to attack healthy trees; Georgévitch (1929) stated that oak roots were only invaded by rhizomorphs when they were injured.

Other later investigators held that the organism was able to attack undamaged roots, though the susceptibility

to attack might vary due to other factors. This view was supported by various workers including Day (1929) and Thomas (1934); the former published a paper discussing the pathogenicity of A. mellea, in which he gave a review of the literature. He emphasised once again, that the environmental conditions were of great importance in determining the resistance or susceptibility of the trees to this fungus when it was present in the soil. Reitsma (1932) also suggested that infection by the fungus only took place when the host was reduced in vigour by the influence of some external factor which might, at the same time, stimulate the activity of the parasite. This conclusion was reached while carrying out some inoculation experiments, which however gave negative results in most cases.

The fungus was noted by Falk (1930) to be present in three conditions, as a parasite, as a semi-parasite in the moribund roots of trees, and as a saprophyte, but no opinion was given as to the factors determining which condition.

At this point it is interesting to note a report of the abundant development of A. mellea in roots of trees destroyed by fire, and its subsequent spread, thought to be due to the altered conditions (Guyot,1933). Ellis (1929) observed that rhizomorphs travelled as far as 22 yds. from their base, though Wallace (1935) thought that their range for effective penetration might only be short,perhaps a

few feet.

Some very interesting observations were made by Leach (1937) on the parasitism of A. mellea in the tea plantations in Nyasaland, and he suggested a method of biological control. The tea plants were found to have a rich starch content in the pith, and it was seen that the fungus developed best in this region of greatest carbohydrate supply. As the infection of the tea comes from infected roots remaining when the virgin forest is removed it was thought that if the original trees were depleted of their starch before felling, the A. mellea would not be able to develop in the stumps, and thus the source of infection would be removed. Experiments in ring-barking carried out to test this view, were successful in lessening the attack on felled trees; it was also presumed that the amount of soil infection in the plantations was reduced. In 1939, additional information was produced from further experiments on the same lines. Cultures of A. mellea on sterilised wood blocks showed that the fungus was able to penetrate the bark, whereas similar cultures on wood from a tree which had been ring-barked the previous year failed to establish themselves through the bark. It seemed advantageous, therefore, to deplete the roots of their reserve food material quickly, so as to make them unsuitable for the parasite. Roots of most species of native tree in the forests were found to be attacked by A. mellea, but in many cases the lesions were localised in

the living tree, the fungus only spreading in the roots when the trees were felled. These roots then became the foci of infection; if the trees were ringed before felling the conditions for the development of this fungus in the roots would be unfavourable. It was also found that A. mellea would not invade tissue already infected with other fungi e.g. Rhizoctonia lamellifer, or prunings which had become infected with saprophytes. The roots of trees which died quickly were invaded by saprophytes preventing the invasion by A. mellea, while slow-growing moribund roots were susceptible to the latter fungus and served to spread the disease.

Rivera (1940) investigated root rots caused by Rosellinia necatrix and A. mellea. He conducted experiments on the susceptibility of vine layer roots to these fungi at various temperatures, and found that the roots became infected at 25°C., but not at higher temperatures. Growth of A. mellea on straw gave similar results and further tests showed that the mycelium in living vine material and on straw, could be killed by exposing the tissues to temperatures of 42°C. and over, with no detrimental effect on the living plant which grew well at high temperatures. A method of control of infection by A. mellea might be devised from these findings. The two fungi were able to develop saprophytically in several types of soil.

Bliss (1941) added more information to the results

of artificial inoculation experiments with A. mellea. He found that infection in soil took place only from woody inocula, the potency depending on the food reserve in the wood. Further papers in 1941 and 1946, gave observations on the relationship between soil temperature and Armillaria root rot. In these experiments, nine species of plants were used, including both ornamental and economic types; these fell into groups with a different temperature for root development at its optimum. Investigations were made of the temperature requirements of both the host plants and the fungus in culture; the latter developed rhizomorphs most abundantly in the range 19.7° - 24°C. In non-sterile soil, however, the fungus grew with maximum rhizomorph formation at the lowest experimental temperature i.e. 10°C., which was inconsistent with the result for formation on agar. In the actual inoculation experiments, it was seen that the plants were least susceptible to fungal attack at the temperatures promoting optimum root growth. From information about the soil temperatures of various regions, it was apparent to Bliss that the occurrence of A. mellea was related to such temperatures; he thought that the optimum temperature for the growth of any host plant would determine the amount of infection possible in the various seasons. These results and conclusions appeared to bear out previous findings, including those of Rivera (1940).

Other interesting observations on the occurrence of various strains of the fungus and its symbiotic habit are described in the following papers.

Childs, Leroy and Zeller (1929) reported the existence of two physiologic strains of A. mellea, only one of which was parasitic. The oak strain was parasitic on orchard trees, while the fir strain apparently was not. Van Vloten (1936) isolated a number of strains of the fungus and stated that they showed distinctive characteristics when grown in culture. Three types of rhizomorph development were noted in pathogenicity experiments with potato, the most virulent strains being those which produced the greatest number of rhizomorphs. The strains showed no host specialisation and were equally pathogenic to the original hosts and to other hosts used in the experimental work. Some strains, however, formed no rhizomorphs and no infection tool place when mycelium from these non- rhizomorphic strains was used.

Strains of A. mellea which did not produce rhizomorphs were recorded by Dade (1927) on cacao, and by Bottomley (1937), on pine; in both cases the spread of the disease took place by contact between infected and healthy roots.

Studies were carried out on A. mellea in Japan in 1939. Here Hamada isolated the organism from the orchid Galeola septentrionalis, with which it existedas a fungal symbiont, and identified it as A. mellea although no fructifications were available. The fungus invaded the

cortex of the orchid, growing very actively in the summer months when the temperature was about 25°C, and optimum for the development of A. mellea. The orchid grew best in the cool winter months and it was then, presumably, that the fungus was ingested. The host reacted in various ways to the invasion of the fungus and the whole relationship was apparently that of a mild parasitism by the fungus balanced by the periodic ingestion of the mycelium by the host.

There are fewer detailed references in the literature relating to the method of invasion of the host by the fungus. Observations have been confined mainly to the fact that the rhizomorphs were the agents responsible for the spread of the disease to other hosts, but in 1926, Zeller added some further information to that readily ascertainable fact. He concluded that the fungus was able to invade the tissues of the host through wounds, or at the point of emergence of the lateral roots, and also where diseased and healthy roots came into contact. Mycelial invasion was noted to take place and it was suggested that the organism was able to affect the host and induce the production of some toxic substance in infected roots, and that this, in turn, acted on the neighbouring healthy roots thus allowing their penetration by the fungus.

Dade (1927) described the "collar crack " disease of cacao due to A. mellea. He showed that the fungus had many wild hosts in the Gold Coast, which made eradication very difficult to effect thoroughly in this area. Spread

of the disease took place by contact between infected and healthy roots; this was the only method of infection possible as no rhizomorphs developed in the soil of this region, though they were able to develop under laboratory conditions. The absence of rhizomorphs was thought to be connected with the soil type. Dade further indicated that there appeared to be a relationship between the humidity of the atmosphere and the intensity of infection.

Day (1927) made contributions to the study of the penetration by A. mellea into the host. He found that the fungus in the filed attacked various conifers, including pine and larch, in a similar way. The rhizomorphs first attached themselves to the roots and this attachment was made by hyphae which penetrated the dead cork cells and developed there to some extent, holding the rhizomorph firmly in position. The fungus then appeared to exert some toxic influence on the tissues of the host, the presence of which had previously been suggested by Zeller (1926). Day noted that the host often reacted to invasion by developing secondary cork layers which might be effective in preventing the entrance of the rhizomorphs. He came to the conclusion that certain hosts were more susceptible than others to attack by A. mellea, but that the disease did not always cause the death of the host. The fungus attacked apparently

healthy trees and any discrepencies in the supposed resistance of a species was attributed to the influence of external factors.

Thomas (1934) from his experimental investigations, on the attack of A. mellea on various hosts, drew similar conclusions to those of Day (1927). He used a varied selection of host plants, including fruit trees and root crops, and observed that the rhizomorphs were able to penetrate directly into the host tissues of both resistant and susceptible species. The rhizomorphic branch was observed to penetrate as a unit, the break-down of the cork cells suggesting the presence of a suberin-destroying enzyme, penetration presumably being by chemical and mechanical action. Death of host cells was seen to occur in advance of the penetrating rhizomorph. Thomas found no apparent relationship between the anatomical organisation of the host and its susceptibility to the fungus; neither was there any relationship between growth of the fungus on expressed sap and the susceptibility of the host. Resistant plants were only distinguished by their ability to prevent the establishment of the fungus, though small wounds occurred which healed rapidly. Resistance to the organism appeared to be an antagonistic effect, produced by the host when it was in a healthy state.

Rayner (1930) investigated the pathogenicity of A. mellea to conifers in pure culture in sand, She obtained infection of seedlings of Corsican pine and of

Douglas fir, using agar inocula of the fungus. Rhizomorphs did not form in the sand medium, and invasion of the host material took place by mycelium. Rayner agreed with Day that susceptibility of the host to attack by A. mellea was greatly influenced by the environmental conditions.

III MATERIAL AND METHOD

The sources of the Armillaria mellea material, and general information which applies to most of the experimental work, are given here. Special methods used are described under the headings of each section or subsection to avoid continual back reference.

a) Sources of the organism

The strains of A. mellea cultured in this work were as follows.

Five strains obtained from the Centraal-bureau voor Schimmelcultures, Baarn :-

1) Strain Mrs. Gregor Wilson (text abbreviation "GW"), isolated 1931 in Edinburgh from the pileus of fructification on bulb of Iris sp.; still producing rhizomorphs.
2) Strain Dr. Mounce (text abbreviation "M") isolated 1927 by Mr. MacCallum in Quebec, Canada, from Picea mariana. In 1936, Dr. Reitsma found fructifications of this strain on cherry agar.
3) Strain Miss Catherine Cool (text abbreviation "C") isolated 1912 in the Netherlands from pileus of fructification; still producing rhizomorphs.
4) Strain Dr. Rant (text abbreviation "R") isolated 1917 by Dr. Rant in Java from root of Cinchona sp.
5) Strain Dr. Reitsma, number 2 (text abbreviation "R_2") isolated 1932 by Dr. Reitsma in the Netherlands from wood of Quercus sp. ; remained luminescent until 1942.

One strain obtained from the Cryptogamic Botany Department of the University, Manchester :-

6) Strain from oil palm (text abbreviation "O") isolated 1949 in Manchester from root of oil palm brought from Belgian Congo.

b) Stock cultures and experimental inocula.

Stock cultures of these six strains were grown on two per cent. malt agar slopes, each tube containing 10cc. of medium. Inoculation was effected by the transference of a tuft of aerial mycelium, or, where there was mainly sclerotial development (strains GW and O) by a portion of sclerotium. After a month at 25°C., the cultures were kept at room temperature.

Cultures on water agar with no added materials were made as sources of inoculum for the experimental work . From stock cultures, a small portion of agar with mycelium was transferred to the centre of a water agar plate which was incubated at 25°C. The fungus developed only mycelium, neither sclerotium nor rhizomorphs being formed. From the advancing edge of the colony, the experimental inocula were cut with a cork-borer of 5mm. diameter and transferred with a sterile inoculating needle. In this way the inocula consisted of a uniform size of discs with approximately equal amounts of mycelial growth, while little nutrient material was carried over to the new cultures.

c) Materials and media

In the experimental work, the substances used were "Analar" chemicals unless otherwise stated. Boots "Extract of Malt" was used in the stock cultures and in many of the experiments.

Petri dishes of 9 cm. internal diameter were the usual containers for plate cultures and contained 20cc. of medium. Erlenmeyer flasks of Pyrex glass, of 250cc. and 100cc. capacity and containing 50cc and 25cc. solution respectively, were used for liquid cultures. For critical experiments with synthetic media, the glassware was cleaned with a chromic acid mixture and thoroughly rinsed in distilled water.

Sterilisation of Petri dishes was effected by heating in an oven at 150°C. for 2 hrs. Media used were usually autoclaved at 15 lbs. pressure for 20 mins.

The following list gives the conponents of the various media used in the experimental investigations :

1) malt agar

malt extract	20	gm.
agar	20	gm.
tap water	1	litre

pH after sterilisation was 5.

2) peptone-glucose-saccharose agar

peptone	10 gm.
glucose	30 gm.
saccharose	20 gm.
potassium dihydrogen phosphate	1 gm.
magnesium sulphate	0.5 gm.
calcium nitrate	1 gm.
agar	20 gm.
distilled water	1 litre

pH after sterilisation was 5.8.

3) Knop's solution

calcium nitrate	0.8 gm.
potassium nitrate	0.2 gm.
potassium dihydrogen phosphate	0.2 gm.
magnesium sulphate	0.2 gm.
ferric phosphate	a trace
distilled water	1 litre

Knop's agar - as above, with 2 per cent. agar. pH - 6.6

4) Pfiefer's medium

glucose	40 gm.
di-potassium hydrogen phosphate	4 gm.
asparagine	4 gm.
ammonium hydrogen phosphate	2 gm.
magnesium sulphate	2 gm.
calcium carbonate	0.25 gm.
agar	15 gm.
distilled water	1 litre

pH after sterilisation was 4.5.

5) Tubeuf's medium

ammonium nitrate	10 gm.
potassium phosphate	5 gm.
magnesium sulphate	1 gm.
lactic acid	2 gm.
agar	15 gm.
distilled water	1 litre

pH after sterilisation was 4.2.

6) Yeast-peptone

yeast extract (Difco)	1.5 gm.
peptone	5 gm.
glucose	10 gm.
agar	20 gm.
distilled water	1 litre

pH after sterilisation was 6.2.

7) yeast-asparagine

yeast extract (Difco)	3 gm.
asparagine	5 gm.
glucose	10 gm.
agar	20 gm.
distilled water	1 litre

pH after sterilisation was 6.2.

8) water agar

agar	20 gm.
tap water	1 litre

pH after sterilisation was 7.

9) Schopfer's medium

glucose	5 gm.
asparagine	0.5 gm.
magnesium sulphate	0.25 gm.
potassium dihydrogen sulphate	0.75 gm.
agar	10 gm.
distilled water	500 cc.

pH after sterilisation was 5.8.

10) Brown's medium

glucose	2 gm.
asparagine	2 gm.
potassium phosphate	1.25 gm.
magnesium sulphate	0.75 gm.
agar	15 gm.
distilled water	1 litre

pH after sterilisation was 7.

11) bean extract agar

dry beans(powdered)	50 gm.
distilled water	1 litre
(steamed for one hour and filtered through gauze before adding agar and autoclaving)	
agar	20 gm.

IV EXPERIMENTAL RESULTS

A. PHYSIOLOGY

a) Germination of spores

The germination of the spores of A. mellea appears to have received little attention. This may be due to the fact that no importance has been attached to the spores as agents of infection of plants. Only occasional references have been made to the conditions suitable for germination and these have been included in investigations mainly concerned with other aspects of the growth of A. mellea.

Hiley (1919) and Reitsma (1932) stated that spores could germinate on nutrient agar and form typical colonies but they gave no greater detail. Germination took place on sterilised manure at 18° - 20° C., according to Gard (1923,1928), and in distilled water and various nutrient solutions; 0.13 per cent. lime water inhibited germination

It was thought that a more detailed study of the germination of the spores would be an interesting contribution to the knowledge of the cultural requirements of A. mellea. The effect of heat, pH, and nutrition, were determined in the following investigations.

Method

The spore material was obtained from fructifications of the fungus collected from Auchincruive in Ayrshire, and Alderley Edge near Manchester.

The fructification was supported by a glass stand in a deep covered Petri dish and the spores were collected as they were shed from the pileus. Presumably other organisms which might be present on the material, would not collect on the base of the dish and a reasonably clean suspension of spores could be expected. The spores were washed from the dish with sterile distilled water into test-tubes which were then stored at 4°C. The material was always returned to this temperature after samples had been removed for experimental purposes.

A modification of the Van Tieghan cell, similar to that used by Webb (1919), was employed for the observation of germination under various conditions. A slide with two glass rings held in position by vaseline was placed in a sterile Petri dish. Firstly a small amount of the solution which was being tested, was pipetted into the centre of the ring. The spore suspension for germination trials was prepared by mixing equal quantities of the stock spore suspension and the solution to be tested. The resultant mixture was transferred to the underside of the sterilised coverslip by means of the loop of a sterile inoculating needle, and then the coverslip with the hanging drop was laid on the top of the vaselined ring.

The nutrient solutions were prepared as liquid media. Various hydrogen-ion concentrations were obtained by adding sterile N/5 caustic soda or N/5 hydrochloric acid

to sterile distilled water. Owing to differences in the original pH of the distilled water, no standard amount of these reagents could be determined which would give the same pH value on repetition of the series.

Germination capacity of the spores in the various solutions was determined by counting the number of spores which had germinated out of ten lots of a hundred. These were expressed as a percentage.

Experiment 1. Germination of spores in various media at different temperatures.

The solutions used for germination tests were as follows:

1) distilled water
2) Knop's solution
3) peptone-glucose-saccharose solution
4) 2 per cent. malt extract solution
5) 5 per cent.glucose solution
6) 1 per cent. peptone solution

The method employed was that described previously. Three temperatures were used, and counts were usually made after 24 and 48 hrs. The summarised results are given in Table 1, but the details of individual readings are shown in Table 3, at the end of the section.

Plate 2 shows stages in the germination of spores on peptone-glucose-saccharose medium after 24 hrs.

Table 1

Germination in various media at different temperatures.

medium	percentage germination of spores					
	after 24 hrs.			after 48 hrs.		
	4°C.	20°C.	25°C.	4°C.	20°C.	25°C.
distilled water	0	0	0	0	0	2.4
Knop's	0	0	0	0	0	0
peptone-glucose-saccharose	0	2.0	32.9	0	26.3	76.5
2% malt	0	1.8	13.9	0	9.6	31.6
5% glucose	-	0	2.0	-	-	-
1% peptone	-	0	2.5	-	-	-

It was found that the spores germinated in most nutrient media after 24 hrs. with an increase in germination of 48 hrs. The solution containing peptone, glucose and saccharose gave the greatest result. At 25°C the percentage germination was consistently higher than at the other temperatures.

It was noted that development of the germinating spore beyond the germ tube stage was dependent on the nutrient supply, no continuation of growth occurring in distilled water.

Experiment 2. Germination of spores at various pH values.

Quantities of distilled water with pH values ranging from 1.5 to 8 were prepared with 0.5 intervals between the values. It was seen in the previous experiment that distilled water supported little of no growth of the spores and it was thought that this might have been due to the

reaction of the water. Following the usual procedure, the results seen in Table 2 were obtained in germination tests of 24 hrs. at 25°C.

Table 2

Germination of spores at various pH values.

pH	percentage germination
1.6	1.6
2.0	2.2
2.5	1.2
3.0	18.3
3.5	22.1
4.0	14.7
4.5	0
5.0	0
5.5	0
6.0	0
6.5	0
7.0	0
7.5	0
8.0	0

The results showed that germination of spores did not always take place in distilled water after 24 hrs. Acidity apparently favoured germination, the percentage increasing as the pH rose to 3.5 and then decreasing suddenly with further increase in pH.

Details of readings are again at the end of the section, and are shown in Table 4.

Experiment 3. Effect of exposure to heat on the germination of spores.

A spore suspension in peptone-glucose-saccharose medium was prepared, this medium having been found to favour germination. Tests were made at three temperatures to determine their effect on the germination of the spores.

Spores incubated at 30°C. did not germinate. Two suspensions held in water baths at 35°C and 40°C. for 10 mins. and then incubated at 25°C., showed no germination. Controls placed at 25°C. germinated as found previously.

Conclusions

It would appear that spores require some stimulus before they will commence growth; this might be provided by an acid reaction of the medium, or by some undetermined substance or substances such as were present in the peptone-glucose-saccharose solution.

As might be expected, germination was conditioned by temperature with the optimum at 25°C.

TABLE 3

Germination of spores in various media at three temperatures after 24 and 48 hours. Number of counts per treatment - 10 sets of 100 spores.

Medium		Percentage germination of spores																			
		24 hrs										48 hrs									
distilled water	4°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	25°C	0	0	0	0	0	0	0	0	0	0	6	4	1	1	4	0	0	5	2	1
Knop's	4°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	25°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
peptone-glucose-saccharose	4°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20°C	4	1	0	2	1	4	3	2	3	2	28	21	25	32	27	28	22	30	27	23
	25°C	24	32	28	29	37	31	31	42	37	38	83	79	79	78	71	73	80	77	75	70
2% malt	4°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20°C	1	1	3	2	2	1	2	1	3	2	12	10	8	10	9	13	10	8	7	9
	25°C	16	16	9	15	11	13	14	10	15	20	35	29	26	29	28	35	35	30	35	34
5% glucose	20°C	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
	25°C	3	3	1	2	2	2	1	1	3	2	-	-	-	-	-	-	-	-	-	-
1% peptone	20°C	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
	25°C	4	1	5	1	1	2	1	2	2	6	-	-	-	-	-	-	-	-	-	-

Table 4

Germination of spores at various pH values after 24 hrs. at 25°C. Number of counts per treatment-10 sets of 100 spore

pH	percentage germination									
1.6	0	2	4	3	0	3	2	1	1	0
2.0	3	2	1	3	4	3	1	1	2	2
2.5	2	0	1	3	0	0	3	1	1	1
3.0	3	15	20	27	17	21	23	16	20	15
3.5	22	25	24	29	20	21	28	10	23	19
4.0	16	14	11	10	10	25	11	15	16	19
4.5	0	0	0	0	0	0	0	0	0	0
5.0	0	0	0	0	0	0	0	0	0	0
5.5	0	0	0	0	0	0	0	0	0	0
6.0	0	0	0	0	0	0	0	0	0	0
6.5	0	0	0	0	0	0	0	0	0	0
7.0	0	0	0	0	0	0	0	0	0	0
7.5	0	0	0	0	0	0	0	0	0	0
8.0	0	0	0	0	0	0	0	0	0	0

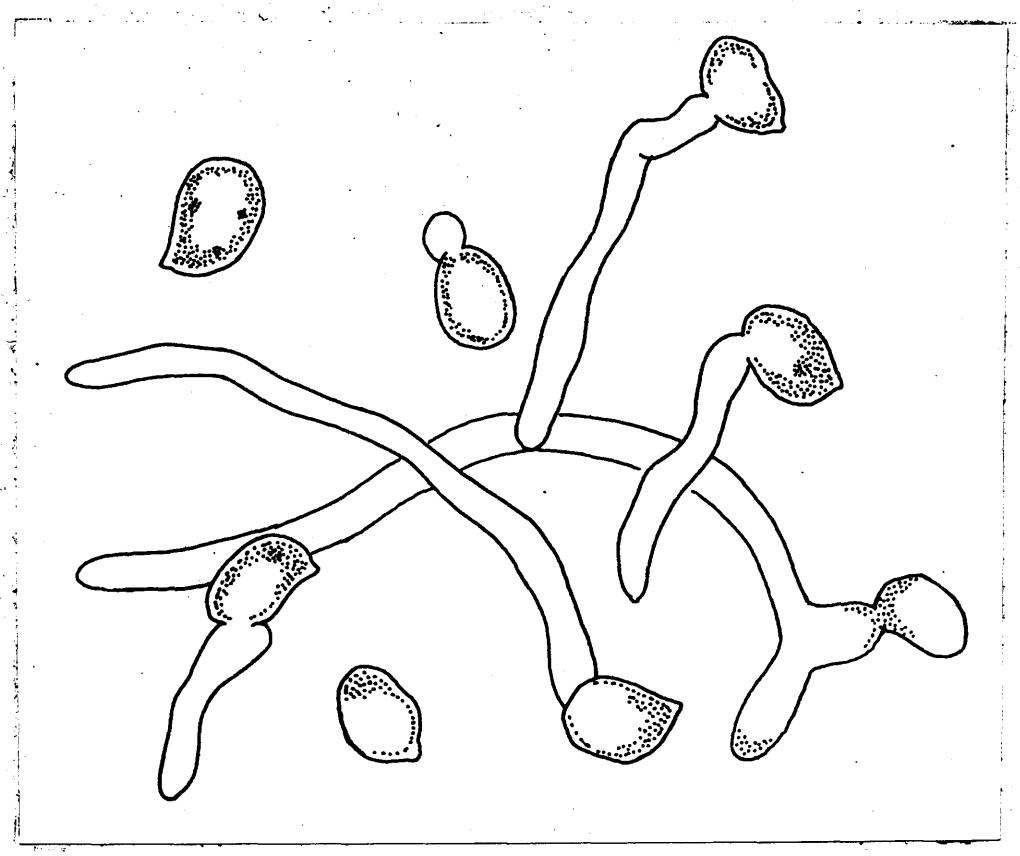

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Plate 2. Germination of spores of A. mellea in the peptone-glucose-saccharose solution after 24 hrs. at 25°C. Note the various stages of germination.

b) Effect of light on growth

It is a well-known phenomenon that light has as an effect on the growth and appearance of many fungi. Among the most interesting of the numerous investigations were those for Phycomyces spp., of the Oomycetes (Wolf and Wolf, 1949), and Polyporus schweinitizii, among the so-called"higher"fungi, (Cartwright and Findlay,1946). For A. mellea, little work of this nature appears to have been undertaken. Reitsma(1932) and other workers, mentioned briefly that light intensity had an effect on the sporophore formation; Edgecombe (1941) grew this fungus at three light intensities and stated that the type of growth varied with the amount of light.

Experiments were devised to determine any effect of light on growth of A. mellea. Even though sporophores do not form readily in culture, it was thought that the fungus might show other obvious changes in form when grown in light; as aerial rhizomorphs are present in culture, there was the possibility of some phototropic response.

Method

The Gregor Wilson (GW) and oil palm (O) strains were used in these investigations.

Use was made of a constant temperature room held at approximately 20°C, illuminated by fluorescent light functioning for 12 hrs. per day. The fungus was grown on

both liquid and solid malt extract media, the preparation and inoculation of which are described under section III in "Materials and media".

Experiment 1. Growth of A. mellea in darkness and light.

The strain GW was grown at room temperature on 24 agar plates, half of which were left in daylight and half covered to act as controls in the dark.

The results of this preliminary investigation , obtained after 2 months, were very interesting as a marked difference was apparent between the growth of the two sets of cultures; those in light showed a limited development of the fungus and this can be seen in Plates 3 and 4.

A sclerotial mat was present in both sets of cultures but in light it was of a duller brown and did not have outgrowths covering the areas of the agar culture which were above branching rhizomorphs, as no rhizomorphs were formed in the light. In the dark the latter were present and profusely branched, with a few aerial tips projecting above the sclerotium.

Experiment 2. Growth of two strains in darkness and light.

From the previous experiment it was seen that light induced marked differences in the appearance of the fungus. This experiment was repeated in liquid culture in order to obtain a quantitative result. The strains GW and O were each grown in 12 x 250cc. flasks, six of which were placed in light and six kept dark. The experiment was

conducted in the constant temperature room and the results obtained after one months growth.

The appearance of the colonies was greatly altered by the exposure to light and this is illustrated in Plates 5 - 8. In the dark, a dense white mycelial mass with sclerotial patches was formed for both strains, while the reduced fungal development in the light was mainly of a sclerotial nature.

The growth of strain GW was again much reduced in light, but sclerotial formation again took place in both light and dark. In the dark, the liquid surface was covered by fungal growth, and many branching rhizomorphs had developed in the medium. In light, some short brownish mycelium was present on the sclerotium and short branched rhizomorphs formed with very few aerial tips.

The growth of strain O showed similar features to those described for strain GW.

Table 5 shows the growth of the strains as expressed by their weights in culture.

Table 5

Quantitative growth of two strains in darkness and light. Number of cultures per treatment - 6. Growth period-3 weeks

strain	cultural conditions	wet weight (in gm.)	dry weight (in gm.)
GW	light	10.602	0.315
	dark	20.573	0.352
O	light	9.125	0.278
	dark	22.670	0.380

The results in Table 5 show that the two fungal strains developed a greater mass when grown in the dark. The wet weights varied very much from dark to light conditions; this might indicate a greater water content of the mycelium in the dark, as the dry weights do not vary accordingly.

Experiment 3. Growth of two strains under light exposures of varying duration.

The strains GW and O, again used, were each grown on 12 malt agar plates in the constant temperature room. These cultures were in four sets for each strain, and in the course of the experiment each set was exposed daily to light for a different length of time. The duration of these were:

1) 12 hrs./day
2) 6 hrs./day
3) 1 hr./day
4) continuous darkness

After two weeks' growth under these conditions, the sets of cultures were observed to show differences. For both strains, growth in the dark showed the development of features already described, while apparent inhibition of mycelial spread and of rhizomorph formation was again seen in the cultures exposed for 12 hrs. daily. The two sets of cultures with intermediate amounts of light had corresponding differences in development and the series as a whole gave a range in the growth and development of

the colonies easily related to the amount of light.

Experiment 4. Growth of one strain under light exposures of varying duration.

Strain GW was grown on liquid malt extract in 15 x 100cc. flasks incubated in the constant temperature room. The cultures were divided into five sets, each of which was exposed daily to one of the following periods of light:

1) 12 hrs./day
2) 12 hrs./week
3) 1 hr. /week
4) 1 min/ week
5) continuous darkness

At the end of 4 weeks, cultures showed a range in growth similar to that already described on malt agar. There was, however, some slight development of rhizomorphs in the longest period of light.

Table 6 gives the weights determined at the end of the experiment.

Table 6

Growth of one strain under light exposures of varying duration. Number of cultures per treatment - 3. Growth period - 4 weeks.

light	wet weight (in gm.)	dry weight (in gm.)
12 hrs./day	3.046	0.162
12 hrs./week	6.760	0.216
1 hr. /week	8.247	0.232
1 min/week	10.736	0.252
control in darkness	11.737	0.252

It is seen from this table that there was an apparent increase in the wet and dry weights of the colonies as the exposure to light decreased. As in Experiment 2, there was a greater variation in the wet weight than in dry weight with the same possible interpretation.

Experiment 5. Growth of one strain in daylight and the subsequent development of the culture in the dark.

Twelve cultures of strain GW in malt extract in 100cc. flasks were grown at room temperature. Six were placed in daylight on the bench, and six were kept in the dark as controls. After 2 weeks' growth, three cultures from each set were exchanged, final observations being made after a further 2 weeks' growth (see Plate 5).

It was observed that cultures in the light were rounded in shape with woolly mounds of short, pale-brown mycelium, below which appeared the elements of a sclerotium. No rhizomorph formation was present, although initials were seen below each colony.

In cultures grown in the dark, a sclerotium had formed beneath a thin layer of mycelium and extended farther than in the "light" cultures, partly due to attachment to the extending rhizomorphs. The latter had developed profusely and were branched and white, though the tips had become brown and smooth where they grew above the surface of the medium.

Cultures which had been transferred from light to

darkness had an intermediate appearance between the two extremes, the sclerotium being slightly woolly and having a fairly regular edge. Rhizomorphs had developed but were less numerous and less extensive than in the "dark" cultures, while the aerial tips were sturdier.

Cultures transferred from darkness to light were nearly indistinguishable from those grown in the light for the whole period, but they had less aerial mycelium and were yellow-brown in colour. Rhizomorph initials, apparent before the exchange, had not developed.

These differences were matched by variation in the weights of the colonies as shown below:

Table 7

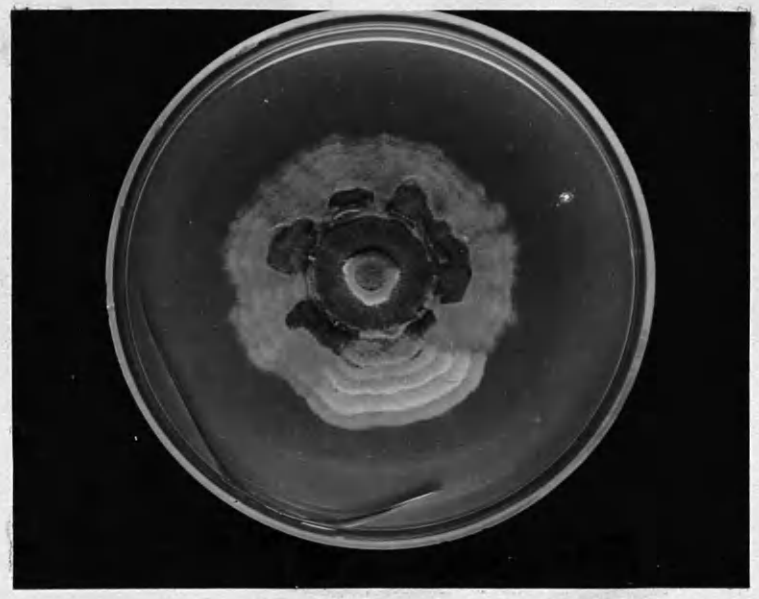
Growth of strain GW in light with subsequent transferance to darkness. Number of readings per treatment - 3. Growth period - 4 weeks.

cultural conditions	wet weight (in gm.)	dry weight (in gm.)
light	5.707	0.167
darkness, with exchange to light	3.506	0.133
darkness	7.528	0.204
light, with exchange to darkness	3.975	0.163

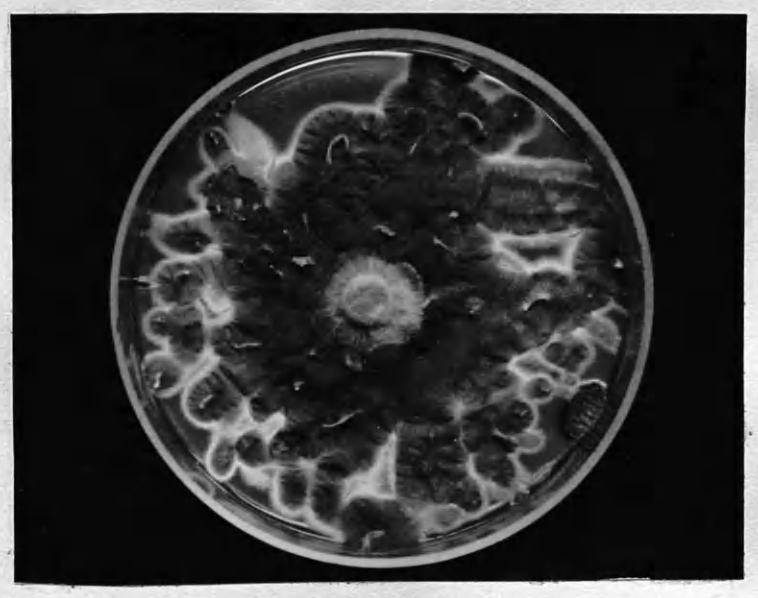
Comparison of the wet weights indicates that growth in the darkness is apparently greater than that in the light, while the cultures which had been exchanged show similar growth amounts. The dry weights do not confirm this result, although development in darkness was appreciably greater.

Conclusions

It is apparent from the foregoing experiments that light has a modifying influence on the form of the colony developed by A. mellea. In daylight and artificial light, there was reduced formation of sclerotium and rhizomorphs. In full daylight, however, rhizomorphs failed to develop although initials were sometimes present. The transference of colonies from light to darkness stimulated further development of the rhizomorphs from the initials. Transference from darkness to light, however, caused inhibition of further growth of partly developed rhizomorphs.



a)



b)

Plate 3. Two month old culture of strain GW grown in
a) daylight
b) darkness

a)

b)

c)

d)

Plate 4. Month-old cultures grown at room temperature
a) strain GW in artificial light
b) strain GW in darkness
c) strain O in artificial light
d) strain O in darkness

a)

b)

c)

Plate 5. Month-old cultures of strain GW grown at room temperature

a) in daylight
b) in darkness
c) in daylight for 14 days, and then in darkness for a further 14 days.

c) Effect of temperature on growth

Previous work has shown that the optimum temperature for the growth of A. mellea is 25°C.(Reitsma, 1932),though a second optimum of approximately 21°C. was noted (Wolpert, 1924). Since the strains cultured in the present investigations were isolated from both tropical and temperate regions, it was thought that there might be differences between the optimum temperatures for their growth.

Method

Strains of the fungus were grown on both liquid and solid malt extract media, prepared as described under Materials and Media" (Section II.), and inoculated with the standard agar discs and tufts of mycelium, respectively. Available temperatures were 30°C., 28°C, 25°C., 20°C,and 4°C. Linear growth was measured on solid media along two diameters of the colony at right angles to each other, the readings being made at regular intervals. Growth in liquid culture was again assessed at the end of four weeks' growth, by taking the wet and dry weights of the colonies.

Experiment 1. Growth of five strains of A. mellea at four temperature.

The strains GW, M, C, R, and R_2 were each grown on 24 malt agar plates, of which 6 cultures were incubated at each of the four temperatures, 4°C., 20°C., 25°C., and 30°C. (Cultures at "20°C." were kept at room temperature which varied between 16° and 20°C.) The six replicate readings obtained every 48 hrs. from 6 replicate colonies of each

strain at each temperature were averaged. Rates of growth were calculated and plotted against temperature (Fig.1).

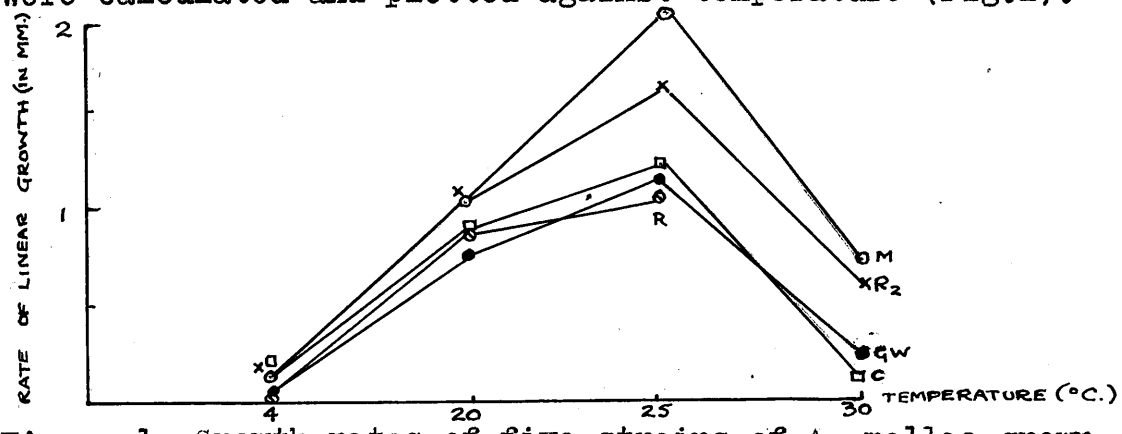


Figure 1. Growth rates of five strains of A. mellea grown on malt agar at four temperatures.

Details of growth at each temperature are shown in graphs at the end of the section (Figs. 2-6).

From the graphs it can be seen that all strains showed the greatest diametric growth at 25°C., it was also noted that the most profuse development of mycelium and rhizomorphs took place at this temperature. In cultures of strains GW, C and R_2, rhizomorphs appeared after only one week; these extended over the medium in the case of strain C, whereas they were represented only, or mainly, as initials in strains GW and R_2, respectively. Sclerotia were also present in these three strains.

At 20°C., rhizomorphs occurred in cultures of all strains except R and R_2, but they grew slowly and extended only a few millimetres beyond the edge of the sclerotium.

At 30°C., only one strain, GW, showed any sign of Rhizomorph formation; here rhizomorph initials were noted after six weeks, but their further development could not be

studied as the cultures dried out.

At 4°C., only the initials of rhizomorphs were formed (strains GW, M, and C) but they developed no further even after 10 weeks.

Experiment 2. Growth of two strains of A. mellea at four temperatures.

Strains GW and R_2 were each grown in 24 x 250cc. flasks of malt extract solution, 6 flasks of each strain being incubated at one of the following temperatures: 4°C., 20°C., 25°C., and 30°C. After five weeks the data in Table 8 was obtained.

Table 8

Growth of strains GW and R_2 at four temperatures. Number of cultures per treatment - 6. Growth period - 5 weeks.

temperature	strain			
	GW		R_2	
	wet wt. (in gm.)	dry wt. (in gm.)	wet wt. (in gm.)	dry wt. (in gm.)
4°C.	0.1074	0.0049	0.0905	0.0010
20°C.	6.3510	0.2783	9.6686	0.2806
25°C.	11.3083	0.3284	10.5165	0.2846
30°C.	0.0506	0.0022	0.0789	0.0080

From comparison of both wet and dry weights, it seems that the strains grew best at 25°C., but developed almost as fully at 20°C.

The appearance of the cultures at these four temperatures was markedly different. At 25°C., strain GW developed more sclerotium and more extensive rhizomorphs than at other temperatures. At 20°C., the rhizomorphs developed aerial tips, and there was no formation of

dense white mycelium about the sclerotium as at 25°C.

Strain R_2 had similar rhizomorph and sclerotial growth at both 20° and 25°C., though the fungal mass at the higher temperature was larger and more heaped-up; many dark brown guttation drops were present.

At 30°C. no growth of either strain occurred; at 4°C. a small amount of mycelium formed round the inoculum.

Experiment 3. Comparative growth of two strains at four temperatures.

This experiment was designed to determine whether the tropical strain O grew better at higher temperatures than the temperate strain GW. The temperature of 4°C. was not used here.

A preliminary test was carried out to find whether growth occurred at 30°C., as it had been found previously that this temperature was unsuitable for the development of other strains. Strains GW and O were each grown in 9 x 100cc. flasks placed in sets of three at 20°C., 25°C., and 30°C., and after 4 weeks' growth the results in Table 7 were obtained.

Table 9

Growth of strains GW and O at three temperatures. Number of cultures per treatment - 3. Growth period - 4 weeks.

temperature	strain			
	GW		O	
	wet wt. (in gm.)	dry wt. (in gm.)	wet wt. (in gm.)	dry wt. (in gm.)
20°C.	13.10	0.31	17.06	0.34
25°C.	15.24	0.34	27.72	0.36
30°C.	0.05	0.002	0.068	0.002

It was seen that no significant growth was madeby either strain of A. mellea at 30°C. For the main experiment, therefore, the slightly lower temperature of 28°C. was chosen and in addition the strains were grown at a room temperature varying from 17°to 21°C. Cultures were grown in 250cc flasks with results as shown in Table 10.

Table 10

Growth of strains GW and O at four temperatures. Number of cultures per treatment - 3. Growth period - 4 weeks.

temperature	strain GW		O	
	wet wt. (in gm.)	dry wt. (in gm.)	wet wt. (in gm.)	dry wt. (in gm.)
room	9.730	0.377	11.332	0.371
20°C.	16.929	0.470	17.601	0.522
25°C.	14.932	0.440	25.477	0.569
28°C.	0.366	0.077	4.622	0.198

The results for the strain GW do not support those previously found and in this case the growth at 25°C is very similar to that at 20°C., even from consideration of the wet weights. The strain O, again, showed more growth at 25°C. than at any of the other temperatures.

Conclusions

These strains grew slightly better at 25°C. and it seems probable that all strains of A. mellea can be described as having this optimum temperature forgrowth, and this is supported by the evidence of previous investigators.

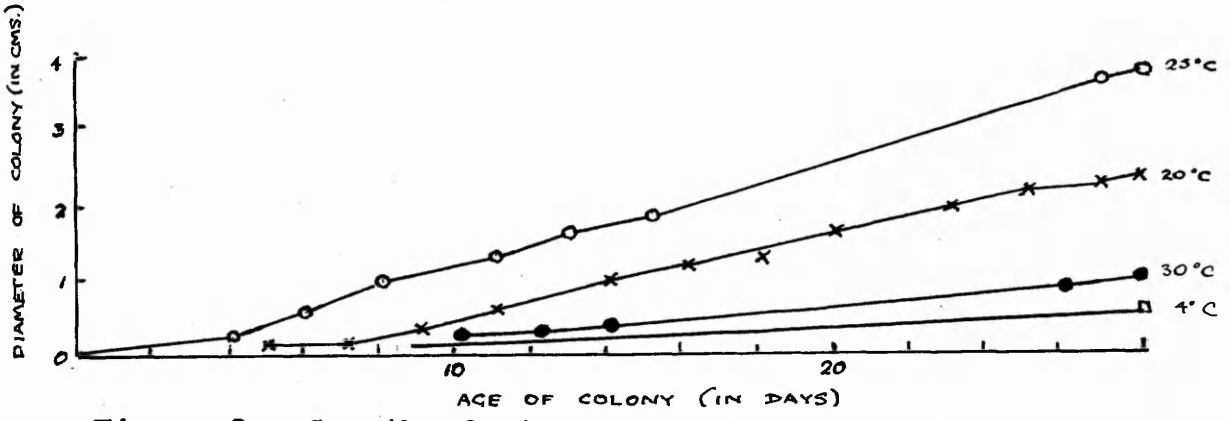


Figure 2. Growth of strain GW during one month.

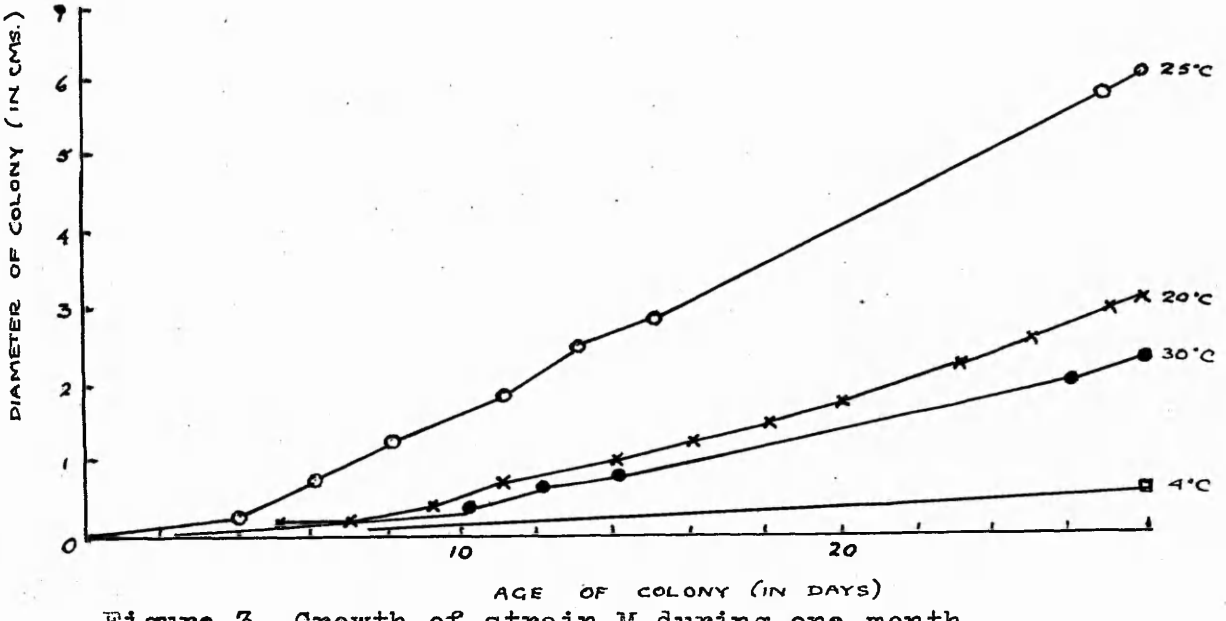


Figure 3. Growth of strain M during one month.

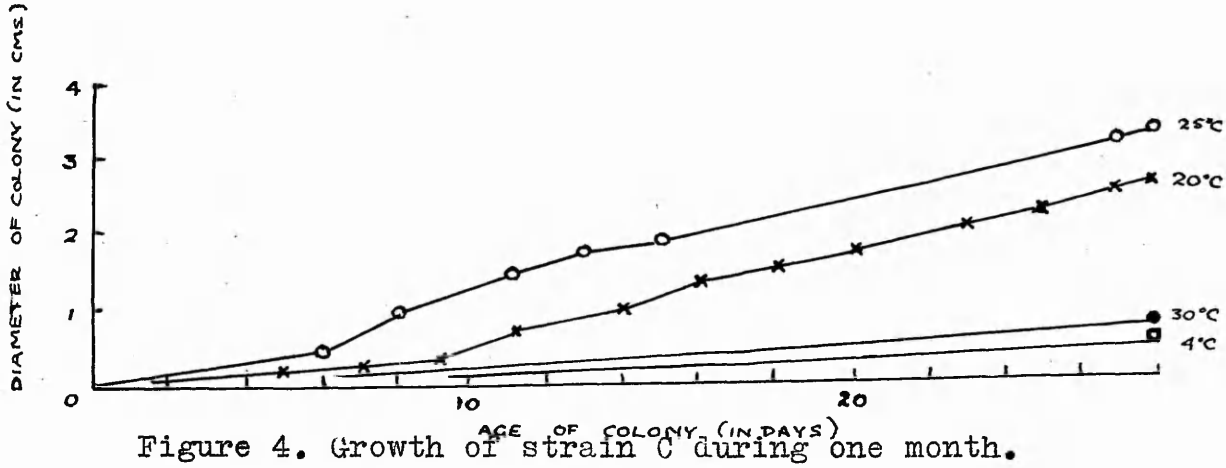


Figure 4. Growth of strain C during one month.

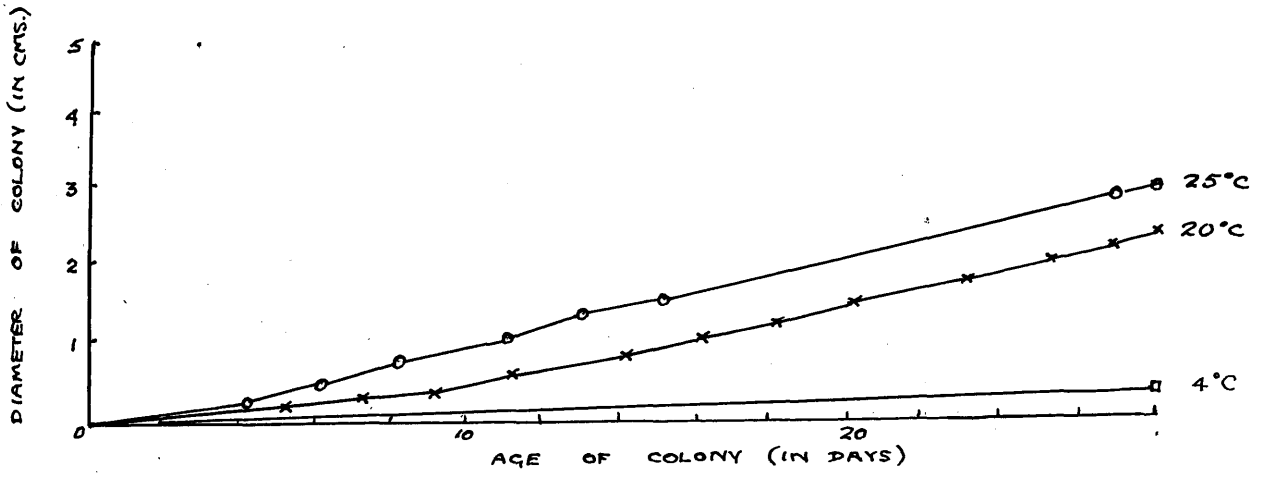


Figure 5. Growth of strain R during one month,

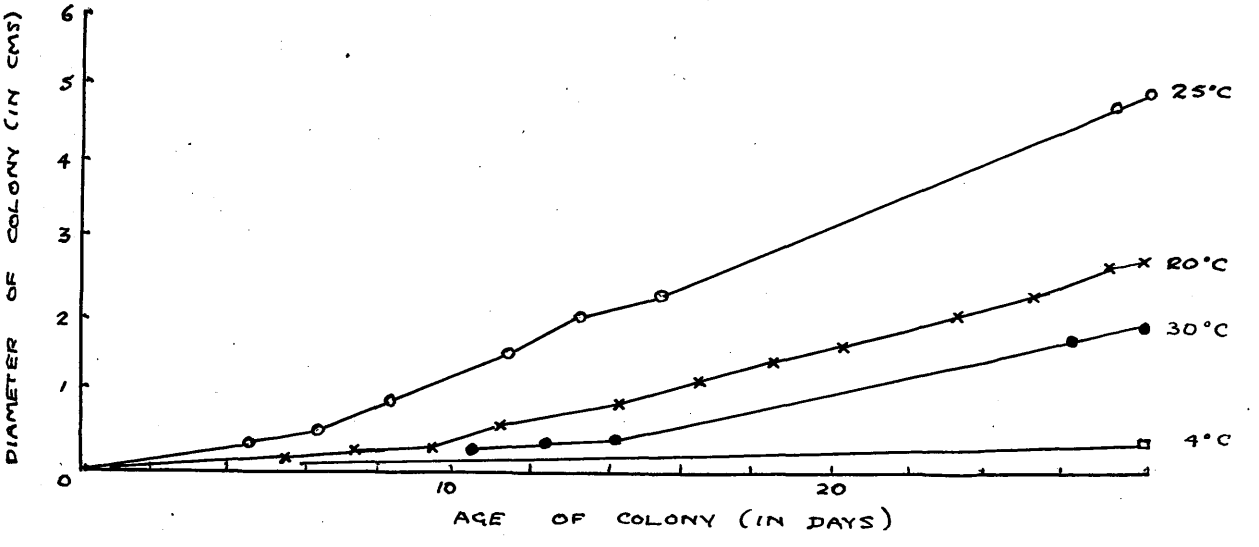


Figure 6. Growth of strain R_2 during one month.

a)

b)

c)

d)

Plate 6. Month-old cultures of strain GW grown at
a) room temperature
b) 20°C.
c) 25°C.
d) 28°C.

a)

b)

c)

d)

Plate 7. Month-old cultures of strain O grown at
a) room temperature
b) 20°C.
c) 25°C.
d) 28°C.

d) Effect of Hydrogen -ion concentration on growth

This experiment was carried out to determine any variation in growth of the fungus which might occur when the pH value of the medium is varied. Other workers (Reitsma,1932; Wolpert,1924) have shown that A. mellea grows better on an acid medium, the most suitable reaction being pH 5.

The method and presentation of results were similar to those of Experiment 1, Section c),but cultures were grown at a constant temperature on media at different pHs. Adjustment of the pH of the medium was achieved by the addition of either conc. hydrochloric acid or 40 per cent. caustic soda.

The results in Fig.7 do not support previous findings probably because this measures only linear spread and not total growth of the fungus, these appearing to be favoured by different reactions.

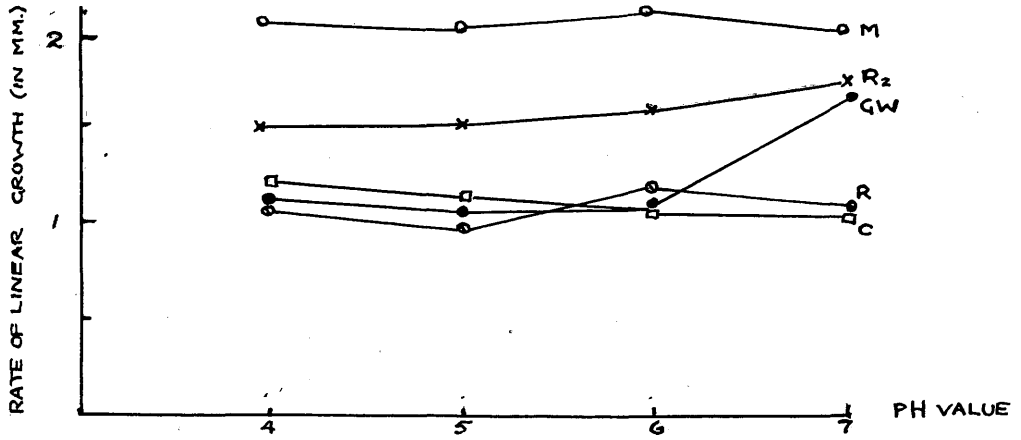


Fig. 7. Linear growth rate of five strains of A. mellea at pH values 4, 5, 6 and 7, grown at 25° C. for 4 weeks. Rate per day.

e) Effect of various media on growth.

Investigators have grown A. mellea on various synthetic and natural media. Wolpert (1924) used Richard's solution and a peptone solution for his investigations of the pH optima of the fungus; Reitsma (1932) and Hamada (1941) found growth to be favoured by media containing glucose and peptone, and Reitsma found that good development occurred on various natural media including cherry decoction and bread. Other workers have cultured the fungus on malt agar, while pursuing investigations of growth rate and other features of development (Benton and Ehrlich, 1941), while in similar experiments, Edgecombe (1941) used a basic medium to which prune extract had been added.

In the following experiments, various synthetic and natural media were used, and the results include the effect of yeast extract, sugars, and organic or inorganic nitrogen on growth of A. mellea.

Experiment 1. Growth of six strains of A. mellea on malt extract agar.

The following gives a description of two-month-old cultures of strains GW, M, C, R, R_2 and O grown on malt agar at 25°C. Illustrations are given in Plates 9 and 10.

1) The Gregor Wilson strain: The colony showed a very definite zonation when viewed from above and below in the Petri dish. On the upper surface the central region

which was apparently composed of sclerotial material, had overlying masses of aerial mycelium disposed in concentric rings of varying density; zones were also seen in the outer mycelial margin of the colony, the thicker parts of which contained a brown pigment. The colour of the aerial mycelium varied from nearly white to a light brown. Saltation areas were clearly evident as wedge-shaped portions of the colony near the outer edge.

On the lower surface the colony presented a more regular appearance, the zonation at the centre being due to the varying thickness of the sclerotium, and that at the margin to the density of the mycelium in the agar. Again light and dark circles of mycelium alternated, and the causal pigment did not appear to be evenly distributed in these zones. Small rhizomorph initials were seen below the central sclerotium; in some cultures these grew out to form spreading rhizomorphs.

2) The Mounce strain; The colony was formed of very thin mycelium which became even thinner towards the outer edge. The upper surface had sparse aerial hyphae, particularly over the original inoculum. Usually a very small and limited sclerotium was formed below this mass. Rhizomorph initials were present as extremely short extensions from the underside of the sclerotium. Zonation of the colony resulted from variable accumulation of the mycelium in the agar in concentric circles.

3) The Cool strain : The colony is similar to that formed by the strain GW, but the mycelium was denser and the zonation less apparent. The upper surface showed a woolly mass of mycelium, thick at the centre but thinning towards the edge where the sclerotium showed through. Rhizomorphs extended in the agar beyond the limit of the central sclerotium; these developed on their outer surface, mycelium which formed a sclerotial cover on the agar. The various parts of this cover later joined to form a larger sclerotium bearing aerial hyphae. The hyphae in the agar beyond the sclerotium was again zoned concentrically.

4) The Rant strain : The colony showed a nearly uniform mass of dense white aerial mycelium, with some variation however at the thin, narrow hyphal edge, and at the central raised portion above the inoculum. The upper surface showed a faint zonation due to very slight concentric differences in the level of the surface. No rhizomorph or sclerotium formation took place.

5) The Reitsma 2 strain : This strain presented a concentrically zoned appearance due to the differential growth in the aerial mycelium. This growth resulted in a broad ring round the centre, with other less obvious zones outside it. The sclerotium below had a rather narrow edge of hyphae with rings of varying density. The dark brown skin of the sclerotium developed alternating bands of light and dark pigmentation; from the centre there developed fluffy, upright, projections like rhizomorphs of a

light brown colour. The undersurface of the colony had a large white central area, the rest being pigmented, with thin folds of white tissue which may develop into short rhizomorphs.

6) Oil palm strain : The colony was formed from a central sclerotial region which had rhizomorphs growing out and then giving rise to other sclerotial regions. Some development of aerial hyphae was seen on the younger parts. Seen from below, the rhizomorphs were white under the sclerotium but brown when in the agar. They were sturdier then in the other rhizomorph-producing strains, and had numerous aerial tips projecting above the agar.

Experiment 2. Effect of six carbohydrates on the growth of five strains.

Strains GW, M, C, R, and R_2 were each grown on variations of Brown's medium on 18 agar plates. These were in 6 sets, each containing one of the following carbohydrates in 1 per cent. concentration: glucose, fructose, maltose, sucrose, lactose and starch.

The media were steam-sterilised for 20 mins, on three consecutive days. The pH values of the media, initially between 7 and 8, were adjusted to approximately pH 5 before sterilisation, and were determined again afterwards. The final pH values for all the media lay between pH 5.6 and 6.1.

Observations were made after an 8 week period. The following table lists the various strains under the features

as shown in colony development, and briefly gives a picture of the growth on the six carbohydrates:

Table 11

Features of five strains of A. mellea on six carbohydrates Number of cultures per treatment - 3. Growth period -8 weeks

carbohydrate source	densest mycelial development	greatest mycelial spread	sclerotial formation
fructose	R_2, M, GW	-	GW, R_2, M, C
glucose	-	R_2	GW, R_2, C
maltose	R	GW	GW, C
sucrose	R	-	GW, R_2, M, C
lactose	-	R, C, M,	GW
starch	R_2	C	GW, R_2, C

The general growth of all colonies afforded no comparison between the carbon sources employed, and was poor compared with similar colonies on malt agar. On the six media, a thin mycelium developed with sparse aerial hyphae.

Some small differences could be seen but no rhizomorphs were present and in only a few instances was there any formation of a sclerotium. This sclerotium was usually small, but never formed on the lactose medium

Experiment 3. Growth of one strain on six carbohydrate sources.

Strain Gw was cultured in 18 x 100cc flasks at 25 C. on a peptone-glucose-saccharose liquid medium. The flasks were in 6 sets, each containing 5 per cent of one of the following carbohydrates: lactose, maltose, glucose, sucrose,

and starch; one set was used as a control with no carbohydrate present. Observations were made after 2 weeks' growth and are shown in Table 12.

Table 12

Features of growth of cultures of strain GW grown on five carbohydrate sources. Number of cultures per treatment -3. growth period - 2 weeks.

medium	rhizomorphs	sclerotium	wet wt. (in gm.)	dry wt. (in gm.)
lactose	-	+	0.112	0.011
maltose	-	+	0.134	0.023
glucose	+	+	3.450	0.313
sucrose	+	+	0.543	0.051
starch	-	+	0.264	0.042
control	-	+	0.167	0.011

From both wet and dry weights the growth on glucose was greatest, with that on sucrose being the next. Growth was poor on the other media but sucrose was only slightly better. It was observed that the amount of sclerotial material and browning of the medium varied according to the amount of growth.

Experiment 4. Growth of one strain on four nitrogen sources

Strain GW was cultured in 24 x 250cc. flasks at 25 C. on peptone-glucose-saccharose medium. The flasks were in four sets, each containing one of the following nitrogenous sources in the given concentrations : 0.5 per cent. peptone. M/2 potassium nitrate, M/4 ammonium nitrate, M/4 ammonium sulphate.

Observations were made after 2 weeks' growth and are shown in Table 13.

Table 13

Features of growth of cultures of strain GW grown on four nitrogen sources. Number of cultures per treatment - 6. Growth period - 2 weeks.

nitrogen source	rhizomorphs	sclerotium	wet wt. (in gm.)	dry wt. (in gm.)
peptone	+	+	3.234	0.301
KNO_3	-	-	2.066	0.029
NH_4NO_3	-	-	1.061	0.050
$(NH_4)_2SO_4$	-	-	0.714	0.026

The amount of growth shown on the last three nitrogen sources is poor compared with that on peptone. No differences were apparent in their development.

Experiment 5. Growth of five strains on media containing combinations of glucose, yeast extract, and peptone.

It was decided to grow A. mellea on media of more certain composition than that containing malt extract, and possibly gain some information about the nutrient requirements of the fungus.

Sixteen agar media were prepared containing glucose, yeast extract, peptone and mineral constituents, as indicated in Table 14. Sterilisation was effected by steaming for 20 mins. on 3 successive days. Triplicate plates of each medium were inoculated in the usual manner with each of strains GW, M, C, R and R_2 of A. mellea. The experiment was incubated at 25°C. and observations were made after 3 weeks.

Table 14

Composition of media containing glucose, yeast extract, peptone, and mineral constituents.

initial agar medium	additions			
5% glucose	-	.15% yeast	.5% peptone	.15%yeast .5%peptone
1% glucose	-	.15% yeast	.5% peptone	.15%yeast .5%peptone
water	-	.15%yeast	.5% peptone	.15%yeast .5%peptone
Knop's	-	.15%yeast	.5% peptone	.15%yeast .5%peptone

The final observations after 3 weeks showed that variations in the medium used had a marked effect on the development of the fungus, as shown by the type of colony.

The combination of 5 per cent. glucose, 0.5 per cent. peptone and 0.15 per cent. yeast extract, gave the densest colonies for all the strains, and usually the greatest formation of sclerotia and rhizomorphs also. The addition of peptone stimulated the formation of sclerotium for strains GW and R_2, and the addition of yeast plus peptone, allowed sclerotial development of strains GW, M, C and R_2. The four rhizomorph-forming strains developed rhizomorphs on media with yeast and strain GW also on medium with glucose plus peptone.

The general growth and development of all strains was poor on media with no glucose; colonies of strains GW, M and C, showed dense mycelium and aerial growth when yeast and peptone were incorporated in the medium. No

sclerotium or rhizomorphs were formed except in strain GW, which formed a sclerotium on media having yeast and peptone present.

There was no difference in the colonies growing on the initial media, water agar and Knop's agar.

Experiment 6. Effect of three components of yeast extract on growth of one strain.

In this experiment, three components of the vitamin B complex, which is present in yeast extract, were tested for their effect on the development of the fungus. The basic medium contained 5 per cent. glucose and 2.5 per cent. sulphate of ammonia. The amounts of the vitamin B components were calculated from an analysis of yeast extract supplied by the makers. The quantity was that in a 0.15 per cent. yeast extract solution.

Triplicate flask cultures of strain GW were grown in 100cc.flasks. The experiment was incubated at 25°C and observations, as detailed in Table 15, were made after 4 weeks.

It was seen that no sclerotial formation took place in the solutions lacking the complete yeast extract. Rhizomorphs originated from various points in the mycelial mass, but were always very tiny and did not extend. Even the combination of the three components of the vitamin B complex did not stimulate growth similar to that found on the full yeast extract.

Table 15

Features of cultures of strain GW grown on various components of yeast extract. Number of cultures per treatment - 3. Growth period - 4 weeks.

medium	mycelium	sclerotium	rhizomorphs
yeast extract (0.15%)	present on sclerotial surface; has guttation drops, and medium is brown in colour also.	rounded mass	many and branched (short)
nicotinic acid (0.4185mg./l.)	fringe of mycelium extending from the inoculum into the medium; some slight thickening of the inoculum surface.	-	very small
riboflavine (0.0285mg./l.)	as for nicotinic acid	-	very small
thiamine (0.0048mg./l.)	as for nicotinic acid	-	rhizomorph points
nicotinic acid riboflavine thiamine	as for nicotinic acid	-	small
control	as for nicotinic acid	-	small and with aerial tips

Experiment 7. Growth of one strain on various agar media

Strain GW was grown on various media in order to determine their effect on sclerotial and rhizomorph formation. Triplicate plates of the following media were inoculated in the usual manner, and incubated at 25°C. Observations were made after 3 weeks and the details recorded in Table 16.

clarified malt extract agar, 2 per cent.
peptone-glucose-saccharose agar
Knop's agar
water agar
Pfiefer's medium
Tubeuf's medium
0.15 per cent. yeast extract, 0.5 per cent. peptone
0.5 per cent. asparagine, 0.3 per cent. yeast extract
Schopfer's medium

Table 16

Features of cultures of strain GW grown on various media at 25°C. Number of cultures per treatment - 3. Growth period 3 weeks.

medium	mycelium	sclerotium	rhizomor
2% malt extract	extension of the zonation of mycelium; that above rhizomorphs fusing	below a thin mycelial cover	many and branching
p.-g.-s.	whitish mycelial cover	brown and zoned	initials
Knop's	thin, even and white	-	-
water	thin, even and white	-	-
Pfiefer's	brownish, submerged in the agar	small and central	-
Tubeuf's	thin and whitish, radiating from the inoculum	-	-
yeast-peptone	whitish-brown, slightly fluffy; zoned surface	below mycelium	branching
asparagine-yeast	thin, brownnand zoned, having a thinner edge; medium browned; some guttation drops present	small and central	initials
Schopfer's	brown, with whitish dense irregular fringe.	small and central	-

It can be seen that the media, with a few exceptions, stimulated the formation of a sclerotium. Rhizomorphs were formed only on the malt and yeast-peptone media while undeveloped initials of these organs only on the peptone-glucose-saccharose and yeast-asparagine media.

Experiment 8. Growth of one strain on various liquid media

In order to obtain quantitative comparison of growth the strain GW of A. mellea was grown on the liquid media listed in Table 17. Triplicate cultures were grown on 50cc. lots of each medium in 250cc. flasks, using the standard inocula. The experiment was incubated at 25°C. for one month, and the wet and dry weights then determined.

The cultures showed a considerable variation in appearance and bulk according to the medium used. Table 17 shows the wet and dry weights.

Table 17

Growth of strain GW on eight liquid media at 25°C. Number of cultures per treatment - 3. Growth period - 4 weeks.

medium	wet weight (in gm.)	dry weight (in gm.)
2 per cent. malt exract	6.616	0.317
5 per cent. malt extract	20.796	1.041
10 per cent. malt extract	24.386	2.365
peptone-glucose-saccharose	18.532	1.252
Knop's	0.052	0.001
Tubeuf's	0.089	0.003
0.15 per cent. yeast extract 0.5 per cent. peptone	8.778	0.320
0.5 per cent. asparagine 0.3 per cent. yeast extract	1.921	0.094

From these results it was apparent that greatest development as determined by weight, had occurred on 10 per cent. malt extract, then on peptone-glucose-saccharose,

5 per cent. malt extract,2 per cent. malt extract, and then other media supported relatively less growth. These results were in accordance with the observed differences as illustrated in Plates 10 and 11.

Experiment 9. Growth of five strains on apple root portions

Apple roots were obtained and cultures made on portions of them. Roots about half an inch diameter were washed free from soil and cut into portions approximately three inches long. Some portions were surface-sterilised by dipping into absolute alcohol, transferring to 0.1 per cent mercuric chloride solution for three minutes , and then washing thoroughly in sterile water. Similar pieces of root were sterilised by autoclaving for 1 hr. at 15 lbs. pressure.

The surface-sterilised(living) and autoclaved (dead) portions of root were then added to duplicate malt agar slope cultures of each of five strains of A. mellea. Final observations were made after incubation at 25°C for 2 weeks.

Strains GW, M, C and R_2 had formed a dense mass of fluffy white mycelium on the exposed surfaces of the root, while strain R formed rather less mycelium on the cut ends. This occurred on the living root portions, mycelium appearing in all strains after one week. There had been no formation of rhizomorphs in the root, and invasion of roots had taken place by hyphal growth alone. No further observations were made as contaminants developed.

The dead apple root portions were invaded by A. mellea within a week, the mycelium being apparent before that of the corresponding living root cultures. The mycelium was concentrated at the ends of the roots, forming a white cover and a sclerotial rim where it touched the tube. The sides of the roots against the fungal colonies were surrounded by mycelium which had formed small white tufts on their surfaces. In some instances, the fungus had formed rhizomorphs under the bark of the root, these rhizomorphs sometimes penetrating outwards and becoming aerial. In only one tube had the invasion of the root taken place by rhizomorph penetration. Table 18 summarises the condition of each strain of A. mellea in the dead root cultures.

Table 18

Features of growth of five strains of A. mellea on dead apple root portions. Number of cultures per treatment = 2. Growth period - 2 weeks.

	GW	M	C	R	R_2
rhizomorph penetration	+	-	-	-	-
mycelial penetration	-	+	+	+	+
mycelial tufts on root	+	+	+	+	-
mycelial cover on root	-	-	-	-	+
sclerotium against tube	+	+	-	-	+
rhizomorphs under bark	+	+	+	-	+
aerial rhizomorphs	+	+	+	-	+

Experiment 10. Growth of five strains on apple and cherry root extracts.

From the previous experiment it was seen that the fungus developed better on dead roots. It was decided to test the growth of A. mellea on extracts from both apple and cherry roots.

The outer tissues of the roots, consisting of phloem, cortex and bark layers, were cut into shavings to facilitate the extraction of substances. 200gm. of this material were steamed in a litre of distilled water for 3 hrs. The resultant extract was filtered through coarse filter paper and the filtrate made up to a litre by the addition of distilled water. A sterile agar medium was prepared from the extract by the addition of 2 per cent. agar followed by the usual sterilisation. The final pH value was 4.4.

A similar wood medium was prepared from the wood of apple roots and the pH in this instance was 5.3.

Six plates of each medium were inoculated in the usual way with each of the strains GW, M, C, R and R_2. The experiment was incubated at 25°C and observations made after 2 weeks.

In all the cultures there was an appreciable growth of mycelium after 2 weeks' incubation. The cultures on the bark extract medium showed much denser growth for all the strains than the cultures on the wood extract medium; sclerotial formation was better on the bark medium, the sclerotium being covered by fluffy masses of mycelium.

Development of rhizomorphs was also more extensive on the bark medium. On the wood medium the mycelium was thinner but the central area was sometimes rather dense. Table 19 shows briefly the chief features of the cultures formed.

Table 19

Features of growth of five strains of A. mellea on extracts of apple wood and bark. Number of cultures per treatment - 6. Growth period - 2 weeks.

		GW	M	C	R	R_g
mycelial growth	bark	-	+	-	+	-
	wood	+	+	+	+	-
sclerotium	bark	+	-	+	-	+
	wood	-	-	-	-	+
rhizomorphs	bark	+	-	+	-	+
	wood	-	-	+	-	-
pigment in medium	bark	+	+	+	+	+
	wood	+	-	+	+	+

This experiment was repeated with cherry bark and wood extracts, and results were essentially similar to those described above.

Conclusions

In the different strains of A. mellea, great variation in growth form due to differences in mycelial, sclerotial and rhizomorph formation occurred and was influenced by the various media used. Media containing the greatest quantities of dissolved nutrients, such as 10 per cent. malt extract and peptone-glucose-saccharose solutions, supported maximum growth of the fungus with development

of sclerotium and rhizomorphs.

As found by other workers, the most favourable carbon and nitrogen sources for growth of A. mellea were glucose and peptone, respectively.

It was noted that the addition to the medium of yeast extract stimulated the growth of the fungus. The best development of sclerotium and rhizomorphs took place on glucose-peptone media containing yeast. It is probable that some of the constituents of the yeast extract might be responsible for these effects, but the experiment with three components of the vitamin B complex showed that the latter was not responsible constituent of the yeast extract.

The fungus could invade dead material more rapidly than live, and live roots, though excised and probably moribund, seemed to resist invasion. Development of rhizomorphs of A. mellea in the cortex of dead roots was probably due to the provision of a suitable food base by the dead cortical cells.

The better development of mycelium, sclerotium and rhizomorphs on bark extract, indicated that bark contains more substances suitable for the growth of the fungus than does the wood. This is in agreement with the fact that development in the field is usually extensive in the outer tissues of the host before invasion of the wood takes place.

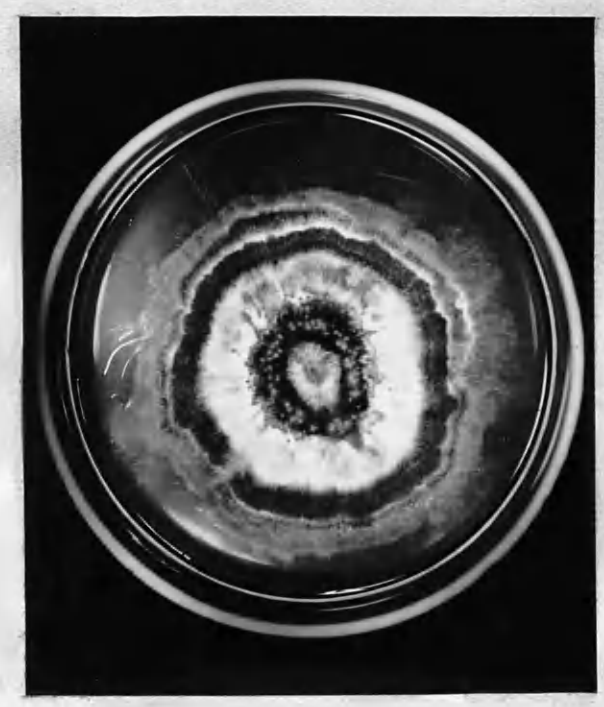
a)

b)

c)

Plate 8. Two-month-old cultures of A. mellea grown at 25°C
a) strain GW
b) strain M
c) strain C

a)



b)

c)

Plate 9. Two-month-old cultures of A. mellea grown at 25°C
a) strain R
b) strain R_2
c) strain O

a)

b)

c)

d)

Plate 10. Month-old cultures of strain GW grown at 25°C on
a) 2% malt extract solution
b) 5% malt extract solution
c) 10% malt extract solution
d) peptone-glucose-saccharose solution

a)

b)

c)

Plate 11. Month-old cultures of strain GW grown at 25°C on
a) Knop's solution
b) yeast-peptone solution
c) yeast-asparagine solution

f) Influence of the inoculum on colony development

For stock cultures, mass transfers of fungal material were made and although there were variations of colony type within each strain, it was always possible to distinguish between strains. This was especially so in the rhizomorph-forming strains, which had variations in the initiation and extension of the rhizomorphs and it was thought that the inoculum used may have influenced the subsequent development of the colony.

The following investigations were made to determine whether there was any relationship between the type of inoculum and the colony formed.

Experiment 1. Effect of inocula from different zones of a colony on the subcultures developing from them.

The strain R_2 showed definite zonation in agar culture the sclerotium which formed having light and dark zones concentrically placed about the original inoculum. The colony was usually circular and the rhizomorphs which sometimes developed had no sclerotial covering. A colony having no rhizomorphs was used for this experiment, (see Plate 12).

Inoculum discs were cut from a colony on 2 per cent. malt agar, the diameter of 3 mm. enabling them to be taken from within the margins of the zones. These inocula were numbered 1 - 8 from the centre outwards. Duplicate cultures from each of the six dark and two light zones were grown on malt agar plates at 25°C.

After 18 days the observations shown in Table 20 were made.

Table 20

Features of growth of subcultures of strain R_2 grown at 25 C. Number of cultures from each zone - 2. Growth period - 18 days.

	inoculum							
	1 (d.)	2 (d.)	3 (l.)	4 (d.)	5 (d.)	6 (l.)	7 (d.)	8 (d.)
diameter of sclerotium (in cm.)	3.15	2.8	3.1	3.1	3.0	2.95	2.82	2.8
rhizomorphs initials	--	--	-x	-x	-x	--	-x	-x
rhizomorph extension	xx	xx	--	x-	x-	x-	x-	--

There was apparently no relationship between the development of the colonies and the sources of their inocula. This was still the case after 5 weeks' growth. The main features of a typical colony formed in the sub-cultures is shown in Plate 13.

Experiment 2. Growth of one strain of the fungus using various inocula.

The strain chosen for this investigation formed all the varying types of fungal growth shown by A. mellea. Strain GW was grown at 25°C. in 48 plates of 5 per cent. malt agar. The cultures were in 8 sets, each having one of the types of inocula detailed in Table 21.

In all cultures, considerable growth had taken place after 4 weeks, and each type of inoculum had produced a typical growth of the fungus, although there were variations within each set of six cultures. A sclerotium was always

formed but the differences in rhizomorph development and extent were the most striking features. These are summarised in Table 21 and illustrated by Plate 14.

Table 21

Features of the growth of strain GW at 25°C. using varying inocula. Number of cultures per treatment - 6. Growth period - 4 weeks.

form of inoculum	form of subculture	
	point of origin of rhizomorphs	rhizomorph growth - x^n
hyphal tip	colony centre	x
mycelium in agar (disc)	edge of disc	xx
tuft of aerial mycelium	centre	xxxx
sclerotium with rhizomorphs (disc)	edge	xxxx
sclerotium, no rhizomorphs (disc)	edge	0
rhizomorph tip from agar (disc)	edge	xxxxx
aerial rhizomorph tip	centre	xxxxx
rhizomorph portion from agar (disc)	edge	xxx

In all cultures there was development of sclerotium .

Conclusions

The size attained by a colony depends on its rhizomorph development and the subsequent formation of attached sclerotium. The formation of rhizomorphs does not depend on the presence in the inoculum of rhizomorph initials, but their abundant development did occur from inocula containing rhizomorph portions.

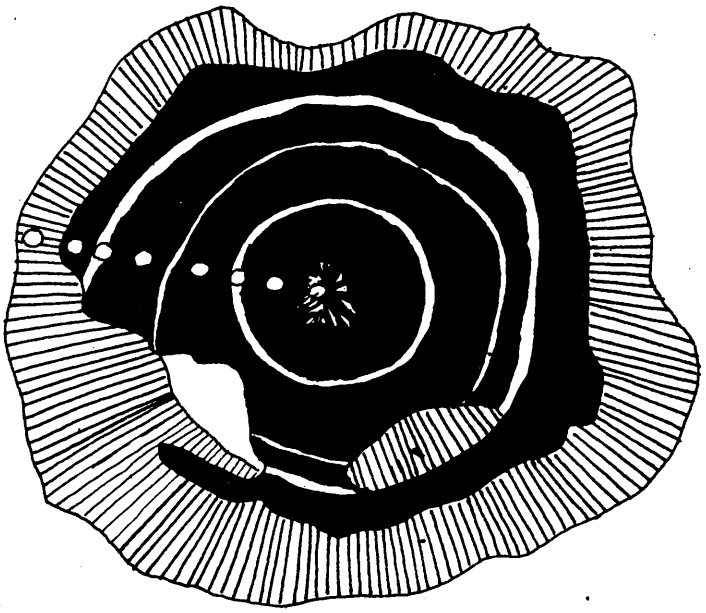


Plate 12. Diagram of a six-week-old colony of strain R_2. Note the concentric zones of light and dark sclerotial material with an outer hyphal margin. The light circles represent positions from which inocula were taken.

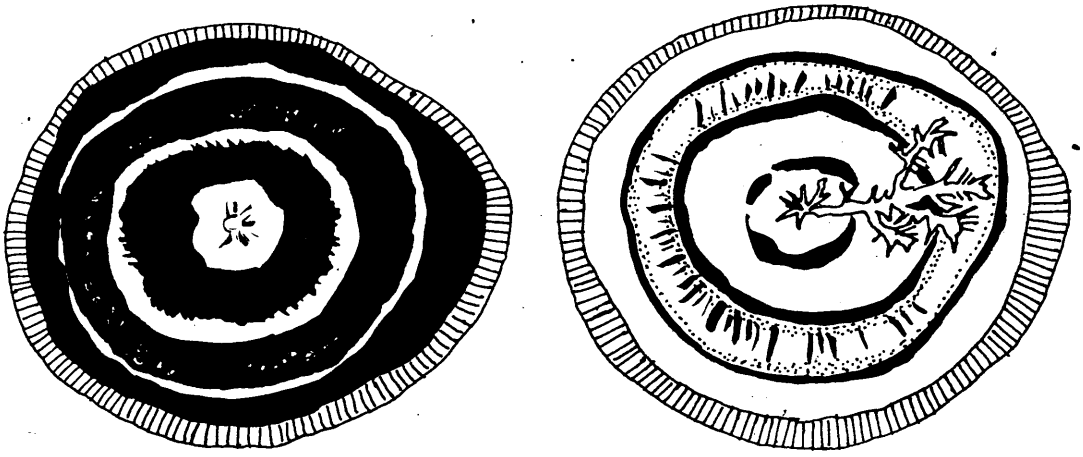


Plate 13. Diagram of a five-week-old colony of strain R_2, a subculture from that shown in Plate 12, and having similar zonation.
a) upper surface
b) lower surface. Note the branching rhizomorph.

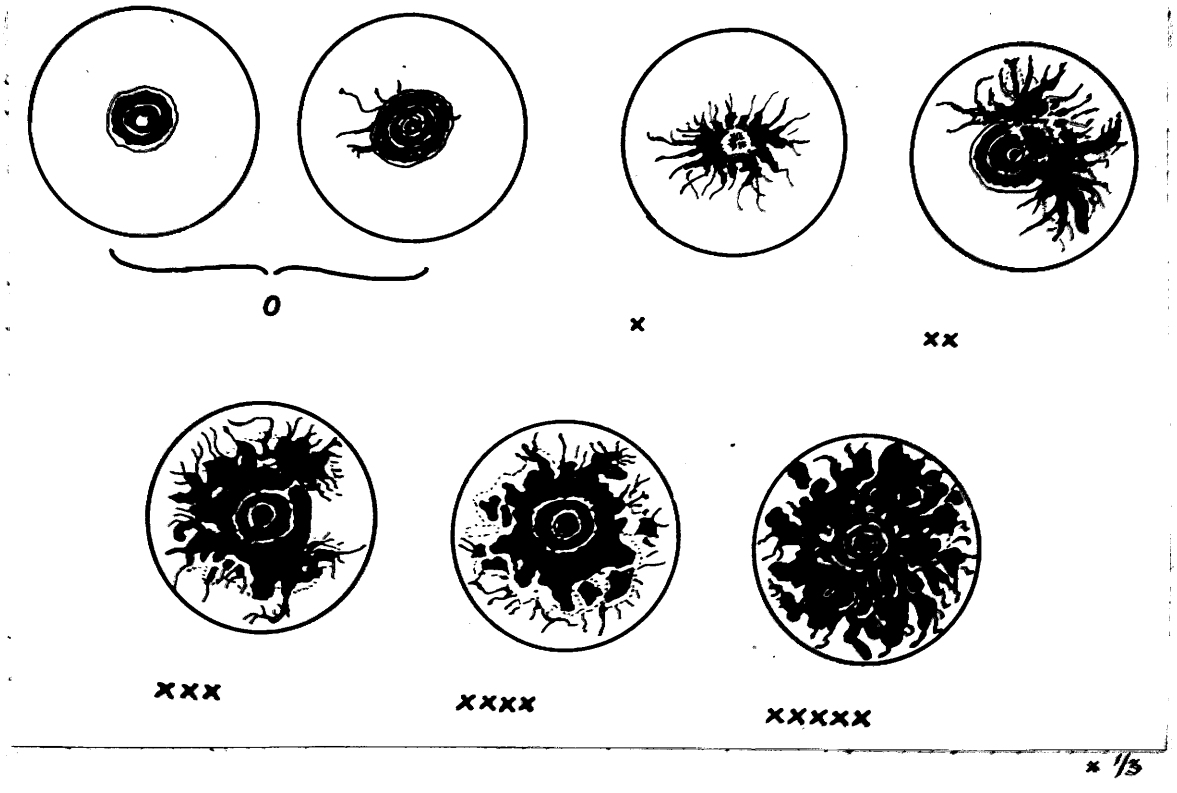


Plate 14. Diagram to show variation in rhizomorph development in month-old cultures of strain GW.

g) Culture with other fungi

There appear to be few records of the influence of other micro-organisms on the growth and development of A. mellea which can exist both saprophytically in the soil and parasitically on various host plants. This fungus can, presumably, compete successfully with other soil organisms for the available nutrients, and in order to do this, it may produce some antibiotic substance or substances. A. mellea may itself be influenced by other soil organisms, which produce similar substances.

As mentioned in the Review of Literature, it was stated by Hiley (1919) that the presence of another wood-rooting fungus on dead stumps of trees would prevent the development of A. mellea. A similar observation was made by Leach (1939) when he found that the organism would not spread in host tissues which were already infected by other fungal parasites or saprophytes. After fire, which had presumably destroyed other micro-organisms, A. mellea was found to develop in the remaining stumps (Guyot, 1933). One common soil fungus, Trichoderma lignorum is known to inhibit its growth (Weindling, 1934; Bliss, 1941). No details of these observations are given and little is known of the antagonism between A. mellea and other organisms which are able to inhabit a similar substrate.

In the following investigations, wood and soil inhabiting fungi were cultured with A. mellea.

The fungi listed below were used in the investigations:

From the Departmental Culture Collection, Glasgow.

1) Phycomyces nitens (1)

2) Phycomyces nitens (2)

3) Aspergillus oryzae

4) Helminthosporium sativum

5) Monilia fructigena

6) Mucor hiemalis +

7) Mucor hiemalis -

8) RRhizopus stolonifer

9) Gibberella zeae

10) Aspergillus niger

11) Stachybotrys

12) Penicillium notatum

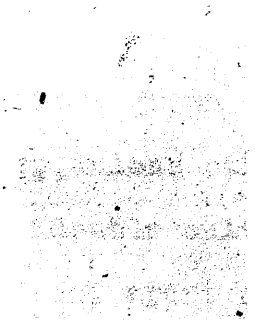
13) Trichoderma sp.

14) Trichoderma viride 1

15) Trichoderma viride 211

16) Trichoderma viride 213

17) Trichoderma koningi 41



From The Forest Products Research Laboratories, Aylesbury.

(the type numbers are given with the fungi)

1) Ustilina vulgaris (95)

2) Polyporus squamosus (103a)

3) Polyporus sulphureus (29a)

4) Polyporus schweinitzii (102b)

5) Stereum purpureum (88)

6) Stereum sanguinolentum (27a)

7) Fomes pinocola (98)

8) Fomes applanatus (20)

9) Coniophora cerebella (11a)

10) Poria vaillantia (14)

11) Merulius lacrymans (12)

12) Trametes pini (45b)

A preliminary test determined that the fungi were able to grow well on 2 per cent. malt agar at pH 5, which is the most suitable for the growth of A. mellea; all except Stachybotrys had a greater growth rate than the latter. The strain GW was used in these investigations.

For the experiments, the cultures were grown on plates inoculated in the standard manner, unless otherwise indicated, and incubated at 25°C. The "double cultures" consisted of two inocula placed about 2 cm. apart on the same plate.

Development of cultures was assessed in arbitrary units (xx - normal growth of A. mellea). Comparison was made with cultures of the fungi growing separately in culture under similar conditions.

Experiment 1. Growth of A. mellea and seventeen soil fungi in double culture.

Triplicate cultures of each of the seventeen soil fungi listed previously were made with A. mellea. Observations made after 14 days showed that in most plates the soil fungus had made good growth and had covered the medium A. mellea grew in its usual manner in about half the plates;

in the others, growth was somewhat reduced with the other fungus extending over it. The results are shown briefly in Table 22.

Table 22

Features of growth of strain GW and 17 soil fungi. Number of cultures per treatment - 3. Growth period - 2 weeks.

fungus	growth of A. mellea	growth of other fungus	fungus inhibited	comments
P. nitens 1, 2	xx	xxxx	-	some strands of P. over A.
A. oryzae	0	xxxx	A.	A. oryzae over most of A.
H. sativum	0	xxx	A.	H. over A.
M. fructigena	xx	xxx	A. & M.	reduced growth on adjacent sides
M. hiemalis - -	xx	xxxx	-	M. over most of A.
G. zeae	x	xxx	A.	G. over A.
R. stolonifer	x	xxxx	A.	R. over A.
A. niger	x	xxx	A.	no overgrowth
Stachybotrys	xx	xx	S.	S. growth reduc adjacent to A.
P. notatum	xx	xx	A. & P.	reduced growth of both
Trichoderma	0	xxxx	A.	T. over A.
T. viride 1	0	xxxx	A.	T. over A.
T. viride 211	0	xxxx	A.	T. over A.
T. viride 213	0	xxxx	A.	T. over A.
T. koningi 41	0	xxxx	A.	no overgrowth

In some instances the development of A. mellea in double culture as compared with that of the fungus itself, showed a definite reduction of growth. Only in the case of Stachybotrys did the A. mellea inhibit the growth of a another fungus.

Experiment 2. Growth of A. mellea with delayed inoculation of the soil fungi.

A.mellea was allowed to grow alone for two weeks, in order to establish itself before the plate was inoculated with another fungus. Any substance produced would therefore have time to diffuse into the agar where it could affect growth of a second organism. After two weeks' growth the results of the double cultures were assessed as shown in Table 23.

In all the cultures the colonies of A. mellea developed normally and a zone with little or no mycelium was left between the two organisms. The obvious inhibition of the growth of A. mellea noted in the first experiment had not taken place and in some cases the inhibitory effect had been reversed.

Continued observation of the cultures showed that growth of A. mellea was eventually inhibited by the other fungi, as seen in the first experiment.

(Table 23 is given on the following page)

Table 23

Features of growth of strain GW with delayed inoculation of 17 soil fungi in double culture. Number of cultures per treatment - 3. Growth period - 2 weeks.

fungus	growth of *A. mellea*	growth of other fungus	fungus inhibited	comments
P. nitens 1, 2	xx	xxxx	-	some P. strands over A.
A. oryzae	xx	xxxx	A.	narrow intermediate zone of sparse myc.
H. sativum	xx	xxx	A. & H.	wide zone between A. & H. of sparse myc.
M. fructigena	xx	xxx	A. & M.	intermediate zone with no myc.; dark edge on M. colony
M. hiemalis -	xx	xxxx	-	hyphae of M. on edge of A.
M. hiemalis -	xx	xxxx	M.	narrow inter. zone, no fungus
G. zeae	xx	xxxx	-	some strands of G. over A.
R. stolonifer	xx	xxxx	R.	clear inter. zone with a little myc.
A. niger	xx	xxx	A.	a little A. niger on A.; poss. A. inhibited
Stachybotrys	xx	x	S.	wide inter. zone.
P. notatum	xx	xxx	A. & P.	clear zone
Trichoderma	xx	xxxx	-	some T. on A.
T. viride 1	xx	xxxx	A. & T.	mutual inhib.
T. viride 211, 213	xx	xxxx	x	some T. on A.
T. koningi 41	xx	xxxx	-	some T. on A.

Experiment 3. Growth of A. mellea on agar containing growth products of the soil fungi.

A greater concentration in the medium of growth products of a second fungus might have a more definite effect on A. mellea . The 17 soil fungi were cultured on malt agar plates for a week then the agar was reversed and an inoculum of A. mellea was placed on the former underside. Observations were made after another weeks' growth and these are shown in Table 24. A. mellea was affected to varying degrees by the agar cultures. No growth had occurred on plates containing Trichoderma spp. and some re-establishment of the latter took place on the reversed agar. Some growth of A. mellea was apparent in the other cultures, but was reduced.

Experiment 4. Effect of substances from an A. mellea culture on growth of the soil fungi.

Colonies of A. mellea, grown for 12 days on top of pieces of sterilised cellophane 2 cm. square lying excentrically on malt agar plates, were transferred to fresh agar and held at 4°C. for 24 hrs. The colonies did not grow at 4°C. and the medium gained from them any diffusible materials After the 24 hrs. the cellophane was removed and substituted by inocula of the 17 soil fungi; control inocula were placed on the adjacent halves of the plates. Observations were made after 3 days (see Table 25.) Only seven of the fungi showed any retardation of growth, the three most outstanding being M. fructigena, G. zeae and A. niger

The experiment was repeated with these fungi and similar results were obtained.

Table 24

Growth of strain GW on agar containing growth products of the soil fungi. Number of cultures per treatment - 3. Growth period - 1 week.

fungus	growth of *A. mellea*	comments
P. nitens 1	0	no myc. margin
P. nitens 2	x	no margin of aerial myc.
A. oryzae	x	definite myc.spread
H. sativum	x	no myc. margin
M. fructigena	x	narrow myc. margin
M. hiemalis -	x	no myc. spread
M. hiemalis -	x	no myc. spread
R. stolonifer	x	narrow myc. margin
G. zeae	x	no myc. spread
A. niger	x	*A. niger* growing onto *A.*
Stachybotrys	xx	small colony of *S.* (below only part of *A.*)
P. notatum	x	some *P.* myc.over *A.*
Trichoderma	0	*T.* over *A.*
T. viride 1	0	*T.* over *A.*
T. viride 211	0	*T.* over *A.*
T. viride 213	0	*T.* less dense than above
T. koningi 41	0	*T.* over *A.*

Table 25

Growth of 17 soil fungi as affected by substances from *A. mellea* in the medium. Number of cultures per treatment-3. Growth period - 3 days.

fungus	diameter of soil fungus colonies (in cm.) expt.	control	inhibition	comments comparison of exp. colony to control
P. nitens 1 } 2 }	2.25	4.5	-	reduced growth
A. oryzae	2.0	2.2	-	slightly less growth
H. sativum	1.6	1.7	-	similar coloni
M. fructigena	0.55	0.85	-	reduced growth
M. hiemalis-	5.3	5.55	-	similar colonie
M. hiemalis-	5.05	5.75	-	similar colonie
R. stolonifer	0.95	1.15	-	whole plate covered, only dense centres measured
G. zeae	2.3	2.65	-	reduced growth
A. niger	1.8	2.2	-	reduced growth
Stachybotrys	0.45	0.6	-	slightly less growth
P. notatum	1.0	1.05	-	similar growth
Trichoderma	whole plate covered		-	less dense centre
T. viride 1	whole plate covered		-	thicker central myc.
T. viride 211	plate nearly covered		-	similar colonies
T. viride 213	whole plate covered		-	similar colonies
T. koningi 41	plate nearly covered		-	similar colonies

Experiment 5. Growth of A. mellea and twelve wood-rotting fungi in double culture.

A. mellea was grown, as in Experiment 1, with each of twelve wood-rotting fungi listed. The cultures were grown for two weeks before the observations in Table 26 were made.

In most of the cultures, A. mellea grew in a normal manner, but showed restricted development when surrounded by the other fungus (see Plate 16a).

A clear zone was left between A. mellea and a few of the other colonies, especially that of P. sulphureus (see Plate 16b).

Mycelium of P. schweinitzii and P. vaillantia grew over the A. mellea and the latter showed slightly less growth than normal (see Plate 16c).

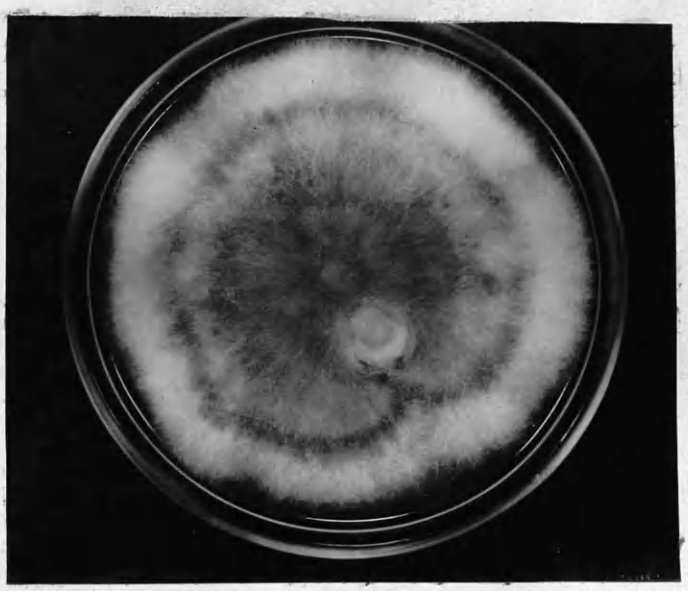
Conclusions

The foregoing experiments with A. mellea and other fungi in culture showed that some inhibition did occur, usually of A. mellea. When A. mellea was allowed to develop first, growth of some of the other fungi was reduced, but this effect soon disappeared. Other fungi could not grow intermixed with the colonies of A. mellea This result is in agreement with the observations of other investigators. Trichoderma spp. and two wood-rotting fungi, however, could extend over A. mellea colonies.

Table 26

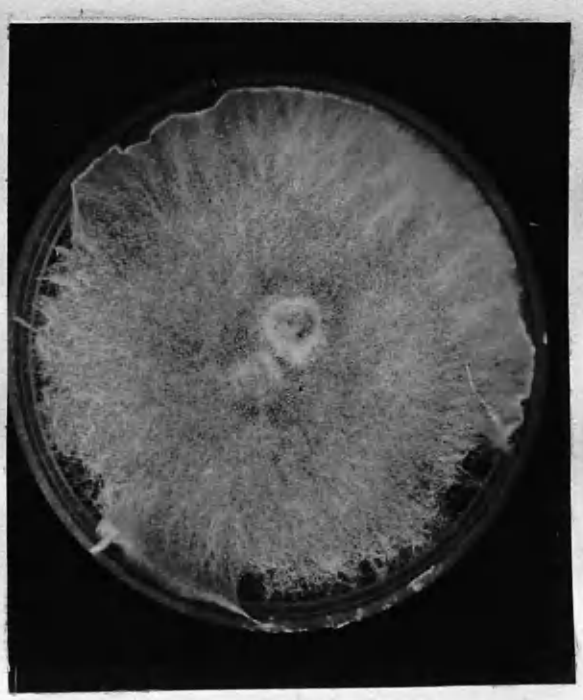
Growth of A. mellea with 12 wood-rotting fungi in double culture. Number of cultures per treatment - 3. Growth period - 2 weeks.

fungus	growth of A.mellea	growth of other fungus	fungus inhibited	comments
U. vulgaris	x	xxx	A.	A.colony nearly surrounded
P. squamosus	xx	xx	-	colonies still apart
P. sulphureus	xx	xxxx	P.	thin P. myc. in clear area round A.
P. schweinitzii	XX	xxxx	-	a little P. myc. on A.
S. purpureum	x	xxxx	A.& S.	A. colony surrounded & less dense myc. in inter.zone
S. sanguinolentum	xx	xx	S.	S. growth less on side next A.
F. pinicola	xx	xxx	A.	A. growth less on side next F.
F. applanatus	xx	xxx	-	A. nearly surrounded
C. cerebella	xx	xxx	A.	A. growth less on side next C. thin C. in inter. zone
P. vaillantia	xx	xxxx	-	P.over A. with poss. less growth of A.
M. lacrymans	xx	xxx	M.& A.	reduced growth on adjacent sides
T. pini	xx	xx	A.	A growth less next T.



a)

b)



c)

Plate 16. Two-week-old double culture of A. mellea grown at 25° C. with
a) Coniophora cerebella. Note that it surrounds the A. mellea inoculum.
b) Polyporus sulphureus. It leaves a clear zone.
c) Poria vailantia. It overgrows the A. mellea

h) Isolation of monospore cultures

Fructifications of A. mellea collected in the field (see Section a)) were used as a source of spore material.

No record has been found of the growth of monospore cultures of this fungus;it was considered that some interesting information about the development of colonies and the constancy of appearance of the fungal strain might be obtained.

A spore suspension in water was prepared and streaked over the surface of a malt agar plate. Germination of a number of spores took place in 24 hrs. at 25°C. The viable ones could be isolated, by locating under the low-power objective of a microscope and ringing round with a cutting-objective. Complete isolation of each spore was checked before transference to a malt agar slope.

After further incubation at 25°C. it was noted that only some of the isolated viable spores had developed into colonies. Colonies had developed from 17 out of 40 Auchincruive isolates, and out of 45 Alderley Edge isolates only 8 developed. The reason for the lack of growth in some cultures was not determined.

The appearance of colonies varied, but sclerotia and rhizomorphs were produced in all of them.

i) Production of fructifications

Some investigators found that fructifications of *A. mellea* could be produced in culture but that various strains behaved differently in this respect (Van Vloten, 1936). The conditions for their production are not exactly known; Reitsma (1932) found that fructifications developed when c cultures of peptone-glucose-saccharose media or twigs were placed in the daylight for several months.

Attempts were made to produce fructifications on peptone-glucose-saccharose solution, as above.

Experiment 1. Production of fructifications on a synthetic medium.

Following the method described by Reitsma (1932), strains GW, M, C, R, R_2 and O were each cultured in 3 x 250cc. flasks on peptone-glucose-saccharose solution. They were incubated at 25°C. for a month, and then transferred to a window bench facing North for a further growth period.

In June, fructifications appeared on a culture of strain O which had been growing for 7 months and had dried up(see plate 15). Varying sizes of fructifications were produced on the sclerotial mass, some very small; no annulus was present on the stipe though other typical features were noted.

Conclusions

Fructifications are formed in culture on synthetic medium by a tropical strain of *A. mellea*. This agrees with the findings of Reitsma (1932) except that the fructifications were formed after a growth period November - June, and

not in the autumn. The tropical strain may have a growth periodicity differing from that of the strains he cultured and the production of fructifications may depend on some such factor.

x 1/2

Plate 15. Fructifications of strain O produced after 7 months' growth of the fungus on peptone- glucose-saccharose solution under daylight conditions. Note the drying-up of the medium; various stages in the development of fructifications are seen.

B) INVESTIGATION OF PATHOGENICITY.

Many of the recorded observations of the pathogenicity of A. mellea are only concerned with noting the host plant, or adding a new district to those already known to harbour the disease. In Britain, there are numerous hosts, the main ones being pine, spruce, larch, beech and oak (Butler and Jones, 1949). Other plants such as apple, pear, raspberry, gooseberry and potato, may become infected in ground which was previously covered by wood and consequently harbours the fungus as a saprophyte.

Once invaded, living or dead parts of plants constitute foci of infection and a base from which the fungus can spread (Hiley, 1919; Dade, 1927), though completely rotted material does not appear to provide a suitable base for the extension of rhizomorphs (Leach, 1939). It is known that rhizomorphs can extend for great distances in soil, and one method of control is to dig a trench round the infected plants to prevent spread and further infection by means of rhizomorphs. Wallace (1935) suggested that the effective range for attack by rhizomorphs might be only a few feet.

Leach (1939) found that trees resistant to A. mellea might act as a barrier to the spread of infection by preventing establishment and subsequent passage to other hosts; this resistance was destroyed when trees were felled. Investigators have not determined what makes a plant

susceptible or resistant to infection by the fungus.

It has always been stated that rhizomorphs are the agents of infection exceptin the districts where none are formed and infection takes place by contact between deseased and healthy roots, with presumed entry by mycelium (Dade,1927). One instance has been reported of infection by spores (Van Poeteren, 1939). Detailed investigations of attack by A. mellea have been confined to that by rhizomorphs; Day (1927) and Thomas (1934) came to similar conclusions about the method of penetration (see review).

It was thought that further investigations of actual invasion,including that by spores and hyphae, would be of interest. The following experiments were carried out to determine both the relationship of the food-base to the virulence of A. mellea, and the method by which the fungus attacks the selected host.

Material and Method.

The host plants used in these experiments had to be grown in pure culture in order to eliminate the presence and effects of other organisms. The "Monarch Long Pod" and "Field Tick" varieties of broad bean, Vicia faba, were chosen for this purpose. All the strains of A.mellea available, were cultured, while spore material was again obtained from locally collected fructifications.

The bean seeds were surface-sterilised with mercuric chloride. Dry beans were shaken for 2 mins. in absolute alcohol in a flask; the alcohol was replaced by 0.1 per cent. mercuric chloride solution and the flask agitated for a further 6 mins. Six changes of distilled water were used to remove all traces of the chloride. The beans were soaked for approximately 24 hrs. in sterile water and then placed on agar plates at 25°C. to germinate. After a few days, when it was obvious that no contamination had developed, they were usually transferred to boiling tubes, each containing 50cc. of nutrient agar medium. Previous tests had shown that this was suitable for growth of bean roots, and that the seeds contained enough stored food for several weeks' growth, providing that the medium was not allowed to dry up.

A. mellea was cultured in the tubes with the seedlings standard agar discs being used as inocula. Pathogenicity could be tested in this way, and observations made of the mycelium and rhizomorphs, and their contact with the roots of the seedlings.

a) Relation of nutrient supply to virulence of A. mellea.

In this series of experiments, various media, several strains of the fungus, and "Monarch Long Pod" variety of V. faba were used in order to investigate the effect of the external food supply on the ability of the fungus to attack the bean seedling.

Experiment 1. Culture of bean seedlings with one strain of A. mellea.

Twelve tubes containing 2 per cent. malt agar (pH5.4) were simultaneously inoculated with bean seeds and strain GW, and were incubated at 25°C.in darkness. After one week, they were placed at room temperature in the light for another 4 weeks, during which time observations were made.

Rhizomorphs of the fungus and roots of the bean developed in close association with each other in the agar. It was sometimes possible to observe contacts between roots and rhizomorphs,but actual penetration was visible in only a few associations. Rhizomorphs grew very close to some roots, but apparently were not always stimulated to invade. Plate 17 illustrates the culture method and rhizomorph-root contacts.

Other instances of root penetration may have occurred in the depths of the medium, where they were not visible. it was not possible to assess the number of fungus-root contacts,even when the cultures were removed from the tubes and the surrounding agar melted. The agar came away

easily from the roots, but adhered firmly in the form of a sheath round the rhizomorphs. This sheath was held in position by fine hyphae, spreading from the rhizomorphs like root hairs; these were characteristic of the fungus in agar culture. Some roots remained attached to the rhizomorphs by means of this agar sheath.

The roots presented a brown, withered appearance where they were in contact with sclerotial or myeelial cover on the agar, and microscopic examination of these areas revealed the presence of mycelium. As a similar brown discolouration was present in some roots not in contact with the fungus, it may be partly due to the oxidising system of the seedling.

Experiment 2. Culture of bean seedlings with each of the five strains of A. mellea.

In these cultures, the inoculation of the tubes with A. mellea was made about 2 weeks before the germinated beans were added. This allowed growth of the fungus in the agar prior to the development of roots.

Duplicate cultures of strains GW, M, C, R and R_2 were prepared as before, and kept at 25°C. in darkness.

Observations made 10 days after the bean was included are summarised in Table 27.

It was seen that the number of associations and contacts increased with the development and extension of rhizomorphs.

In cultures with poorly developed rhizomorphs, it

was noted that invasion of the root tissue, as indicated by a necrotic appearance, was effected by mycelium alone.

Table 27

Features of cultures of bean seedlings(10 days old) with five strains of *A. mellea*. Number of cultures of each strain - 2.

strain	growth of *A. mellea*	growth of *V. faba*	comments
GW	sclerotial mass with abundant rhizomorphs	long root and many laterals	rhiz.-root contacts; a few penetrations
M	mainly mycelial, but rhizomorphs develop from small sclerotium	long root and many laterals	no rhiz.-root contacts
C	mycelial cover on sclerotium; abundant rhizomorphs	long root, and some laterals	rhiz.-root contacts; a few penetrations
R	only mycelium develops	main root, some laterals	-
R_2	mycelial cover on sclerotium; many rhizomorphs	long root, and some laterals	rhiz.-root contacts; no visible penetrations

Experiment 3. Effect of four media on pathogenicity of *A. mellea*.

From the foregoing experiments there is evidence that the form of the fungal growth exerts a deciding influence on the virulence of *A. mellea*. Four media were chosen to test whether the source of fungal nutrient had an effect on the pathogenicity. The pH values were adjusted to 5, and the media sterilised by steaming on three successive days. Strain GW was cultured as it produced abundant rhizomorphs on suitable media, and these

effected penetration of the roots. The media used, were:

1) Knop's medium with 2 per cent. agar

2) Knop's medium with nutrient source and agar

3) peptone-glucose-saccharose agar

4) "Monarch Long Pod" bean extract agar.

Cultures were prepared as before with the addition of the germinated bean after the fungus had grown for 2 weeks. Eight cultures on each medium were incubated at 25°C. Observations made after 14 days are summarised in Table 28.

Table 28

Features of cultures of bean seedlings and strain GW on four media. Number of cultures per treatment - 8. Growth period - 2 weeks.

medium	mycelium	sclerotium	rhizomorphs	comments
Knop's	thin white cover	-	-	apparently myc. invasion
Knop's - nutr^{s}	light brown tufts on scler.	covers agar surface	abundant	mass of rhizs, round roots; penetrations; poss. hyphal attack
p.-g.-s.	fluffy brown cover on scler.	as above	abundant and much branched	mass of rhizs.; no visible penetrations; poss.myc. attack
bean	alittle aerial myc. on scler.	as above	some short ones	contact of bean with upgrowth of scler.; poss.myc. attack

On Knop's and bean agar, the seedlings made good growth, the shoot emerging from the tube and the root

system ramifying throughout the agar. Brown patches, sometimes spreading, appeared on the roots where they made contact with sclerotium or mycelium.

On agars containing peptone-glucose-saccharose and Knop's medium plus nutrients, the bean seedlings grew fairly well, but the shoots did not extend beyond the tube and the roots were entwined with the mass of rhizomorphs.

b) Spores as agents of infection of roots

Seeds of the "Field Tick Bean" were allowed to germinate on plates of water agar, after the usual sterilisation. When main roots several centimetres in length had developed, they were surface-inoculated with drops of spore suspension in peptone-glucose-saccharose solution. It was thought that the latter would facilitate germination of the spores. The position of inoculation was marked, and after a day at 25°C. the area was examined for traces of mycelial growth and possible invasion of the tissue of the root.

No evidence of germination was found.

c) Method of fungal penetration into roots

Both varieties of bean and various strains of A. mellea were used in these investigations. The method of culture was that described under "Material and Method". After two weeks' growth the agar containing the bean root and the fungus was removed from the tube; in some instances the outer layer had to be melted to facilitate extraction. Portions of the agar which contained root and rhizomorph contacts were cut out and prepared for microscopical examination by the following method:

1) fixation - Carnoy's fluid (acetic acid: absolute alcohol - 1:2).....30mins.

2) dehydration - absolute alcohol
five changes of .....15mins.

3) clearing - absolute alcohol:xylol -1:1 .....30mins.
xylol .....60mins.

4) embedding - paraffin wax (M.P.52°C.)
infiltration overaa period of 3days
transfer to fresh was and embed

5) sectioning - at 11μ.
sections held on slides by egg albumen

6) staining - Delafield's Haematoxylin .....10mins.
(procedure as described by Johansen in "Plant Micro-technique")

7) mountant - Canada balsam

Some microscopical features of fungal growth are shown in Plates 18 and 19. Plate 18, a) and b) shows sections of sclerotial material of strain GW from the

surface of an agar culture, where the hyphae dilate into the typical "bladder cells" of the sclerotium. In Plate 19,a) and b), drawings give the details of longitudinal and transverse sections of the walls of rhizomorphs of strain C in agar culture. The hyphae run longitudinally in the rhizomorph and give rise to the spreading hyphae in the agar.

One of the sections examined (see Plate 20) came from a culture of "Monarch Long Pod" and strain GW. This was a portion of root which had along one side, a sheath of agar, overgrown by sclerotial material; this sclerotium appeared to be attached to the root. Hand sections were cut transversely and mounted in Lactophenol erythrosin.

It is seen that the "bladder cells" of the sclerotium form in the agar,a dense layer interrupted by the surface of the root. A mass of fungal hyphae. present at one side, is connected with both portions of the sclerotium and the intermediate part of the bean root. The tissues of the root adjacent to the fungus are invaded by hyphae which extend as far as the endodermis and occupy about half the cortex. The farther side of the root from the fungus was not infected.

Plate 21 shows a similar portion of material, but from a culture of "Field Tick Bean" and strain O. Here the layer of sclerotial material next to the root is

complete and hyphae have developed in the agar as previously. Penetration of the root tissue by hyphae is confined to a few outer layers, the affected cells being filled with dense mycelium. It is possible that this is a preliminary stage to the previously noted occupation of the major part of the cortex by the fungus, as other sections examined showed hyphae infecting the whole cortex.

Material consisting of "Field Tick Bean" root with adhering rhizomorphs of strain O was also examined. Various sections showed that although rhizomorphs were present in close proximity to the roots, they themselves did not necessarily penetrate the tissue. In Plate 22 hyphae arise from the pseudo-parenchymatous wall of the rhizomorph and usually invade the tissue of the host. In this particular section, part of a rhizomorph is cut longitudinally and the root transversely. From the lower side of the rhizomorph, an initial has formed against the root which is mis-shapen and torn in this region. Cells of the piliferous layer are separated, and cells below the point of contact with the initial are crushed. The hyphae invade the cortex and are aggregated outside the endodermis on the opposite side to this contact, to form a semi-circle of sclerotial tissue which encloses non-infected cells. This latter area is possibly similar to the xylostroma known to be formed in wood by this fungus.

Plate 23 shows a transverse section of both root (of"Monarch Long Pod") and a rhizomorph of strain GW; there is bridging of the gap between the two sections by hyphae from the latter. These hyphae grow through the connecting agar matrix and accumulate in a mass on the surface of the root. The outer cells of the root contain hyphae; penetration by these is drawn in detail in Plate 24.

One of various longitudinal sections of a root (of Monarch Long Pod") with a rhizomorph of strain M is shown in Plate 25. The rhizomorph above the root has been cut transversely and fron its lower surface two initials are developing. These were cut in median longitudinal section and had not made contact with the root. Hyphae extend from the rhizomorph to the root surface and penetration could be observed (see Plate 26, a) and b).) In these drawings, the hyphae are shown penetrating the cell walls of the host tissue. Some of the outer cells have separated from the root and the intervening spaces have been filled by mycelium.

In all the sections, there was dense staining of the cell walls of the root, especially in areas where the fungus was present and the walls appeared to have some thickened regions.

In the sections examined, no mycelium was present in

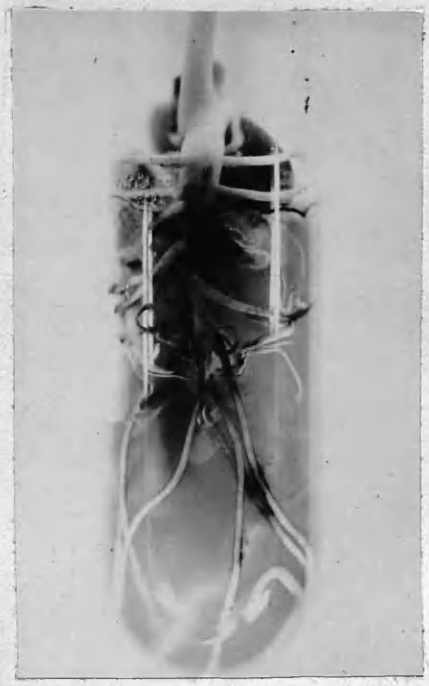
the vascular system of the root, but it was thought likely that invasion would take place eventually.

Conclusions

It would appear that when the fungus grows on a favourable medium and forms numerous rhizomorphs, the growth of the bean seedling is adversely affected. Where the medium only supports mycelial or sclerotial growth, the seedling are able to grow nearly normally.

Infection of bean roots by A. mellea does not depend on entry of rhizomorphs as is generally described for the attack on cork -covered tissues. Microscopical preparations show that the bean root can be invaded directly by hyphae from an outer fungal mass. This penetration was possible because the bean roots were not protected by a layer of cork and it may have been facilitated by the action of fungal enzymes. Other workers have shown that when rhizomorphs enter as a whole, they probably penetrate by means of pressure as well as by enzyme action. This is supported in the present investigation, which also shows that hyphae invade host tissue in advance of the rhizomorph.

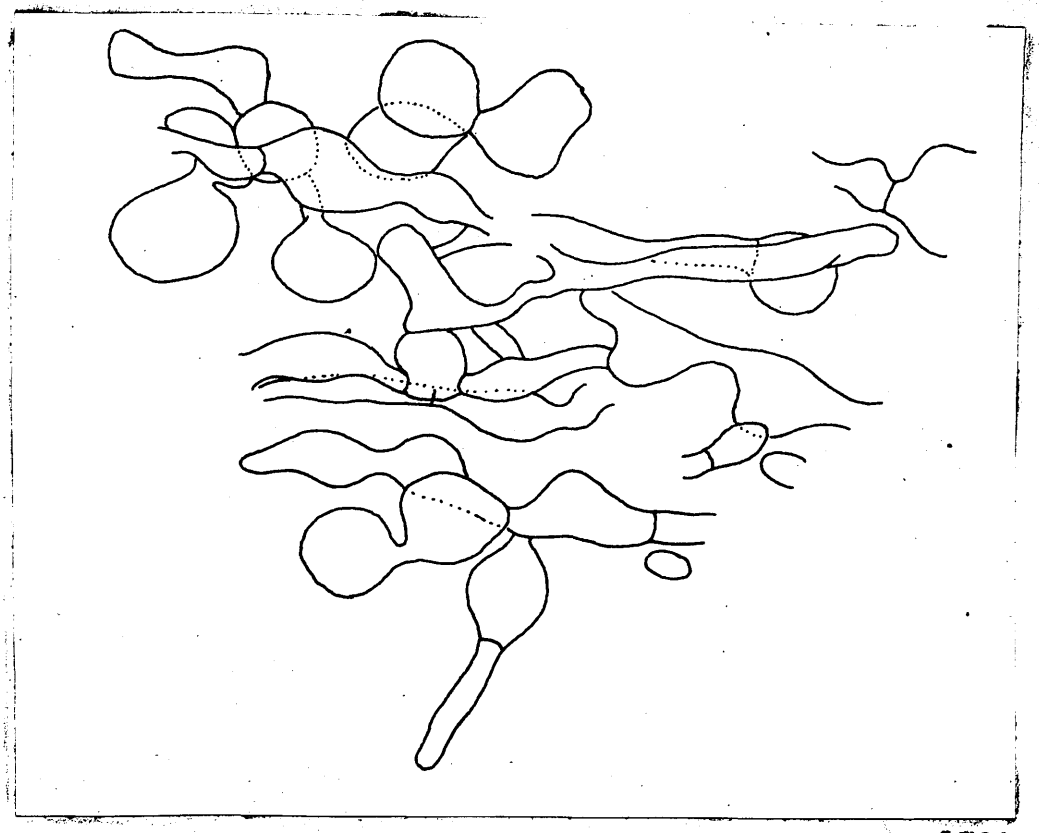
No evidence of germination of the spores was found and conditions may not have been suitable, but it is doubtful whether more suitable conditions exist in nature. It was thought probable that the spores might not infect a host directly, but form a mycelial mass on some suitable medium from which rhizomorphs extend and invade.



a)

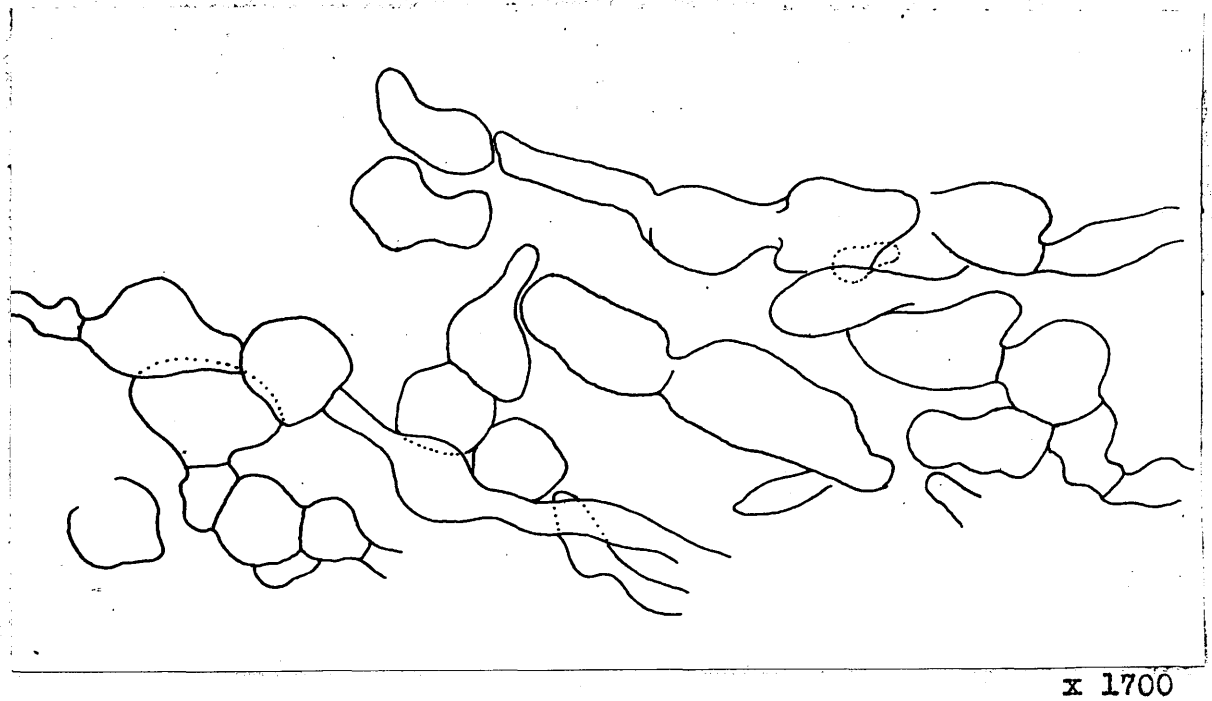
b)

Plate 17 a) and b). Week-old bean seedling in sterile culture with A. mellea
a) Note root and rhizomorph contacts.
b) Note rhizomorph development in the agar and the formation of s sclerotium against the main root.



x 1700

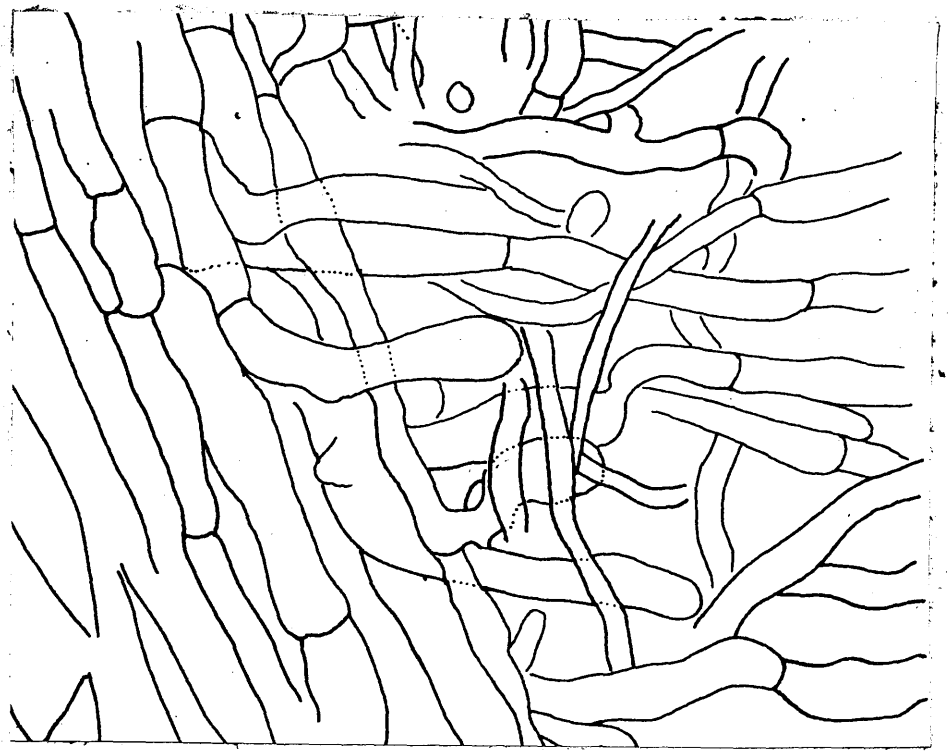
a)



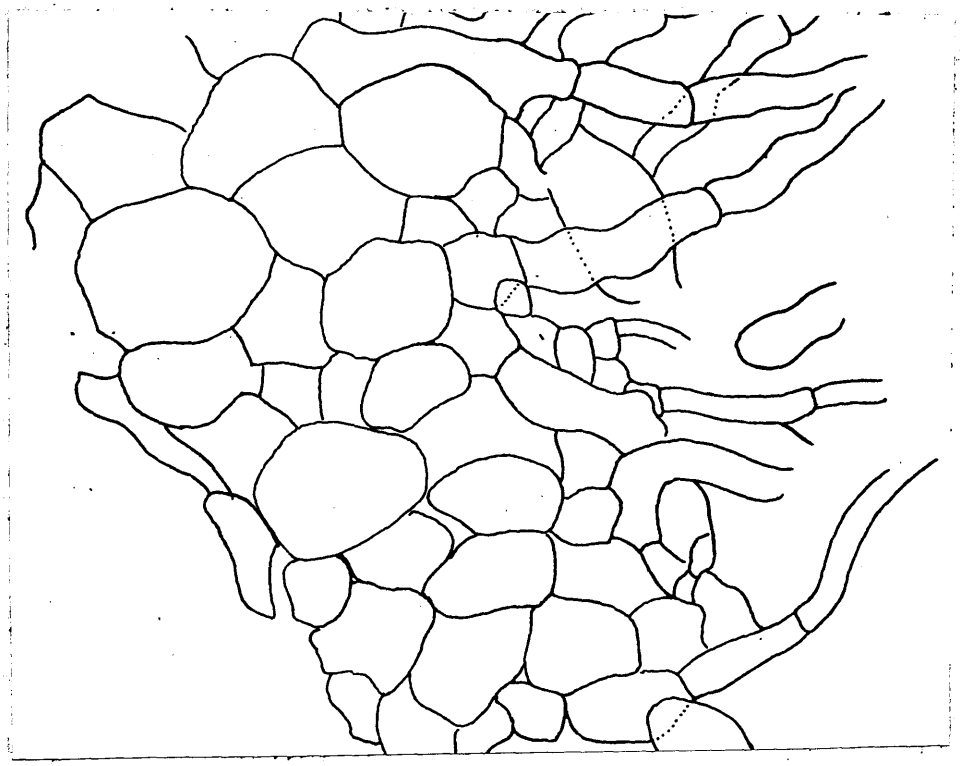
x 1700

b)

Plate 18, a) and b). Mycelium from the surface layer of a malt agar culture of strain GW. Note the presence of distended cells on the branching hyphae.



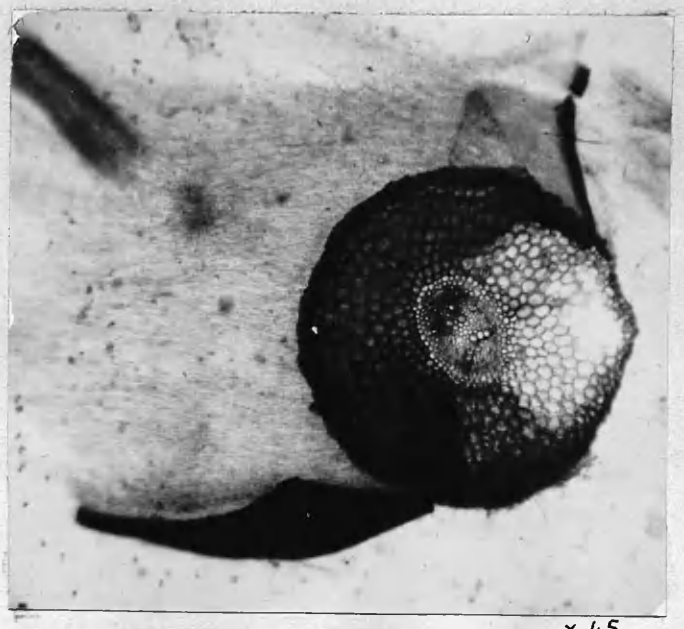
a)



b)

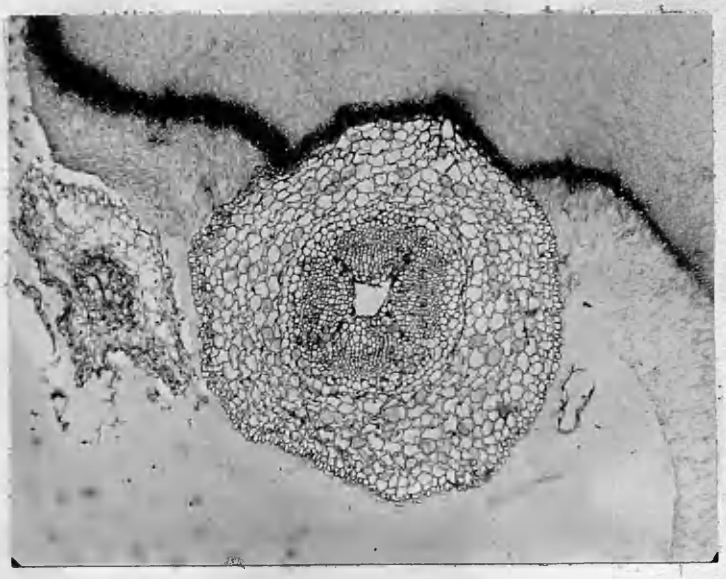
Plate 19. Cells of a rhizomorph of strain C showing the origin of the hyphae which extend from the compact tissue(left) into the surrounding agar(right).

a) longitudinal section
b) transverse section



× 45

Plate 20. T.S. bean root from an agar culture. Hyphae of A. mellea are present in the agar and the invaded portion of the cortex of the root is densely stained. Dark sclerotial tissue forms a line in the agar, interrupted by the root.



x 45

Plate 21. T.S. bean root from an agar culture. Hyphae of A. mellea are present in the agar and some dark sclerotial tissue is developed on the root surface, with little hyphal penetration of the cortex.

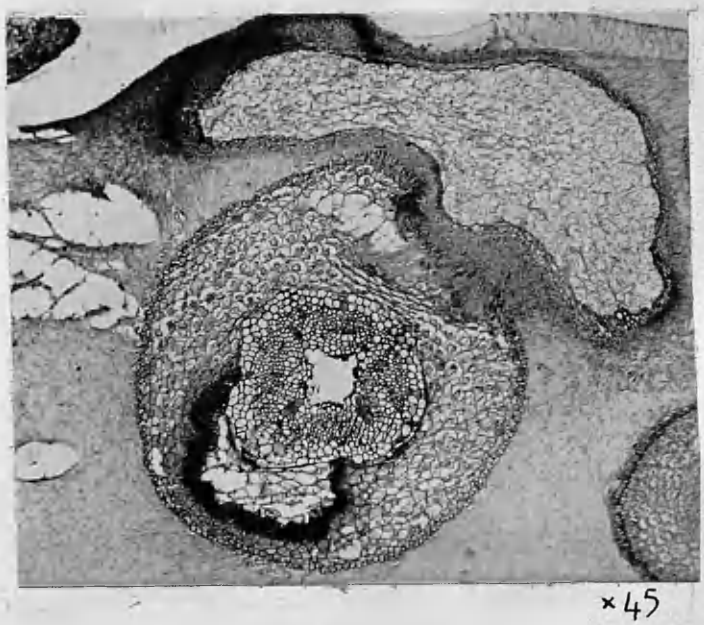
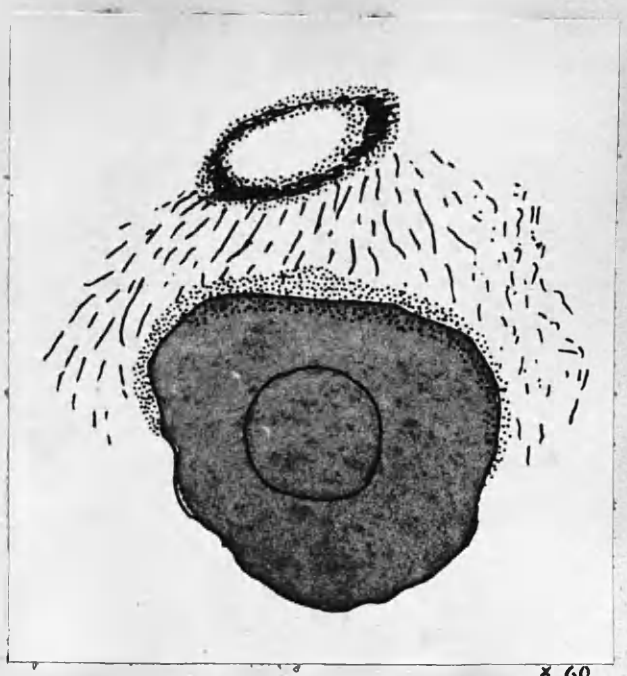
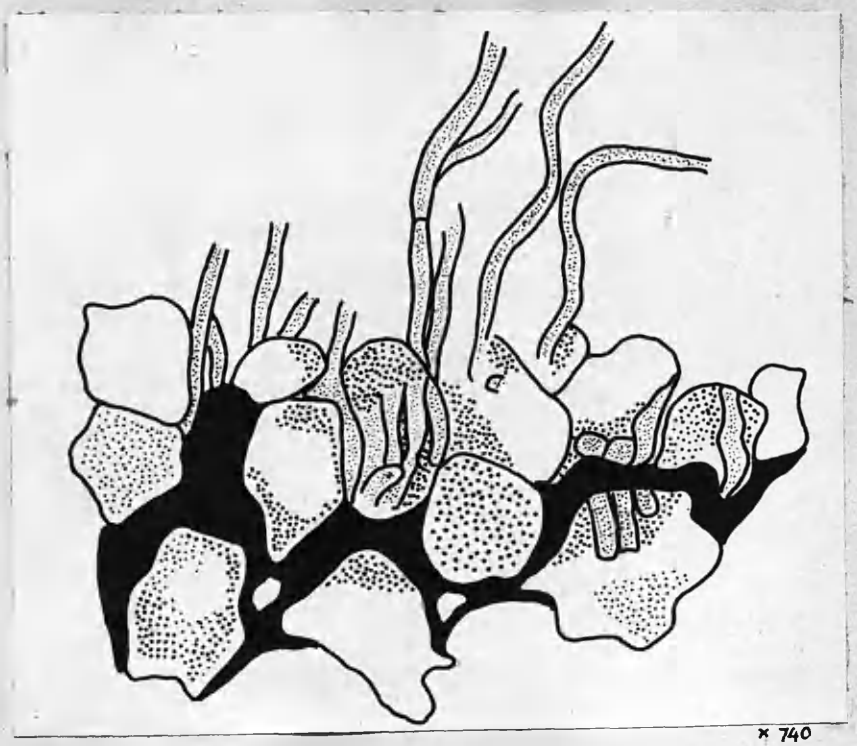


Plate 22. T.S. bean root and L.S. rhizomorph of A. mellea from an agar culture. Hyphae from the rhizomorph are present in the agar and invade the cortex of the root, forming a dark sclerotial line in the lower half. A branch rhizomorph has distorted the root, above, and crushed cells are seen below the torn region.



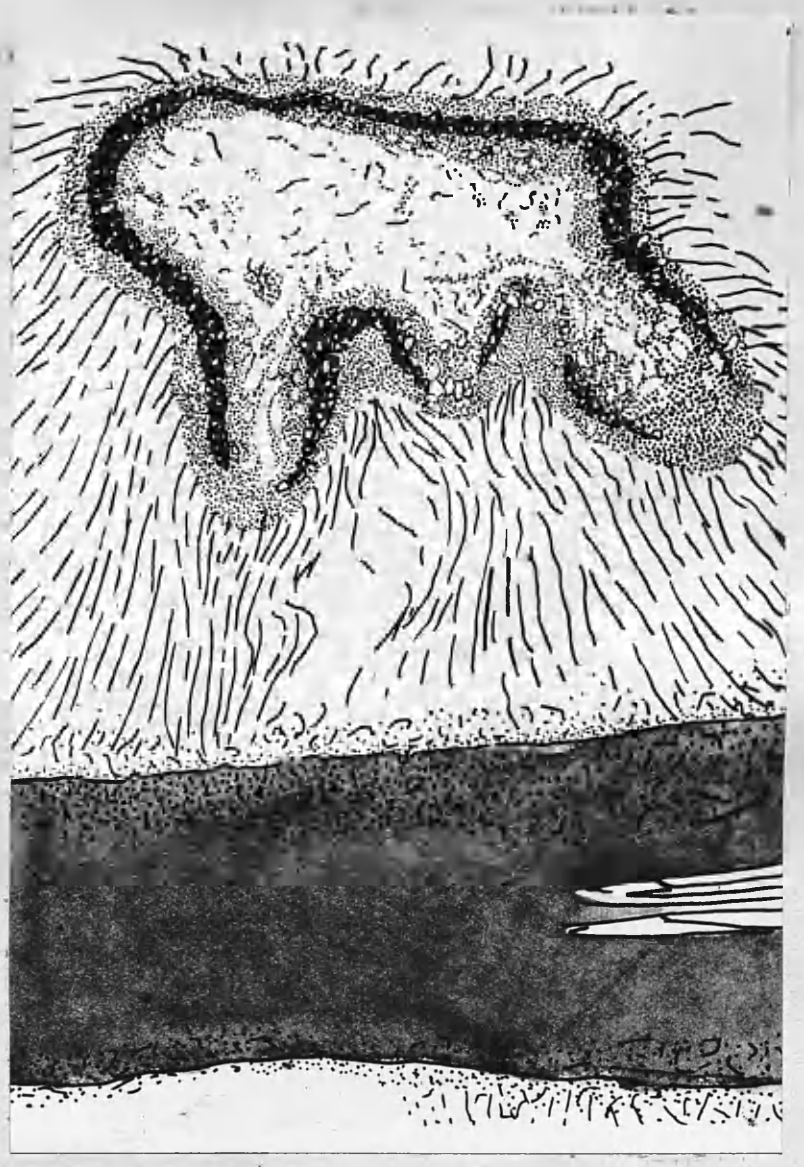
× 60

Plate 23. Diagram of T.S. bean root with an obliquely-cut rhizomorph above. Note the hyphae growing through the intervening agar, and collecting on the root surface before invading the outer tissues.



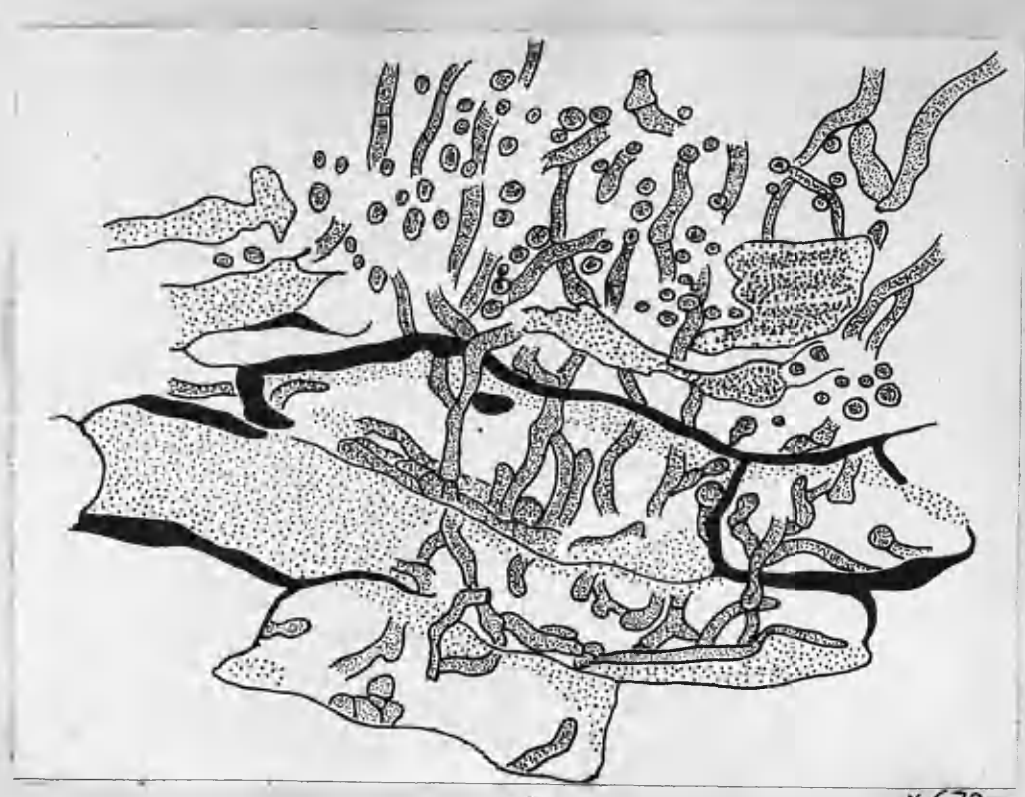
× 740

Plate 24. Details of the hyphal penetration shown in Plate 23. Note the dark staining of walls of the host cells and some contents.



x 67

Plate 25. Diagram showing L.S. bean root (A) with mycelium of strain M collecting on its surface (B) and invading the cortical tissues (C). The hyphae are growing from the outer layers of the rhizomorph (D) which is cut transversely. Note the branch rhizomorphs below the main mass and the development of dense mycelium in the fungal tissue.



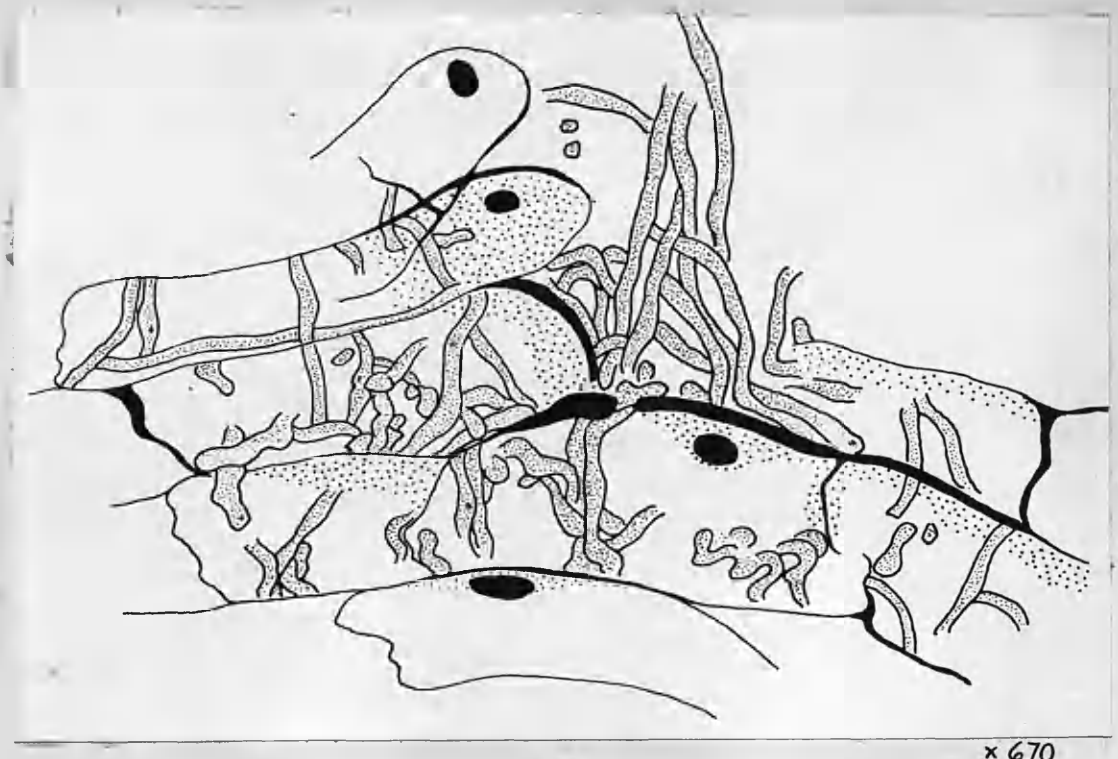


Plate 26, a)and b). Details of the penetration of hyphae into the cells where they accumulate. Note the dark staining of the host cell walls; some host cells are detached by the action of the fungus.

V. DISCUSSION

The conclusions drawn from the various experiments have been briefly given at the end of each section. Some major topics are selected here for further consideration.

Response to light

One of the main findings in this study has been that the fungus shows much greater growth in the dark than in the light. Considerable development of hyphae, rhizomorphs and sclerotia is characteristic of cultures maintained in complete darkness whereas exposure to normal daylight with intermittent darkness, gives only a reduced hyphal development, small sclerotia and no rhizomorphs. It may be that light destroys some substance present in the medium which is required for growth (though this is regarded as improbable), or that it prevents the formation by the organism of a substance necessary for its further growth, or again that it destroys this substance as soon as it is formed. In view of the remarkable growth and development of colonies in darkness, the substance, which may be comparable to growth-regulating substances in higher plants, is apparently one which is metabolised in the dark. The effect of light on colony growth is not a lasting one, as in cultures in the light and subsequently transferred to darkness, the several morphological features of A. mellea develop over the whole colony. In cultures held initially in the dark and then transferred to the light, little further development of the sclerotium

and rhizomorphs took place. These facts suggest that the hypothetical growth-promoting substance is unstable in light. Fuller data on the effects of intensity of light and duration of exposure are desirable. Since the postulated growth-regulating substance (or substances) are apparently involved in the inception and development of sclerotia and rhizomorphs, their isolation would be a matter of interest and importance, the more so as other growth-regulating substances are likely to be involved in the formation of the sporophores for the formation of which light is necessary.

It is a matter of interest that the preventitive and remedial measures which have been prescribed for Armillaria infections e.g. exposing the infected regions to light, are compatible with these findings.

Other factors affeting colony growth

Various factors, such as light, temperature and components of the medium, were found to have an effect on colony development. It was noted that even when conditions were constant, variation in the colonies could be observed, and this was related to the type of inoculum. As with other fungi, standardised inocula are essential where comparative growth studies are being carried out. Other things being equal, inocula consisting of unmatted hyphae yielded closely comparable colonies. Rhizomorph and sclerotial inocula, on the other hand, proved unreliable for consistent results. Where these inocula are used,

differences observed in the subcultures may be due to the presence of rhizomorph initials which can continue growth immediately they are transferred. In that the inception and growth of rhizomorphs take place in the older regions of a colony, it would seem that there is and antecedent phase in which the metabolites required for the formation of these relatively massive structures accumulate and attain to critical concentrations. Accordingly some inocula e.g. those in older regions of the colony, would either have an initial supply of this substance, or be capable of producing it rapidly and hence the rapid formation of rhizomorphs when such materials are used as inocula.

The composition of the medium exercises a marked effect on the formation of all the morphological characters of A. mellea. As many investigators have found, the best substrates for vigorous growth contain balanced carbon to nitrogen supplies, or consist of natural media. Peptone, yeast extract and malt extract have a stimulating effect on the formation of the sclerotial mass and rhizomorphs, but no adaquate explanation of the observed effects has been advanced..On some media, sclerotia and rhizomorphs are never formed; the inference is that the specific substances involved can not be formed from the substances present in themedium. In some media, growth-regulating substances may already be present and available for fungal growth; in others these substances may be produced by the

fungus itself provided the composition of the medium and other environmental factors e.g. temperature and light intensity, are suitable.

Pathogenicity

As indicated above, the nutrient supply to the fungus has a considerable effect on rhizomorph development. This in turn, may determine the amount of infection of a host plant, in that it tends to increase the number of rhizomorph and root contacts. In fact this has been ascertained in laboratory infection experiments. The same feature is probably of importance in the field, i.e. nutrients in the host plant or stump in which the fungus is growing saprophytically, will determine the extent to which the rhizomorphs can spread. Field observations support this view.

Hyphal as well as rhizomorphic infection of the host plant must be considered. This aspect has been somewhat neglected by previous workers. Rapid hyphal penetration of roots, unprotected by cork layers, has been observed by the writer. Hyphae originating from rhizomorphs, sclerotial and hyphal masses, penetrated host cells and ramified through the cortical tissues. The invasion of hyphae in advance of the rhizomorph is of interest and differs from the findings of other investigators. The spread of the disease between plants had previously been noted from the contact of diseased and healthy roots, without the agency of rhizomorphs. The present findings

show the method of invasion by hyphae; conditions in the field may be vastly different from those in controlled laboratory experiments, but it is probable that there also hyphae may normally act as agents of infection.

Experiments showed that spoes applied directly to roots did not cause infection under the conditions employed. Spores of A. mellea have often been regarded as of little significance in the continued presence of the fungus in the soil. In the light of the writer's investigations, some revision of this view now seems necessary. The view now advanced is that spores may actually be of importance in establishing new foci of infection. They germinate readily on acid substrates; moreover, on rich media the strong growth to which they give rise eventually, forms rhizomorphs and sclerotium, Such conditions may well occur in the field. It has also been ascertained that the germination of spores in clumps is more liable to give rise to colony development than single spores, but whether spores are dispersed in clumps under natural conditions is not known.

Control

Light has already been mentioned as inhibiting growth and development of the fungus, and certain temperatures have a similar effect. The strains of A. mellea cultures, grew best at 25°C, and although the tropical strain had a greater tolerance of high temperature than the temperate strain, neither grew at 30°C under the experimental conditions.

Light and temperature factors are employed in present day methods of control. When soil is cleared from about the base of a tree and the affected parts exposed to the air, fungal growth is reduced presumably by the action of sunlight and increased temperature.

The present investigation shows that in culture A. mellea is affected by the growth of several other fungi, Trichoderma spp. particularly having an adverse effect. Trichoderma spp. are present in most soils, and it may be possible to stimulate their growth and thus inhibit the development of A. mellea.

Further investigations

Various aspects of the growth of A. mellea merit further investigation.

The production of rhizomorphs and sclerotia is a variable feature influenced by such factors as temperature, light and composition of the medium. It is suggested that a growth-promoting substance is responsible. Under favourable conditions, such a substance or its precursors may be present in certain media and involved in the metabolism of A. mellea. Its presence in the medium or its production by the fungal colony could probably be detected by the use of another organism; potency and production under various conditions could then be estimated. It would be interesting to add to a medium, aminoacids and growth regulating substances from higher plants in order to investigate their

effect on the growth of A. mellea.

A. mellea does not fructify readily in culture and some particular substance may again be required. Light is considered essential for fructification by A. mellea as well as other fungi, but further experiments are desirable.

The pathogenicity of A. mellea has received much attention, and rhizomorph invasion was usually found. Growth of the fungus and a host plant in soil may show similar mycelial invasion to that found in the author's laboratory investigations. Culture of the fungus in sterile soil with an introduced soil fungus would demonstrate whether antagonism takes place. Growth in non-sterile soil, however, would reveal the relationship between A. mellea and the mass of soil organisms. It would be interesting to grow A. mellea on wood, in double culture with other wood-destroying fungi.

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