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STUDIES RELATING TO SPORE GERMINATION

IN *Agaricus campestris* L. ex Fr.

A thesis submitted to the University of Glasgow,

for the degree of Doctor of Philosophy

in the Faculty of Science

by

DOROTHY MARGARITA Mc TEAGUE

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CONTENTS

Page.

Acknowledgements i

Introduction .... 1.

Methods 7

PART 1.
Effects of physical factors on the germination of spores of Agaricus campestris..... 10

PART 2.
Effects of biological factors on the germination of spores of Agaricus campestris..... 55

PART 3.
Effects of various factors on growth of Agaricus campestris mycelium..................... 110

General conclusion 135

Summary 136

Bibliography 145

Appendix (containing tables of results) 150

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INTRODUCTION.

Introduction.

This thesis describes an investigation of some of the factors affecting spore germination in the cultivated mushroom, Agaricus campestris L. ex Fr., and some subordinate studies of factors influencing hyphal growth. Many workers have investigated these problems in the past with inconclusive and often conflicting results. Much of this work has had limited objectives relating to specific problems of commercial mushroom growing, and few comprehensive physiological studies have been made. This may have contributed to the uncertainty but the gross disagreement between the various reports suggests that they may also have been influenced by variation of some unsuspected limiting factor, not controlled in these experiments.

The work in this thesis has been designed primarily to examine this last possibility. It has involved:

(1) An examination of the effect of physical factors on spore germination on similar lines to those of previous workers.

This work was carried out in order to check and extend earlier work and also to provide information on the most convenient methods for further investigati

In particular it is stressed that it was not intended as a detailed study of the effect of each physical factor. During this phase of the work many interesting points arose which will justify further examination. It was decided that this should not be done at the time, however, as:

(i) It would be irrelevant to the object of the work stated immediately above and,

(ii) It was apparent that this detailed examination would be unprofitable

before the elucidation of any possible effect of an overall limiting factor.

(2) An examination of the effect of biological factors on germination.
It was suspected that the unknown factor which appeared to affect the results of the above studies might be associated with previous reports of stimulation of A. campestris spore germination by mycelium of the same species. Repetition of previous work led to the discovery of the activity of a volatile germination stimulant, and subsequent study was directed towards the isolation and identification of the active substance. This phase of the work was brought to a successful conclusion by the isolation of material which induced the characteristic stimulation and which was found on analysis to be 2:3-dimethyl-1-pentene. The results of this work have been communicated to the press (McTeague et al., 1959).

(3) Some examination of conditions affecting growth, in connection with the maintenance of mycelial cultures for certain germination studies.

The findings of this investigation explain many of the discrepancies of previous work and may have implications, not only in the physiology of A. campestris, but also in the wider field of plant physiology. A general summary of the work recorded in each part is presented at the end of this thesis.

Review of literature on the germination of spores of Agaricus campestris.

Spore germination in A. campestris is of a characteristically slow and variable type. The earliest report of successful germination of these spores is that of Hoffmann (1860), who obtained germination in moist air after seven days. In spite of this, de Bary (1884), reviewing the results

of several workers, cites mushroom spores as never having been germinated with certainty and, for a considerable period, these spores were regarded as exceptionally difficult to germinate. The failure of most of the earlier workers may have resulted mainly from ignorance of the unusually long germination period (many of these workers discarded their cultures when no germination had occurred after a few days), and also of the necessity for sterile conditions, so that inhibitory growth of contaminants did not occur before germination of the A. campestris spores could take place. This last factor could explain the failures of Nylander (1863), who cultured mushroom spores for many months in hot, moist chambers on different substrates, mainly horse manure, and of Reese (1876) and de Bary. Repin (1897), reporting work at the Pasteur Institute, stated that germination could be achieved on damp sand or simply in moist air, as well as on compost or any of the nutritive media used in Bacteriology, but adds that it is necessary to stimulate germination by means of various "artifices". It is likely that they employed aseptic techniques but they did not disclose their methods.

The first detailed study of germination of A. campestris spores to be reported was that of Ferguson (1902), who noted the very slow and variable germination of these spores and tried by various methods to shorten the germination time. This study was continued by subsequent workers such as Falk (1924), Bechmann (1929), Hein (1930), Cayley (1935), Kehl (1942, 1943), Fries (1943), Stoller (1954). Only a broad review of the results achieved will be given here. More detailed consideration of aspects relating to the present work will be given in the appropriate sections.

The principal physical factors affecting germination have been studied

by various workers. Light was found by Hoffmann (1860) and Ferguson (1902) to retard germination. Critical temperature data for germination were recorded by Bechmann (1929). The optimum lay between 18° and 25°C, with no germination above 30°C or below 4°C. Germination was poor between 4° and 16°C. Bechmann found that spores were killed by being kept at -30°C for one hour. Kehl (1942) found that these spores had remarkable heat tolerance and claimed that some tolerated a temperature of 100°C without injury.

The effects of the hydrogen ion concentration of the medium were also examined by Bechmann, who recorded total germination and good further growth at pH's 4 to 7. Poorer germination and further growth was recorded at pH 2.8. The nutrient requirements for germination are disputed and it appears that some workers may be confusing the effects of nutrients in stimulation of germination and in the further growth of mycelium. Several workers record germination in distilled water or even in moist air, but others maintain that nutrient media are essential for germination. Literature relating to this will be discussed in the appropriate sections. Some information on the effect of age of spores on germination is recorded by previous workers and this also is discussed in the appropriate section.

Many attempts have been made to shorten the germination time of these spores by specific treatments. The habitat of the wild variety of <u>A. campestris</u> suggested, by analogy with various coprophilous fungi, that passage through an animal may be necessary for stimulating germination of <u>A. campestris</u> spores. Nylander (1863) and Duggar (1901) considered this essential but could obtain no evidence. Ferguson (1902) fed a rabbit with mushroom spores after several days on a carefully controlled diet of washed

vegetables but with only negative results. Bechmann (1929) found that passage through an animal had no effect on the germination time of mushroom spores.

This line of study was accompanied by a series of attempts at artificial digestion of the spores. Ferguson used various combinations of hydrochloric acid and pepsin solutions and also lactic and hippuric acids. The erratic nature of the results suggested, however, that the germination which occurred was not a result of the particular factors she was attempting to study. Bechmann found preliminary treatment with hydrochloric acid, with 2% pepsin, with combinations of pepsin and hydrochloric acid, with trypsin or a pancreatic preparation had no effect on the germination time of these spores. Falk (1924), however, studying specific acid treatments, reported some acceleration of germination by various organic acids, especially succinic acid.

Other miscellaneous treatments have been tried in order to reduce the germination time required for these spores. Kehl (1942) examined the effects of many "disinfectants" on mushroom spores and found chloroform the most effective in stimulating germination. He reported 95% germination of spores which had been steeped in chloroform for four hours. Bechmann (1929) tried without success to stimulate germination of these spores by various chemical treatments. He claimed some stimulation, however, with irradiated media, stating that 100% germination was obtained in nine days on irradiated wort agar, which had been exposed before inoculation to a mercury quartz arc-lamp of 150 volts for 5 minutes at a distance of 30 cms. Similar spores germinated only after twenty days on similar untreated agar.

The stimulatory effect of mycelium of A. campestris on the germination

of spores of the same species was discovered by Ferguson (1902). Subsequent workers, e.g. Falk (1924) and Cayley (1935) confirmed this report but little further examination of the effect was made. The studies of these and other workers on the stimulation of germination of A. campestris spores by mycelium of the same species and some related reports are of considerable interest and will be discussed in more detail in the appropriate section.

A study of the literature relating to the germination of spores of this species, shows little agreement on the conditions necessary for germination. Throughout the reports of previous workers there is great variability in the time and amount of germination recorded in apparently similar conditions, and it appears probable that germination in these cases is strongly affected by some factor not controlled in their experiments. Many interesting problems are raised by these earlier reports, particularly the stimulation by mycelium of the same species. Although it is more than fifty years since Ferguson's discovery of this effect, it has received little further attention and its mechanism has not been investigated. The following work studies a number of the points not made clear by previous workers and gives particula attention to this stimulation phenomenon.

METHODS.

Methods.

The procedure outlined here relates to most parts of the work recorded in this thesis. Techniques applying only to particular studies will be described in the appropriate sections.

(1) Supply of spores.

Spores of a commercially grown variety of *Agaricus campestris* L. ex Fr. were collected aseptically in glass specimen tubes, (1 cm x 5 cms) in the following way:

A number of specimen tubes were placed upright on a glass plate under a bell-jar closed at the top with a plug of cotton wool, and the entire apparatus sterilised. A well developed sporophore with the veil still unbroken, was peeled and the pileus placed over the specimen tubes in the bell-jar, which was then left undisturbed for several days. Spore fall commenced after about one day and was allowed to continue for four or five days, at the end of which time a dense deposit of spores was visible at the base of the specimen tubes. The tubes containing the spores were then removed from the bell-jar and plugged with sterile cotton wool. Stocks of spores were stored at room temperature.

(2) Preparation of spore suspensions.

The media used in germination tests were inoculated with spore suspensions prepared by transferring aseptically collected spores to small test tubes of 0.5 cm. bore, 5 cms in length, half-filled with sterile distilled water. The suspension was then passed rapidly through the capillary mouth of a pasteur pipette until all the spores were uniformly dispersed. The density used was such that, when inoculated on the surface

of agar discs, spore counts of the order of 1,000 spores per low power field were obtained.

(3) Spore cultures.

Since studies relating to the condition of the medium, recorded in Part 1, Section I, showed better and more uniform germination on solid than in liquid medium, most of the subsequent germination tests were carried out on solid medium. Except where otherwise stated, this was 2% water agar, which had been filtered to facilitate observation of the spores. Non-nutrient agar was chosen in order to avoid possible masking of the effect of other factors being studied by the presence of nutrient.

In the few germination studies in drops of liquid medium, in the earlier parts of this work, the drops were placed on the bases of petri dishes and inoculated with spores. Hanging drop methods were unsatisfactory, since these relatively dense spores tended to accumulate in a mass at the lowest point of the drop. In later studies, recorded in Part 2, drops of liquid medium were confined in vaseline rings, half an inch in diameter, made simply by pouring melted vaseline from a finely drawn-out pipette. Thus the drops were prevented from running together when the plates were handled or inverted for examination of the spores through the base of the petri lids. It was thus possible to use a greater volume of spore suspension in such test drops, and to avoid the rapid drying out which previously caused difficulty.

Cultures were incubated at 24°C, except where otherwise stated. The relatively long time required for germination tests necessitated careful control of conditions to avoid drying and the entry of contaminants. Drying was reduced by placing the inoculated agar discs on the lid of the petri dish

and adding water to the base, but this involved the danger of splashing of the spore cultures, when the petri dishes were handled, and was accompanied occasionally by excessive condensation. Later work avoided these difficulties by enclosing all the plates involved in an experiment in a polythene bag containing a pad of damp cotton wool to maintain humidity.

(4) Assessment of germination.

Germination counts were made at a magnification of x100, using a low power objective, so that spores could be observed through the lid or base of the petri dish, without opening. This allowed frequent examination without risk of contamination.

Results are given throughout as number germinated per 5,000 spores, rather than an expression of percentage germination, on account of the relatively low levels of germination usually present at the time of recording of results. This is one of the difficulties arising from the very slow type of germination characteristic of *A.campestris* spores. The first spores germinate normally after about one week in suitable conditions and others follow during a further two or three weeks. Thus mycelium from the earliest spores grows over the culture medium, obscuring the later germinating and ungerminated spores, so that accurate records can usually be made only at an early stage, while germination is still at a low level.

Part 1.

Effects of physical factors on the germination of spores of Agaricus campestris.

Part 1. EFFECTS OF PHYSICAL FACTORS ON THE GERMINATION OF SPORES OF AGARICUS CAMPESTRIS.

I. Germination of Agaricus campestris spores in relation to the condition of the medium.

1. Nutrient status of the medium.

   (a) Effects of malt concentration on germination.

2. Hydrogen ion concentration.

3. Physical state of the medium.

   (a) Studies on germination of spores suspended in deep culture in liquid media.

      (i) Effects of aeration.

      (ii) Effects of increased concentrations of oxygen.

      (iii) Effects of the presence of nutrient.

      (iv) Effects of spore concentration.

   (b) Germination of spores on solid medium after pretreatment by submergence in water.

   (c) Comparison of germination on the surface of solid medium with germination in hanging drops of liquid medium.

II. Germination of Agaricus campestris spores in relation to concentration of spores.

III. Germination of Agaricus campestris spores in relation to age of spore.

I. Germination of Agaricus campestris spores in relation to the condition of the medium.

The main object of the work recorded in this section was to find the most suitable medium for spore cultures in germination tests. The effects of variation in physical condition, nutrient status and pH of the medium on germination were examined for this purpose, as it was thought that these were probably the primary factors concerned. Detailed study of the accessory problems raised was irrelevant to the limited aims of the present study.

1. Nutrient status of the medium.

Previous workers have held diverse views on the nutrient requirements for germination of *Agaricus campestris* spores. Hoffmann (1860) found that they germinated normally in moist air alone but no subsequent study of germination in these conditions appears to be recorded.

Ferguson (1902), Hein (1930) and Stoller (1954) obtained germination in distilled water and, indeed, Hein records germination levels frequently over 50% in distilled water in three weeks. Stoller observed germ tube formation by about 25% of the spores in suspension in distilled water after seven days, although he found only a low percentage forming colonies.

On the other hand, many workers have considered the presence of nutrients essential for germination. Bechmann (1929) found no germination in distilled water or in Knop's solution, even when the spores were placed on quartz sand to prevent sinking. Cayley (1935), however, recorded germination in Knop's solution. Bechmann found no germination on 2% non-nutrient agar but recorded successful germination on various other nutrient media and he attributed this

to the presence of the nutrients, particularly sugar. Similarly, Kehl (1942 found no germination of spores on blotting paper in distilled water or soil suspension during a period of six months and concludes that nutrient media are essential for germination. However, little detail of the work is given and, in view of Brown's finding (1922) of reduced germination of *Aspergillus* in the presence of damp blotting paper, it is possible that the spores were exposed to inhibitory conditions.

Little systematic study of the effects of increasing concentration of nutrient on germination in other Basidiomycetes appears to be recorded. Ferguson found that spores of most fungi showed poorer germination in distilled water than other media. Good and Spanis (1957), working with spores of *Fomes igniarius*, found germination on malt extract agar increased with malt concentration up to a concentration of 8% malt, but from studies of the effects of the principal components of the malt on germination and from their finding that addition of various concentrations of glucose to a 2% malt medium had no effect on germination, they concluded that the stimulating action of high concentrations of malt was not due to sugar but to some other substance present in it.

Experimental.

(a) Effects of malt concentration on germination.

Preliminary work had suggested that the problem is the presence or absence of any nutrient, rather than the effects of specific components. Malt was therefore chosen for this study since it was likely to provide a complete medium, so that the effect of nutrient concentration in general, rather than of a single nutrient factor could be studied.

Germination was compared on non-nutrient agar, 2%, 3%, 4%, 5%, 7% and 10% malt agar media. Similar discs of each medium were cut and one disc of each placed on the lid of each of seven petri dishes. Each disc was inoculated with spore suspension. Germination at intervals after inoculation is recorded in Table 1 of appendix and may be summarised as follows:

% Malt	Mean germination per 5,000 spores		
	10th day	13th day	18th day
0	0	0.7	500
2	2.6	174.6	"
3	0	60	"
4	0	0.86	"
5	0	29.3	"
7	0.14	14.3	"
10	0	0	"

Germination was first recorded on the tenth day after inoculation on discs of 2% malt agar. By the thirteenth day, germination was extending to the higher concentrations but was completely absent from the discs of 10% malt agar and beginning on only a single disc of non-nutrient agar. The final observation, on the eighteenth day, showed good germination on all media with very uniform germination on almost all the discs from which germination had been absent or had been very low at the previous examinations, apparently independently of malt concentration. Accurate mean values cannot be given for the eighteenth day, except on plain agar (0% malt), since on one or more discs of each of the other media, germination could only be estimated to the 10% level, due to mycelium from the earliest germinating spores obscuring the later germinating spores.

In the above experiment, no adjustment of the pH of the medium was made. Colorimetric measurement of the pH of similar media showed a pH value of 7 for plain agar, while all the malt media lay in the range 5.2 to 5.4. It was possible, therefore, that the differences in germination recorded above between the plain agar and the malt media might include differences due to the lower pH of the malt media.

The effect of this factor was examined in a further experiment, in which five discs of 2% malt agar and five of plain agar, both media being adjusted to pH 6, were placed alternately in a circle on the lid of each of four petri dishes and inoculated with spore suspension. Germination after one week is recorded in table 2 of appendix and summarised below.

Medium	Mean germination per 5,000 spores.
2% malt agar	10.3
Plain agar	5.4

Analysis of variance showed that the difference between germination on malt agar and that on plain agar lay above the 0.001 level of significance. Thus germination was significantly better on the nutrient medium. It was noted, however, that in this experiment, germination began after a similar period on both media.

Discussion.

In this work, the rate of germination has been shown to be more rapid on media containing 2% malt than on media of any other malt concentration. The second experiment shows that the difference may be associated partly with pH since germination began after a similar length of time on plain agar and on 2% malt agar of the same pH, although at a higher level on the malt agar. Thus the slow germination on plain agar in the first experiment may result from the unfavourable effect of the higher pH of this medium than of the malt media (cf. effect of pH on germination, in next section). It is unlikely, however, that differences in germination within the range of malt concentrations studied are due to this factor, since little difference in pH was found within the range of malt concentrations from 2% to 10%.

The poorer germination recorded on the 10th and 13th days on media of malt concentrations above 2% may indicate that the factors in the malt favouring germination are already present in excess in the lowest concentrati of malt used here and that the increase in sugars or other components of malt may exert an unfavourable effect on germination at first. The results of the eighteenth day indicate, however, that spores of this species are capable of germination on all media of the range of malt concentrations provided and even on plain agar without addition of malt.

The total amount of germination on each medium cannot be discussed from these results. Germination had in all cases reached a high level by the eighteenth day but, in general, quantitative comparisons could not be made since growth of mycelium from the earliest germinated spores prevented accurate assessment of germination above the levels recorded. The apparentl

simultaneous germination about the eighteenth day of a high proportion of the spores on media of widely differing malt concentrations, suggests that this was due not simply to the concentration of malt in the medium but to some other factor affecting all media. In this connection, attention may be drawn to the studies recorded in Part 2.

The results of these experiments are contrary to those of such workers as Bechmann (1929), and Kohl (1942), who concluded that the presence of nutrient, particularly sugar, was essential for germination. It is possible that the conflict with these previous results may be due partly to the time at which these authors recorded germination.

2. Hydrogen ion concentration.

The effect of the hydrogen ion concentration of the medium was studied since it appeared that this factor might contribute to the effect of nutrients and to the possibility of biological stimulation by carbon dioxide or organic acids.

Although several references to the favourable effects of acidity are to be found in the literature concerning spore germination in Agaricus campestris, the only direct study of germination at different levels of pH appears to be that of Bechmann (1929). He found a wide range of pH's favourable to germination of spores of A. campestris and recorded 100% germination at pH's 5 and 5.2 after eighteen days and at pH 7 after twenty days. 80% germination was attained in nineteen days at pH 2.8 but further growth was poor.

Various workers (Ferguson, 1902; Falk, 1924; Bechmann, 1929) have studied the effects of specific acid treatments on germination. The work of Ferguson and Bechmann on this problem appears to have arisen from the theory, suggested by Nylander (1863), that spores of A. campestris, like those of some coprophilous fungi, require preliminary digestion in animal intestine before germinating. Their acid treatments attempted digestion of these spores in vitro.

Ferguson subjected spores to digestion with N/1000 or N/10,000 solutions of hydrochloric acid combined with pepsin, for various lengths of time before comparing their germination in nutrient media, but she obtained variable and generally very low germination. Lactic and hippuric acids were also tested but gave very poor germination. A similar investigation by Bechmann showed

no effect of hydrochloric acid treatments on germination.

Falk (1924) found that acid concentration played a decisive part in the initiation of germination. He carried out an extensive study of the effects of organic acids, both by pretreatment and by direct addition of acid to spore cultures. The effects of various concentrations of succinic, malic, lactic and oxalic acids on germination were compared. With acetic acid, no germination was obtained. The other acids showed some germination at particular concentrations but without marked stimulation. Best results were obtained using 0.25 N succinic acid in malt solution, the pH of which changed from 5 to 3.3 on addition of the acid. In drops of this medium, total germination was recorded in nine to ten days, at which time no germination had occurred in 'neutral' malt solution without addition of acid.

Methods.

A series of buffered solutions of pH's 3, 4, 5, 6, 7, 8, and 9, were prepared using solutions of citric acid and disodium phosphate. These solutions were added to six lots of plain agar after all had been autoclaved and when the agar was just above the temperature of solidification. The buffered agar was then poured into petri dishes and allowed to set. Using this method it was possible to solidify the agar at all pH's, instead of only about the neutral point, as happens when the pH is adjusted before autoclaving. The pH of each medium after autoclaving was measured colorimetrically.

Uniform discs cut from similar plates of each medium were placed in randomised positions on the lids of each of seven petri dishes, each petri

dish receiving one disc of each pH. All discs were then inoculated with uniform spore suspension and incubated at 24°C.

Germination on the 7th, 8th, 14th, and 20th days after inoculation is recorded in table 3 of appendix. The results of this experiment may be summarised as follows:

		Mean germination per 5,000 spores			
Day		7	10	14	20
pH	3	0	0	0	0
	4	0	41	376	500
	5	57	137	500	500
	6	31	83	440	500
	7	4	7	56	500
	8	0	0	0	0
	9	0	0	0	0

Analysis of variance showed that differences in germination between spores on media of pH 5 and pH 6 were above the 0.001 level of significance. Germination occurred at all pH's from 4 to 7 and, in all cases, where germination took place, it had reached a high level by the twentieth day. The rate of germination, however, varied with the pH of the medium, most rapid germination occurring at pH 5 and the slowest at pH 7. After twenty days, no germination had occurred at pH's 3, 8 and 9.

The pH of samples from each set of discs was measured at the end of the

experiment. No change was found in the discs of pH's 4, 5, 6, 7 and 8. Agar originally of pH 3, however, showed a final pH value of 4 and the pH of agar originally of pH 9 had fallen to 8. A later examination of the discs originally of pH 3 showed good germination, possibly due to the decreasing acidity of this medium.

Discussion.

The results recorded here agree with those of previous workers who found a slightly acid medium favourable to germination of A. campestris spores. They confirm and extend the findings of Bechmann except that in these experiments no germination was found at a pH as low as 2.8. Further observation at the lowest pH examined was prevented by the drift in pH of these discs at this time. It is possible that, at low pH's, germination may be unfavourably influenced by the phosphate buffer, a factor which has not been studied in relation to germination but which has been shown (Treschow, 1944) to affect growth of A. campestris mycelium. Bechmann also used phosphate buffer, but at a lower concentration (M/10). It is noted, however, that Bechmann determined the pH of his media after sterilisation and that he does not mention any subsequent check of pH. It is therefore possible that some unrecorded drift in pH may have occurred similarly in his experiments.

It is clear from this work that variation within the range of pH from 4 to 7 does not very markedly affect the type of germination but that germination in these conditions is unlikely to occur outside this range.

These results are of interest in relation to studies recorded in later

sections on the influence of living mycelium and of carbon dioxide on germination of A. campestris spores. Thus it appears that changes in pH due to the presence of carbon dioxide are unlikely to stimulate germination to the degree observed in the presence of living mycelium. Similarly, it appears that, while the favourable effect of various organic acids on germination of these spores, noted by Falk (1924), might be due to their influence on pH, the more marked stimulation which he reported to be due to 0.25% succinic acid is probably not a simple pH effect. In the present work, attempts to repeat Falk's experiment with 0.25% succinic acid have, so far, given inconclusive results.

3. Physical state of the medium.

Ferguson (1902), Hein (1930), Cayley (1935), Kehl (1942) and Stoller (195
studied germination of A. campestris spores in drops of liquid medium. Bechmann (1929) carried out some tests on agar media and others in liquid. It appears, however, that the previous workers have not reported any direct comparison of germination on solid and in liquid media. This is a surprising omission in view of the considerable differences in environmental conditions between spores submerged in drops of liquid and those lying on the surface of agar medium.

In this work preliminary experiments were carried out using spores on the surface of agar discs, and in hanging drops of liquid media. From these it was soon appreciated that spores could be held on the surface of solid media in a uniformly dispersed arrangement, and that they could be examined easily. Such media tended to dry out irregularly, however, and the humidity and aeration conditions around the spores could not be controlled precisely. Spores held in hanging drops of liquid media were maintained in a more uniform medium, but they tended to accumulate at the lowest point of the drop instead of remaining evenly dispersed, and the drops also tended to dry out irregularly in the conditions available. It was thought, however, that a more uniform condition might be attained by using relatively large volumes of liquid media in constant aeration and agitation.

The studies in this section were designed primarily to determine whether germination studies were possible in such "deep culture", and to

determine which of the alternative methods would be most satisfactory if this one was found unsuitable. More extensive examination of the other interesting points which arose during the work has been postponed as it was irrelevent to this object.

(a) Studies on germination of spores suspended in deep culture in liquid media.

Various advantages were to be expected from the adoption of these methods. The rather variable drying factor with its accessory effects was eliminated and uniform distribution of nutrient could be obtained. Further, a uniform dispersion of spores could be maintained, in spite of the relatively high specific gravity of these spores, by suitable aeration of the cultures. The oxygen level in such cultures was easily varied by altering the gas mixtures used for aeration of the liquid, thus ensuring the exposure of the spores to known concentrations of oxygen.

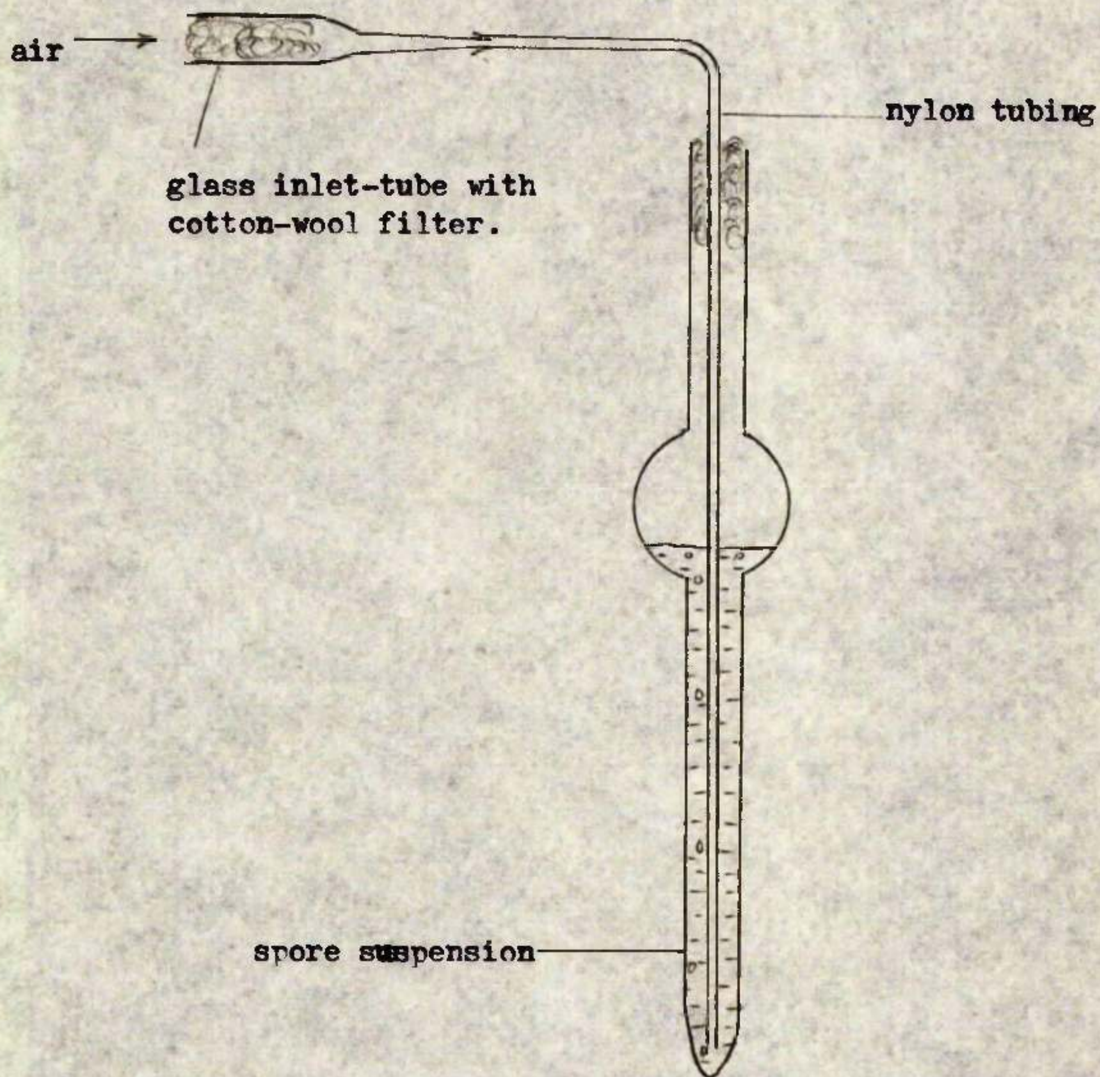
The following factors, which appeared likely to affect germination in suspension were chosen for study: (i) aeration, (ii) increased levels of oxygen, (iii) presence of nutrient, (iv) spore concentration.

Methods.

Since the supplies of aseptically collected spores available were limited, this work had to be carried out with small samples of spore suspension in order to secure sufficient replication while using what were thought to be suitable concentrations of spores. It was necessary also to devise apparatus for the efficient aeration of these small volumes of suspension so that spores could be kept in suspension and prevented from sinking. The type of aeration tube finally chosen is shown in text figure 1, and was made in the following way.

10 cm. lengths of glass tubing of 4 mm. bore were drawn out at one end and sealed. A bulb was blown half way up the tube. Using a capillary pipette, half ml. aliquots of spore suspension were added to each tube so that the

Text figure 1. Apparatus for aeration of small volumes of spore suspension.



tubes were filled to just below the bulb.

When these tubes were aerated, spores were constantly driven away from the narrow end of the tube where air was entering and thus prevented from settling. The bulb half way up the tube prevented spore suspension from being forced out of the tube by the air-stream. During aeration the spore suspension filled the base of the bulb and air was able to escape from the more extensive liquid surface at that point. Air was passed to the base of each aeration tube by means of nylon tubing of 0.5 mm. bore, which was flexible and resistant to autoclaving. This nylon tubing was connected to the aeration system by short glass tubes drawn out to a suitable diameter at one end and packed with a cotton wool filter.

In preliminary experiments in this work, finely drawn out glass inlet tubes were used but these were too fragile and aeration was too often interrupted by breakage of the inlet tubes. The use of nylon tubing provided a system free from leakage and breakage and allowed experiments to run with very little attention for as long as a month.

Loss of water from the aeration tubes was remarkably low, partly due to condensation of water vapour, from the spore suspension, in the bulb and upper parts of the aeration tube. In long-term experiments, any loss of water was compensated for by periodic topping up with sterile water. Compressed air from a cylinder was used in these experiments and was passed through a flask of distilled water, then through a manifold into the aeration tubes. Aeration tubes complete with nylon inlet tubes and glass connections were sterilised by autoclaving. The system was shown to provide aseptic conditions in a preliminary experiment in which samples of malt

extract solution remained free from contamination after two weeks of aeration, and no evidence of contamination was seen in the regular examinations of each culture involved in the work.

The oxygen concentration of spore suspensions used in this work was estimated by the Fox and Wingfield (1938) modification of the Winkler technique. These authors state that the oxygen content of samples of one to two ccs. may be estimated by this method using a microburette. In the present work, however, it was desirable to measure oxygen in samples of less than one cc. Successful estimations using samples of 0.3 cc. were made using the syringe pipette of Fox and Wingfield and carrying out the titration within the barrel of the pipette. This was accomplished by means of very fine stainless steel tubing attached to the micrometer burette by 0.5 mm bore nylon tubing, so that the steel tubing could be inserted into the barrel of the pipette and the solution in the burette injected directly into the solution in the barrel of the pipette, avoiding all contact with atmospheric oxygen. Otherwise, with such small samples, the error resulting from exposure to atmospheric oxygen during titration would be appreciable.

The oxygen content of the reagents used is significant in estimations of oxygen in small samples. This value was found by comparison with samples of freshly boiled distilled water.

Spores were counted by use of a haemocytometer. The spore suspensions were incubated at 24°C and air from a cylinder was led through the thermometer hole at the top of the incubator into the aeration system.

(i) Effects of aeration.

Previous workers have suggested that the germination of spores of various species in solution, even in small drops, is limited by lack of oxygen. This effect was noted by Duggar (1901) and Doran (1922). Similarly, De Bary (1884), Doran and Coyley (1925) reported that germination was generally best at the margins and on the surface of drops of liquid. Doran further demonstrated that, in drops of previously aerated distilled water, equally good germination was obtained whether or not the spores were submerged in the drops and in all subsequent investigations he used aerated, distilled water.

In preliminary experiments germination of spores of A. campestris was found to be poorer in drops of liquid than on solid medium, suggesting that, here too, aeration conditions within the medium may be unfavourable. In the following study it was hoped that aeration of spore suspensions would make such conditions suitable for germination studies.

Experimental.

0.5 ml samples of spore suspension were placed in 20 aeration tubes. Ten of these were aerated with air from a cylinder and ten were left unaerated. Germination after one month is recorded in table 4 of appendix and summarised below, together with the oxygen concentration of the suspensions at this time.

Treatment	Mean germination per 5,000 spores	ccs. oxygen per litre.
Aerated suspension	0.62	5.9
non-aerated	0.69	6.1

Only a low level of germination was found and analysis of variance showed no significant difference in germination between aerated and non-aerated spore suspension.

Oxygen content of suspension:

Each oxygen concentration recorded above is the mean value of five samples. Similar results were obtained for other sets of spore suspensions and for aerated and non-aerated distilled water. Thus, both aerated and non-aerated suspensions had approximately the oxygen concentration of water saturated with dissolved oxygen, which at 21°C is 6.2 ccs. per litre.

Further indication that such volumes of suspensions are normally saturated with dissolved oxygen was provided colorimetrically using the dye, o-chlorophenol indophenol, which has a relatively high oxidation-reduction potential (with an E_o, i.e. the potential of the half-reduced dye, of 0.25), although somewhat lower than the oxidation reduction potential of oxygen. Ten aeration tubes of the type normally used for aerating spore suspensions, were filled with reduced (pink) dye solution to a depth of five centimetres. Five tubes were aerated and five left unaerated. The aerated suspension regained the blue, oxidised, colour in less than a minute. The blue colour returned to the top half-centimetre of the non-aerated solution within a few

minutes and after several hours had penetrated to the bottom of the suspension, which thereafter remained oxidised. This oxidation must have been due to dissolved atmospheric oxygen diffusing through the suspension.

(ii) Effects of increased concentrations of oxygen.

It was clear from the above work that aeration with normal air in these experiments is principally a means of maintaining spores in suspension and does not affect the oxygen concentration of these suspensions which is already at equilibrium with atmospheric oxygen. It appeared possible, however, that the oxygen level in solutions at equilibrium with the atmospheric concentration of oxygen might be inadequate for germination in submerged conditions. To test this the following preliminary experiment was carried out in solutions of enriched oxygen concentration.

In order to raise the oxygen concentration of a solution to a level above that found in equilibrium with the normal atmospheric concentration of oxygen (6.3 ccs per litre at 20°C), it is necessary to expose it to a gas mixture containing a higher concentration of oxygen. In the following experiment, germination was compared in suspensions aerated with the following: Air of reduced oxygen content, giving approximately 0.5 ccs per litre of solution; normal air; oxygen enriched air (approximately 10 ccs per litre of solution), and with pure oxygen (30 ccs per litre). The first of these airstreams was obtained by passing normal air through pyrogallol, and the air supply of enriched oxygen level, by adjusting the flow of oxygen into the airstream so that the resulting gas mixture maintained a concentratio of oxygen between the limits of 30% and 35%. Oxygen levels in the gas streams were measured at intervals during the experiment by means of the

Haldane Gas Analysis apparatus. The oxygen level in the "reduced oxygen" airstream tended to rise during the experiment, since the pyrogallol solution became saturated with oxygen.

0.5 cc. aliquots of a uniform spore suspension were placed in four sets of five tubes. One set was aerated with each of the four gas mixtures described above. Samples of the same spore suspension inoculated on plain (2% agar) at the time of commencement of this experiment showed good germination (about 10%) after one week, but examination of the aerated suspensions after eighteen days showed no germination at any oxygen level. By the 21st day, however, germination was present in all tubes, as recorded in table 5 of appendix, but the results were inconclusive and showed no evidence of any effects of oxygen level on germination. The low level of germination occurring in most cases was similar to that recorded in other experiments with spores suspended in solution. In three tubes, however, a much higher level of germination was recorded, the distribution of these being, apparently, independent of the oxygen concentration in solution. No explanation for this variation can be given at present.

The generally poor germination occurring at all the oxygen levels studied makes it unlikely that oxygen is the factor determining the rate of germination in solution. Since the purpose of studies recorded in this section was to find if factors affecting spore germination could be conveniently studied in suspension, rather than a detailed investigation of the effects of oxygen on spore germination, no further examination of oxygen requirements in these conditions was made at this time.

(iii) Effects of nutrient.

Germination in 1% malt extract was compared with that in distilled water. The pH of the distilled water was first adjusted to the same value as that of the malt extract, i.e. 4.8.

Suspensions of equal concentrations of spores in distilled water and in malt extract were prepared and ½ ml. aliquots of these transferred to aeration tubes, five containing nutrient spore suspension and a further five containing non-nutrient suspension. All samples were aerated for one month. Germination at the end of this period is recorded in table 6 of appendix and summarised below:

Medium	Mean germination per 3,000 spores
1% malt solution	3.95
water	4.10

Analysis of variance showed that the difference in germination between these two media was not significant. In this case, too, the level of germination remained very low and was not increased by the presence of nutrient.

(iv) Effect of spore concentration.

A relatively dense suspension of spores in distilled water was made. The suspension was dark brown in colour and haemocytometer counts showed that it contained approximately 38,000 spores per cu. mm. Dilutions of the suspension were made in distilled water, and three samples of each diluted suspension were transferred to aeration tubes. Germination after one month

is recorded in table 7 of appendix and summarised below:

Concentration	Mean germination per 5,000 spores
1	0.04
1/10	0.39
1/20	0.03

Very poor germination occurred and analysis of variance confirmed that germination in the three concentrations tested did not differ significantly. Although there is insufficient replication in this experiment, the germination levels obtained make it unlikely that repetition with increased replication would result in any improvement in germination. For this reason, no further study of the effects of spore concentration on germination in these conditions was made at this time. The problem was later examined on solid medium in work recorded in another section.

(b) Germination of spores on solid medium after pretreatment by submergence in water.

In the series of experiments recorded above, no explanation of the very poor germination occurring in suspension is found. It was not known whether submergence affected the ability of the spores to germinate. The following experiment, therefore, studied the effects of limited periods of submergence in water on the subsequent germination of the spores on solid medium.

Germination of spores, which had been given various periods of pretreatment in water, was examined after a fixed time on solid medium.

Drops of a uniform spore suspension were placed on five agar discs on the lids of each of five petri dishes and these containers, together with the tube of spore suspension, were incubated at 24°C. At intervals during the next ten days, further samples of spore suspension were transferred to solid medium in a similar manner. Germination counts were made in all cases on the seventh and eighth days after transfer of the spores to solid medium. The results are recorded in table 8 of appendix and summarised below:

No. of days in suspension.	Mean germination per 5,000 spores.	
	7th day	8th day
0	5	39
1	11	51
2	8	38
4	6	30
10	15	53

Empirical observation of these results indicated that similar levels of germination were attained after equal time on solid medium following these different periods of pretreatment in water. In every case, only an early stage of germination was found on the seventh day. During the ten days of the experiment, there was no sign of initiation of germination among the submerged spores.

(c) Comparison of germination on the surface of solid medium with germination in hanging drops of liquid medium.

It was clear from the foregoing work that levels of germination, satisfactory for further studies, were not being attained in "deep culture" by any of the methods used. It was therefore necessary to return to the use of spore cultures on agar discs or in drops of liquid medium, with special precautions to reduce to a minimum the factors responsible for the variability of germination in these conditions, already discussed.

Preliminary experiments had shown germination both in drops of liquid and on the surface of solid medium but suggested better germination on the solid medium. This was examined quantitatively in the following experiment.

Five water agar discs on the lids of each of three petri dishes were inoculated with spore suspension. Further drops of spore suspension were placed directly on the lids of the petri dishes, alternating with the agar discs. Germination after one week, based on counts of 10,000 spores in each sample, is recorded in table 9 of appendix and may be summarised as follows:

Medium	Mean germination per 5,000 spores
2% agar	171
Water	66

From empirical observation, it was clear that better germination occurred on the agar discs than in drops of water.

Thus, independently of the nutrient status of the medium, its physical condition has a considerable effect on the germination rate. This experiment confirmed the indications of many previous experiments that better and more uniform germination occurs on solid than in liquid medium.

Discussion.

It was concluded from the work in this section that solid medium was more convenient than liquid medium for germination studies with A. campestris spores. In spite of the advantages expected, only very poor germination was obtained in submerged conditions.

These results and those obtained in the study of germination of spores after periods of pretreatment by submergence in water, indicate that some condition of germination of A. campestris spores is lacking in liquid medium. Preliminary soaking for the length of time examined here, does not at all shorten the germination time on agar. Thus, it is doubtful whether any of the preliminary processes of germination have occurred during the period of submergence.

It is not clear why submerged conditions should affect germination in this way. Germination in small drops of liquid was poorer than on the surface of agar medium but did attain a moderate level. In larger volumes of liquid, however, only negligible germination has been found and this only after three or four weeks. It appears, from the investigation of germination of spores suspended in liquid, that some factor other than aeration nutrient or spore concentrations of the range studied, strongly affects the germination of these spores. There was no evidence of removal of a germination inhibitor by aeration.

The results of later work suggest that the unfavourable effect in suspension may be due to dilution of some metabolite diffusing from the spores into the medium. Germination may only become possible when a sufficient concentration of such a metabolite has accumulated in solution.

Such a process might be expected to be very slow and the rate of accumulation would be inversely proportional to the volume of liquid. On agar media and in small drops of liquids, such metabolites would accumulate around the spores and relatively little dilution would occur. If this is the case, it might be expected that a higher rate of germination should have been attained in the above experiment in which germination was studied in suspensions of greater density. In this experiment, however, only a very narrow range of concentrations was studied.

Further examination of these problems was thought to be irrelevant at the time. The limited object of the investigation was attained when these experiments demonstrated that deep culture did not provide convenient conditions for subsequent work.

Conclusions.

It has been shown in section I that the nutrient status, hydrogen ion concentration and physical condition of the medium are important in the initiation of germination of *A. campestris* spores. Germination can occur on solid or in drops of liquid medium, in the absence of nutrient or in the presence of a wide range of concentrations of nutrient, and on media of a relatively wide range of pH. Differences in germination between all these conditions, although significant, were relatively small. A marked contrast to all of these conditions was found, however, in the virtual absence of germination of spores suspended in greater volumes of solution. Only very poor germination was found and that only after three or four weeks. None of the treatments examined resulted in raising the germination in these conditions to a level suitable for further study.

In the discussion of germination studies in deep culture, at the end of sub-section (b), it was concluded that germination in these conditions might be unfavourably influenced by dilution of some metabolite diffusing from the spores into the relatively large volume of liquid and hence that the concentration of spores present might be important. This factor was not examined further in liquid medium, for the reasons already given, but is investigated on solid medium in the following section.

XII. Germination of Agaricus campestris spores in relation to concentration of spores.

Previous workers have commented on the difficulty of germinating single spores of *Agaricus campestris* but they do not appear to have made any quantitative study of germination in relation to spore concentration.

Ferguson (1902), describing germination of *A. campestris* spores in drops of culture solution, stated that massing of spores had no effect, as spores scattered through a drop germinated as frequently as those massed in the centre. Colson, however, for her cytological studies, found it necessary to inoculate large quantities of spores in 3% malt agar on which, usually after four days, several isolated centres of growth were obtained. She appears to have been unable to see the earliest stages of germination. Similarly, Kehl (1942) records that spores germinated in culture mostly only when they lie in clumps and stated that those dispersed on the substrate will not germinate properly. Sarazin (1952) stated that spores germinate if sown in sufficiently dense masses on a suitable culture medium.

It has not yet been possible with *A. campestris* to study the germination of spores isolated from a single basidium in the way in which the products of a single ascus have been studied in the Ascomycetes. In genetical work with *A. campestris* the germination of single spores has proved difficult. Lambert (1938) comments that single spore cultures may be obtained when a number of spores are given time to germinate before isolations are made.

Some more detailed study of the concentration effect was made by Sarazin, who found that if, after four or five days incubation of a mass of spores, a dilution is made causing each spore to be separated from others by

four or five times its diameter, a high level of germination (more than 80%) is obtained after a further forty-eight hours. He postulates the stimulation of germination by an excretion diffusing in the medium from the spores.

There is little information on the effect of spore concentration on the germination of spores of other Hymenomycetes. Good and Spanis (1958), studying the germination of spores of Fomes igniarius, found little variation in level of germination within the range of concentrations used.

Somewhat fuller and probably relevant results are available for the Uredospores of rust fungi. The production of a self inhibitor in the germination of rust uredospores was detected by Allen (1955), working with a strain of Puccinia graminis var. tritici. This substance prevented germination when a large number of spores were sown together. The inhibition was reversable, the inhibitor being removed when spores were floated on water.

Yarwood (1956), studying uredospore germination of Uromyces phaseoli, found the number of spores per unit volume, rather than the number of spores per unit area, to be the character determining the amount of germination. Thus it would appear that his findings involved more than a surface aeration effect and suggest, rather the result of dilution of a metabolite of the spores as determined by the volume of solution present. Yarwood found increase in concentration of spores was accompanied by decrease in the amount of germination obtained. On the other hand, growth, as indicated by germ tube length, increased with spore concentration.

The opposite effect has been noted for Myxomycetes. Smart (1937) found no germination of single spores but higher levels of germination in

cultures containing larger numbers of spores. He demonstrated stimulation of germination of these spores by the filtrate from medium in which similar spores had been actively germinating.

Experimental.

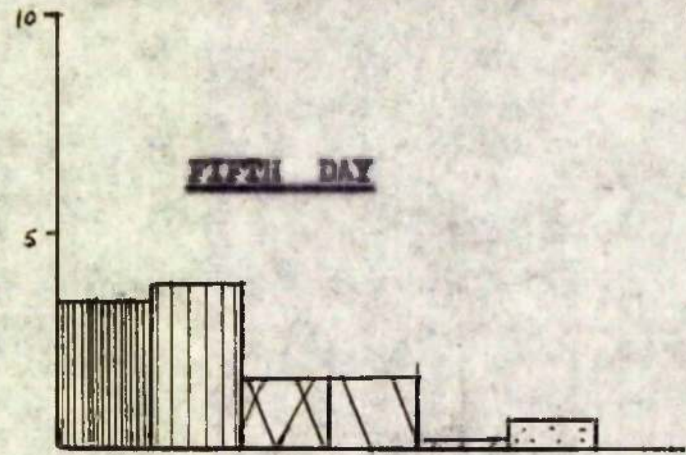
Spore suspensions of seven different concentrations, prepared from the same collection of spores, were inoculated on discs of plain agar on the lids of seven plates, each containing seven of these discs, so that each plate contained one sample of each spore concentration. Some water was added to the base of each plate to prevent drying of the discs. Germination per 5,000 spores on the fifth, sixth, seventh and eighth days after inoculation is shown in table 10 of appendix and summarised in histograms on page 43. Concentrations were estimated as the number of spores per low power microscopic field.

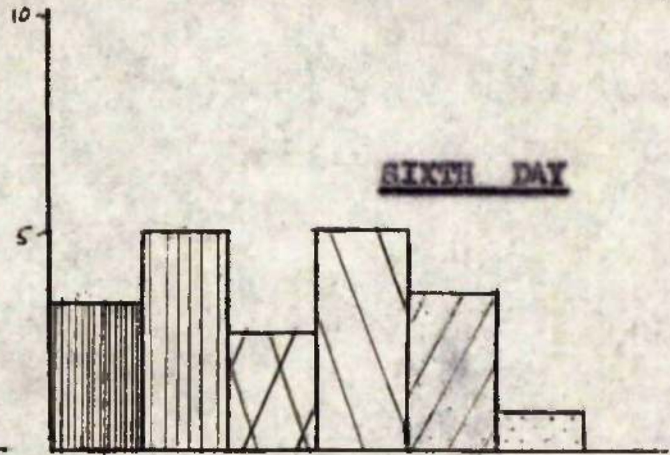
Results.

Fifth day - Empirical observation indicated earlier and better germination at the higher concentrations of spores. Analysis of variance showed that germination was not significantly different at concentrations of 30,000 and of 10,000 spores per field. The differences in germination between concentrations of 10,000 and 5,000 spores per field was above the 0.05 level of significance and that between concentrations of 1000 and 500 lay between the 0.1 and 0.05 significance levels.

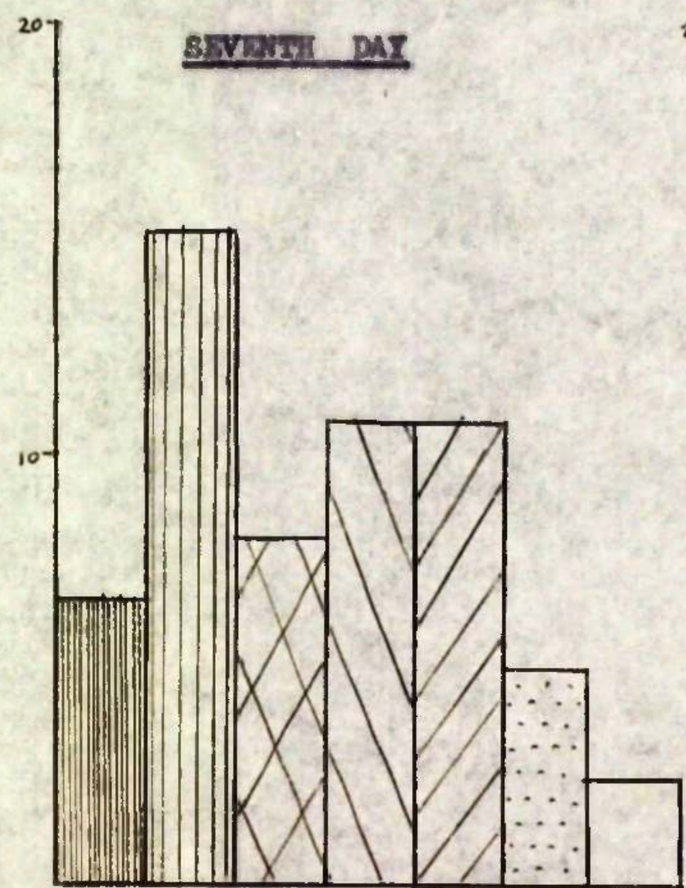
GERMINATION IN RELATION TO CONCENTRATION OF SPORES.

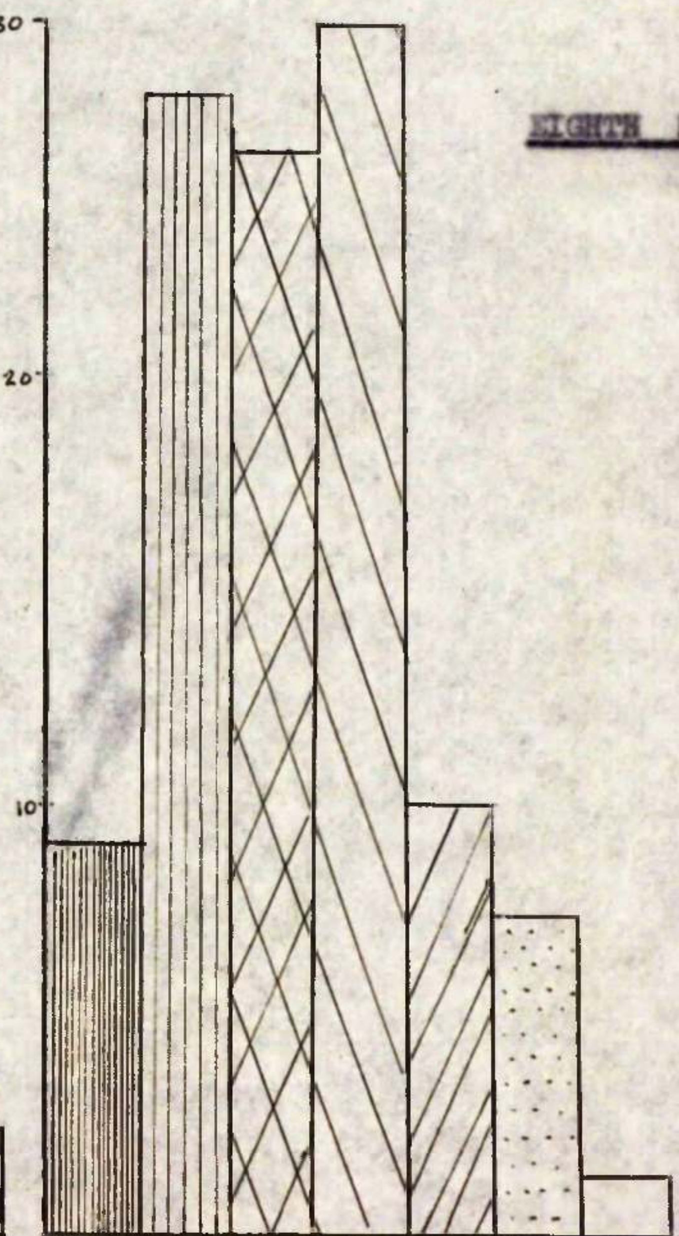
Comparison of mean germination per 5,000 spores on successive days.





Spores per field	
(vertical dense hatching)	30,000
(vertical hatching)	10,000
(cross hatching)	5,000
(diagonal hatching)	1,000
(diagonal hatching)	500
(dotted)	100
(blank)	50





Sixth Day - No significant differences in germination were found between the concentrations 30,000, 10,000, 5,000, 1,000 and 500. Germination at the concentrations of 1,000 spores per field was significantly better than at 100 (over the 0.01 significance level). The difference in germination between concentrations of 500 and 100 spores per field was just below the 0.05 level.

Seventh Day - Germination at the highest spore concentration was significantly less than at 10,000 spores per field, the difference being above the 0.02 level of significance. Germination at this highest concentration was no longer significantly greater than at concentrations of 5,000, 1000, 500 and 100 spores. Significantly better germination occurred at a concentration of 10,000 spores per field than at 5,000, the difference being above the 0.05 significance level. Differences in germination between the concentrations 5,000, 1,000 and 500 were not significant.

Eighth Day - Analysis of variance showed that germination at the concentration 1,000 was significantly better than at 100 or 50 but no significant difference was found in germination between the concentrations from 30,000 to 500 spores per field. On this day germination counts were made in only four plates, since accurate estimates of germination could no longer be obtained from the spore samples of highest concentration, where long hyphae from the first germinated spores were spreading over the densely distributed ungerminated spores.

Discussion.

These results indicate that, in general, the rate of germination of A. campestris spores increased with spore concentration. At the highest levels studied the initially favourable effect was followed by evidence of some inhibitory influence of the massing of spores. At this concentration the surface of the agar was completely covered by spores.

Thus it appears from this experiment that some substance produced by the spores promotes germination. It may also support the hypothesis discussed earlier (p.37) that the generally slower germination found in drops of liquid and the extremely poor germination found in deep culture, really demonstrate the effect of dilution of the metabolites diffusing from the spores.

At the highest concentrations of spores tested here, it is possible that the effect of competition for oxygen, or unfavourable concentrations of carbon dioxide may to some extent offset the stimulating effects of spore density. On the other hand, the postulated metabolite stimulating germination of the spores may exert an inhibitory effect when it is present in concentrations exceeding an optimum level. Little difference was found in the amount of germination recorded, over a range of concentrations from 10,000 to 500 spores per field, from the sixth day onwards. This relatively wide range of concentrations over which no strong concentration effect is seen, may be due to a balance of these stimulatory and inhibitory concentration factors. As far as possible the concentration factor has been standardised throughout the present germination tests. Spore cultures have been inoculated with uniform spore suspensions, rather than with dry

spores, and the use of agar media has generally been preferred to that of liquid media in which spores usually come to lie in a mass at the centre of a drop.

This work shows agreement with the observation by previous workers of the difficulty of germinating small numbers of mushroom spores and may to a considerable extent explain the widely differing results of various authors. Obviously, the quantity of spores used in a germination test may significantly affect the result. In most cases the published results do not give details of the spore concentrations used, nor the method of preparation of spore cultures. Cayley inoculated drops of culture medium by dipping a loopful of spores into it, but not stirring, "so that some spores remained floating". In this case, the total concentration of spores in a drop of medium may have been quite high, whereas the negative results of Bechmann (1929) in germination tests using the same medium, Knop's solution, may have been due to the use of lower concentrations of spores. Bechman's paper gives no direct information about this factor, but his statement, that in tests with distilled water and with Knop's solution, spores were supported on quartz sand, to prevent their sinking, suggests that a relatively greater volume of solution was present, thus diluting any metabolite diffusing from the spores, even if the spores were lying in clumps.

III. Germination of Agaricus campestris spores in relation to age of spore.

The following study was undertaken primarily to find to what extent the use of spores collected at different times was liable to affect germination tests. Since, however, material of a wide range of ages was available, it was clearly of interest to extend this study to spores of much greater age than used in other germination tests recorded in this thesis.

There are several records of examination of the effect of storage of spores of Agaricus campestris on their viability, but only Bechmann (1929) appears to have made a direct comparison of the germination of spores of different ages. He compared the behaviour of fresh spores with that of four, ten and twenty months old spores but found very little difference in their rates of germination or in their further growth. Ferguson (1902) germinated spores which were six months old. Cayley (1935) stated that spores of the wild form of Psalliota campestris remain viable for about six months and those of the cultivated species somewhat longer. Kahl (1942) found no difference in germination power and time and in the growth of young mycelium between spores kept up to two years in a desiccator and fresh spores. Sarazin (1952) stated that, when well desiccated, spores remain viable from three to five years, but that the amount of germination decreases rapidly with age.

Where a long period of viability has been reported for other fungi, the spores tested have usually been obtained from dried herbarium material. From such sources, Fisher (1936) reported germination of 18 years old spores of Tilletia caries (in 12 days) and of 21 years old spores of T. foetida

(in 21 days). Similarly, Lowther (1950), studying the germination of chlamydospores of *T. caries* and *T. foetida*, found no difference in germination between fresh and four to five years old spores, but a marked reduction in the rate and amount of germination in 11 years old spores. McCrea (1923) found spores of *Aspergillus oryzae* alive after being stored for 22 years, and Smith (1929) reported viability of Myxomycete spores up to 32 years old.

Such herbarium material of Hymenomycetes as is usually available would not provide suitable spore samples for germination tests with *A. campestris* spores. Because of the very slow germination characteristic of this species, it is necessary to use aseptically collected spores, otherwise growth of contaminating moulds would take place before the *A. campestris* spores could germinate. Thus, the lack of more extensive information on the viability of spores of this species may be due to difficulty in obtaining suitable samples of greater age than used by the workers mentioned earlier. In the present work, however, aseptically collected spores of *A. campestris*, up to 10 years old, have been obtained through the kindness of Pinkerton's Scottish Mushroom Laboratories, Glasgow.

Experimental.

Standard suspensions in distilled water were prepared from ten collections of spores of ages ranging from one month to ten years. All collections were made from sporophores grown at the Scottish Mushroom Laboratories and as far as can be ascertained were all from the same strain of mycelium. The ten spore suspensions were inoculated in randomised order on ten discs of water agar in each of ten petri dishes, so that each plate contained samples of spores of each age. The plates were incubated at 24°C.

Germination at intervals after inoculation is recorded in table 11 of appendix and may be summarised as follows:

Age of spores	Mean germination per 5,000 spores			
	9th day	11th day	15th day	30th day
10 years	0	0	> 167	> 500
7½ years	0	0	203	> 500
5¼ years	0	0	> 185	> 500
4½ years	0	0	130	> 500
3½ years	0	0	> 153	> 500
2½ years	29	> 273	> 450	> 500
1 year	19	> 155	> 441	> 500
7 months	36	275	> 480	> 500
2 months	380	> 500	> 500	> 500
1 month	84	> 318	> 480	> 500

From empirical observation it was clear that spores of age greater than 2½ years showed significantly slower germination than those of 2½ years and less. Analysis of variance of the results on the ninth day showed that germination of the two months old spores was significantly better than that of any other spores tested but that the differences between other sets of spores of ages up to 2½ years were not significant. Empirical observation of the germination records for the eleventh and fifteenth days also agreed with this.

By the fifteenth day, spores of ages greater than 2½ years showed moderate germination. Analysis of variance at this time showed no

significant differences in germination between spore collections of ages from three and a half years to ten years. By the thirtieth day, all samples of spores of all ages tested showed germination above the 10% level but comparative estimates were made impossible by profuse mycelial growth.

Discussion.

Because of the slowness of the change in germination rate with age in these collections of spores, it was considered unnecessary to examine the change in germination rate of individual spore collections over a period of time. The spores used in the other germination studies recorded in this thesis were less than two years old. It was clear that differences in age of spore of this order were likely to be associated with only negligible differences in germination and that greater variation would probably be found between individual spore collections than within a single collection after such periods of storage.

It appears that viability is only very slowly lost by these spores. Thus no significant difference was found between germination of 1 month, 7 months, 1 year and 2½ years old spores. Similarly, although the older group of spores shows poorer germination, no significant difference was found between germination of spores of ages 3½, 4½, 5½, 7½, and 10 years.

The differences in germination shown here reflect not only changes due to age of spore and any natural variations between individual collections of spores, but also differences due to the conditions of storage. Thus, the significantly better germination of two months old spores than of others may indicate a true difference in germination ability of these spores. It is

less likely to be due to some factor of handling or storage, since the spores were stored together at room temperature. On the other hand, the marked difference in germination between spores of age 2½ years or less and those of greater age than this is probably, to some extent, due to differing storage conditions, the former having been collected and stored in the course of the present work and the older collections having been obtained from the Scottish Mushroom Laboratories. In both cases the methods of collection and storage were similar and, as far as can be ascertained, the sporophores used were of the same strain and from the same source. In all cases the glass tubes containing the spores were stored at room temperature, without special precautions to ensure constant conditions.

Because of the extended germination period and the profuse mycelial growth from the earliest germinated spores, which is characteristic of normal germination of A. campestris spores, it is not possible by this method to determine the final level of germination in any sample and results have not been recorded beyond the 10% level. It is possible, also, with this experimental design, that mycelium from the earlier germinating spores present in the same plate may have influenced the germination of the older spores. In the present experiment, however, the amount of mycelial growth was much less than on the nutrient medium in the earlier experiment and the germination found was not of the more uniform type, which was later recognised to be frequently associated with stimulatory conditions.

It appears that spores of A. campestris must remain viable for a relatively long time. This, together with the slowness of germination suggests a slow rate of metabolism in the resting basidiospores. This idea

is supported by Kehl's (1942) demonstration of the remarkable tolerance of spores of A. campestris to chloroform and carbon tetrachloride, which inhibit the respiration of most living cells. This property allowed Kehl to 'disinfect' spores of this species by exposure to chloroform, a few minutes treatment usually being lethal to other spores present. He claimed that no substantial decrease in germination was found after several days treatment in chloroform and that a harmful effect was first noticeable after twenty to twenty-five days of treatment. Cells which exhibit such properties must have a characteristically slow type of respiration. In studying other methods of spore disinfection, Kehl found that spores of A. campestris could tolerate storage for several months in sodium chloride and also treatment with potassium hydroxide and sodium hydroxide, without obvious injury or decrease in germination ability. Similarly, treatment with concentrated glycerine did not appear to affect A. campestris spores, although, like the above treatments, "disinfecting" the spores by killing spores of other species present as contaminants.

The food reserves in these basidiospores must be considerable and may contribute to this longevity, as well as allowing profuse growth of mycelium from germinating spores in water or on non-nutrient medium. The only information on the nature of the food reserves in spores of A. campestris appears to be the well known presence in the protoplasm of one or two oil drops noted by many workers, e.g. Hoffmann (1860).

It is frequently claimed that the dark pigment in the walls of spores of such species as A. campestris protects these spores from harmful radiation. Kehl (1942) found that spores of A. campestris were remarkably resistant to

exposure to light and heat. He records germination of spores kept for four days at 90°C and found that some spores tolerated a temperature of 100°C without injury. The relatively thick wall probably reduces loss of water from the spore and may even function like the testa of seeds of many higher plants by cutting down gaseous interchange, causing internal accumulation of carbon dioxide and the slowing down of respiration.

The evidence available indicates that the basidiospore of <u>A. campestris</u> is a very resistant structure, able to withstand various treatments which are lethal to the majority of fungal spores. It also suggests that greatly reduced metabolic activity, the character of the spore wall and possibly the food reserves and osmotic properties of the protoplasm, may be among the factors contributing to the resistance and longevity of these spores.

Conclusion.

Part 1 of this thesis has established the conditions suitable for germination tests and assessed the influence of various factors. The implications of the results of individual sections have already been discussed in some detail.

The demonstration of the effects of spore concentration on germination may come nearest to explaining the discrepancies of previous results. The degree of dilution or accumulation of some spore metabolite may be the overriding factor determining germination.

It appeared that this effect might be related to the earlier reports of stimulation of germination of these spores by mycelium of the same species. It was therefore decided not to investigate the spore concentration effect further at this stage but to examine the nature of the reported mycelial stimulation.

Part 2.

Effects of biological factors on the germination of spores of Agaricus campestris.

Part 2. EFFECTS OF BIOLOGICAL FACTORS ON THE GERMINATION OF SPORES OF AGARICUS CAMPESTRIS.

I. Stimulation to germination of spores of Agaricus campestris by living mycelium.

1. Effect of presence of *Agaricus campestris* mycelium on germination of spores of the same species.

    (a) Demonstration of the effect of *Agaricus campestris* mycelium in contact with spore suspension.

    (b) Demonstration of effect of the presence of *A. campestris* mycelium not in contact with spore suspension.

2. Study of effect of sterile medium in which mycelium of *A. campestris* has been growing.

    (a) Germination in sterile solid medium extracted from *A. campestris* cultures.

    (b) Effects of direct addition of liquid culture medium to drops of spore suspension.

    (c) Demonstration that the germination stimulant in sterile medium was volatile at room temperature.

3. Germination of *Agaricus campestris* spores in the presence of other fungi.

    (a) Study of the germination of *A. campestris* spores in the presence of other fungi.

    (b) Study of the effect of variation in the amount of the stimulating organism on the germination of *A. campestris* spores.

II. Analysis of the stimulation effect.

Review of literature.

Methods: (a) Description of apparatus.

(b) Problems involved in design of apparatus

(i) Supply of mycelium.

(ii) Choice of germination chambers.

(iii) Tests of materials suspected of affecting spore germination.

1. Study of the influence of carbon dioxide on germination of *A. campestris* spores.

(a) (i) Preliminary examination of properties of air passing from a culture of *A. campestris* mycelium.

(ii) Effects on germination of passing air bearing the germination stimulant through carbon dioxide absorbent before it reaches the spores.

(b) Effects of increased concentrations of carbon dioxide upon germination of *Agaricus campestris* spores.

2. Examination of methods of isolating the stimulating material.

(a) Distillation of media.

(i) Study of the effect of heat on the germination stimulant in medium from cultures of *A. campestris*.

(ii) Distillation of culture medium.

(b) Ether extraction of media.

(c) Direct isolation of the stimulating material from air which has passed over *A. campestris* mycelium.

3. Chemical analysis of volatile metabolites.

(a) Volatile material from *Agaricus campestris* mycelium.

(b) Volatile material from *Saccharomyces cereviseae*.

I. Stimulation to germination of spores of Agaricus campestris by living mycelium.

The stimulation of spore germination in *A. campestris* by mycelium of the same species was first noted by Ferguson (1902) and confirmed by Falk (1924) and Cayley (1935). Ferguson found that every case of high germination was preceded by germination of one or two spores and noted that cultures into which small amounts of mycelium had been introduced gave almost perfect germination in 144 hours, one week sooner than expected. Cayley introduced mycelium to the edge of drops of Knop's solution in which spores had been left for ten days and obtained in 24 hours more uniform germination than normally occurred. She stated that this stimulus scarcely extended beyond the tips of the hyphae and suggested that a secondary stimulus, set up by germination, extended throughout the liquid.

Other observations have indicated that the growth of *A. campestris* mycelium produces a change in the medium in which it is growing, which stimulates the germination of spores inoculated on this medium. Kehl (1942) observed good germination on an extract of Mushroom "spawn". De Zeeuw (1943) found germination was greatly increased when *A. campestris* spores were inoculated on agar obtained from slope cultures of the mycelium which had been kept in boiling water until the agar was liquified. There appears, however, to be no indication that these workers proceeded further in this study.

Such stimulation by culture medium might be due to a favourable change in the nutrient status of the medium following growth of the mycelium or to the production of a specific germination stimulating substance. Stoller (1955), suggested that the influence of mycelium on the germination of these

spores may be due to oxidation of toxic substances in the medium by oxidases of the mycelium. He demonstrated the production of such enzymes by <u>A. campestris</u> mycelium.

Some study of the effect of mycelium of other fungi on germination of <u>A. campestris</u> spores has been recorded by previous workers. Ferguson found that mycelium of <u>Penicillium</u>, <u>Mucor</u> and <u>Coprinus</u> species, had no effect on germination of <u>A. campestris</u> spores. She, therefore, concluded that the stimulation of germination by <u>A campestris</u> mycelium could not be due merely to the oxygen uptake or carbon dioxide production by the mycelium. Falk (1924), however, pointed to a single case, recorded by Ferguson, where more than 50% germination of <u>A. campestris</u> spores occurred in the presence of mycelium of <u>Hypholoma appendiculatum</u>. He suggested that Ferguson may have been inaccurate in attributing this to the stimulating effect of some metabolite from a few spores of <u>A. campestris</u> which had germinated earlier. Falk (1924) found that <u>Coprinus</u> mycelium stimulated germination of <u>A. campestris</u> spores, even more strongly than did mycelium of the same species. He had demonstrated the effects of various organic acids on germination of these spores and attributed the stimulation by mycelium of <u>A. campestris</u> to the oxalic acid produced by the mycelium. He found, however, that surface mycelia of <u>Coniophora cerebella</u>, <u>Polyporous vaporarius</u> and others, which are stronger acid formers, had no effect on germination of these spores. Falk's hypothesis suggested that the fungi which stimulate germination of <u>A. campestris</u> spores are those which normally grow before <u>A. campestris</u> in the same habitat.

Fries (1943) found that the presence of a wild yeast stimulated

germination of various Basidiomycetes which were difficult to germinate, e.g. Hydnum and Craterellus species, or which previous workers had not succeeded in germinating, e.g. species of Tricholoma, Amanita and Boletus. The presence of Torulopsis sanguinea induced better stimulation than the wild yeast. Fries also demonstrated the stimulation of germination of Boletus spores by mycelium of the same species and of some other species of soil fungi. These spores normally showed only poor or very slow germination.

Thus, while Ferguson's results suggest a unique effect of a specific metabolic product of A. campestris mycelium, the reports of Melk and Fries suggest a more widespread stimulus.

The unique observations by Kohl (1943) suggest a different type of biological activation of these spores. He found good germination of mushroom spores, which, he states, were as common as Penicillium spores in the atmosphere of his laboratory, when they were accidentally introduced into hanging drop preparations of stigmatic mucus of orchids. In earlier work (Kohl, 1942), he had found spore germination poor, reaching 2 to 15% after five to ten but mostly after 20 days. In drops containing stigmatic tissue of the orchid Phalaenopsis, 95% germination occurred after two to five days. This is the shortest germination time anywhere recorded for mushroom spores. In a series of these germination experiments with no sterility precautions, 40% of the preparations remained uncontaminated and in these preparations, 100% germination was observed in a few days, while germination was entirely absent in the contaminated cultures. Kohl concluded that a certain degree of sterility was necessary for germination and suggested the action of the stigmatic fluid might be simply the inhibition of competitors or else might

result from gradual lack of oxygen in the medium or the liberation of carbon dioxide by dying isolated stigmatic cells included in the preparation. He considered it unlikely that the stimulation of germination was due to a specific activator contained in the stigmatic mucilage, since experiments with vitamin amino acids and with the bios component of yeast were all completely negative. Stoller (1954) suggests that Kohl's results may point to the activity of proteinases or cytases and recommends repetition of the work. It is possible, however, that the stimulation induced by the orchid stigmatic preparations may bear some relation to the stimulation of germinatio[n] of Agaricus campestris by the various fungi mentioned above.

In the present work, the results of previous workers have been examined and extended by a more detailed examination of the stimulation induced by the mycelium of Agaricus campestris and other fungi.

Experimental.

1. Effect of presence of Agaricus campestris mycelium on germination of spores of the same species.

(a) Demonstration of the effect of Agaricus campestris mycelium in contact with spore suspension.

This experiment was carried out by methods similar to those of Ferguson (1902) and Cayley (1935).

Drops of aqueous suspension were placed in vaseline rings on the bases of ten petri-dishes. A small piece of malt agar, in which was growing mycelium of A. campestris, was placed at the edge of each drop in five of the plates and a similar portion of uninoculated malt agar at the edge of each drop in the remaining, control, plates.

Germination occurred in one week in the drops with added mycelium, while spores in the control drops remained ungerminated in this time. No quantitative comparison could be made by this method, due to massing of spores in the drop and the obscuring growth of mycelium from the initial inoculum, but the qualitative observation agreed with previous reports.

(b) Demonstration of effect of presence of A. campestris mycelium not in contact with spore suspension.

Ten malt agar plates, five of which were inoculated centrally with mycelium of A. campestris, were incubated for two weeks until good growth was shown in the inoculated plates. The surfaces of all the plates were then covered with sterile cellophane which served the double purpose of

confining the mycelium to the surface of the agar and ensuring uniformity of the evaporating surface. Five water agar discs were placed on the lid of each petri dish and inoculated with spore suspension. Germination levels on the sixth day of the experiment are shown in table 12 (appendix) and summarised below.

	Mean germination per 5,000 spores
Mycelium present	1150
Mycelium absent	8

Empirical observation showed that these differences were significant. Only the normal low level of germination occurred in the control chambers in six days while, in the petri-dishes containing growing mycelium, the much higher level of germination attained is similar to that normally obtained on such media only after fourteen days.

Since there was no direct contact between the mycelium and the spores, this effect must be due to some volatile germination stimulant evolved by the mycelium.

2. Study of the effect of sterile medium in which mycelium of *A. campestris* has been growing.

As a preliminary to further study of the volatile stimulant evolved by *A. campestris* mycelium, agar medium in which mycelium of this species had been growing was examined, to find whether the germination stimulant could be identified in it. The production of this stimulant in liquid medium was then investigated with a view to obtaining larger amounts of more convenient material for analysis. This part of the work initiated studies on growth of *A. campestris* mycelium in liquid medium described in part 3 of this thesis.

(a) Germination on sterile solid medium extracted from *A. campestris* cultures.

The method used was a modification of that of de Zeeuw (1942). Medium from ten one-month old cultures of *A. campestris* on 2% malt agar slopes was liquified by placing the slope cultures in boiling water for half an hour. Liquified medium from below the mycelial mat was then transferred by sterile pipette to a large sterile test tube. Similarly, control medium from uninoculated slopes of the same agar was transferred to another test tube. 10 ccs. of each medium were poured into two small petri dishes, and this, when set, was cut into discs using a ¾ cm. cork borer. Five discs of each medium, alternating, were placed in a ring on the lids of each of five petri-dishes and inoculated with spore suspension. Some water was placed in the base of the dishes to reduce drying of the discs. Germination on the 6th and 10th days after inoculation is recorded in table 13 (appendix). The results may be summarised as follows:

	Mean germination per 5,000 spores	
	6th day	10th day
Culture medium	89	over 500
Control medium	0	31

Empirical observation indicated that the differences shown were significant. Good, uniform germination was found on the discs of culture medium on the 6th day, when no germination had occurred on the control discs. By the tenth day, accurate counts on the culture medium discs were made impossible by the high level of germination, accompanied by dense mycelial growth, while only moderate, and not at all uniform germination was found on the malt agar discs. It appears, therefore, that some metabolite in the culture medium causes more rapid and more uniform germination and, further, that this stimulating material is fairly thermostable.

(b) Effects of direct addition of liquid culture medium to drops of spore suspension.

Methods.

In order to obtain the culture medium used in this work, flasks containing 2% malt extract solution buffered at pH 6.8 were inoculated with standard discs from cultures of A. campestris mycelium on 2% malt agar. The earliest solutions used were buffered with 0.04 M phosphate but, on the basis of results obtained in the growth studies already mentioned, the solutions used in all subsequent experiments were buffered with 1% powdered chalk. Samples of medium were removed from the culture flasks by means of a sterile pipette after two to four weeks growth of mycelium.

Experimental.

The initial experiment in this work was designed to study germination in liquid medium from cultures of A. campestris by a method similar to that used for solid medium. It was found, however, that when culture solution and control solution were added to alternate drops of spore suspension in the same petri-dishes, good germination occurred in all drops in four days.

This observation suggested that the stimulant in the culture medium affected both the drops to which it was added and the control drops. Thus, the stimulant from the drops of culture solution must have diffused into the control drops.

This hypothesis was tested in a further experiment, which was similar to the previous experiment, but with the control solution added to spores in separate containers from the culture solution. Culture solution was added

to drops of spore suspension in each of five petri dishes and control solution to drops in five similar petri dishes. Results are recorded in table 14 (appendix) and may be summarised as follows:

Solution added	Mean germination per 5,000 spores
Culture solution	591
Control solution	8

From empirical observation it was clear that these differences were significant. Good germination was observed on the 6th day in all drops to which culture medium had been added while very little germination had occurred in the control drops by this time. It was clear, therefore, that the results of the preliminary experiment were due to the influence on spores in control drops of germination stimulant volatilising from the drops of culture medium. This was confirmed by the following experiment.

(c) Demonstration that the germination stimulant from liquid culture medium was volatile at room temperature.

Culture solution was added to the bases of five petri dishes each having on the lid three water agar discs inoculated with spore suspension. Similar uninoculated malt solution was added to the bases of five similar petri dishes

Germination records on the sixth day, shown in table 15 (appendix), may be summarised as follows:

Solution present	Mean germination per 5,000 spores
Culture solution	17
Control solution	0

Thus, empirical observation indicated that stimulation of germination had occurred on all the discs in the presence of culture medium but none in the control plates. Thus, liquid in which mycelium of A. campestris has been growing contains a volatile germination stimulant which can influence spores at some distance from it.

Discussion.

These studies have shown that the stimulatory effect of A. campestris mycelium on germination of spores of this species is due to a volatile metabolite of the mycelium which diffuses into the air and into the medium in which the mycelium is growing. Thus it is not a simple nutrient effect as appeared possible from the methods of previous workers. Before further examination of the stimulant, it was clearly of interest to find whether the

effect was specific to _A. campestris_ mycelium or whether other fungi could stimulate the germination of _A. campestris_ spores in this way. This problem was investigated in the following work.

3. Germination of Agaricus campestris spores in the presence of other fungi.

The findings of previous workers in connection with this problem have been reviewed in the introduction to section I.

Experimental.

(a) Study of the germination of A. campestris spores in the presence of mycelium of other fungi.

A preliminary survey was carried out by a method similar to that used in demonstrating the stimulation of germination of A. campestris spores in the presence of mycelium of the same species, the spores being inoculated on agar discs on the lids of petri dishes, the bases of which contained cultures of the test fungi. The following species were tested by this method: Agaricus campestris, Polyporous betulinus, Merulius lacrymans, and Saccharomyces cereviseae. Germination on the fourth, sixth, eighth and fourteenth days is recorded in table 16 of appendix and summarised below:

Species present	Mean germination per 5,000 spores			
	4th day	6th day	8th day	14th day
Agaricus campestris	29	> 500	> 500	> 500
Merulius lacrymans	3	26	110	> 500
Polyporous betulinus	1	15	27	180
Saccharomyces cerevisеae	4	448	1910	4400
Control	0	1	22	> 500

Empirical observation showed stimulation of germination in the presence of all the species tested successfully.

In the presence of S. cereviseae, after an initial delay, almost simultaneous germination of more than 80% of the spores was observed. This is markedly different from the more typical pattern of germination in these spores in which germination normally occurred in discrete groups of spores. The mycelium from these first germinating spores then normally grew all over the medium and obscured the remaining spores.

Species of conidia forming fungi such as Penicillium were tested but no results could be obtained by this method due to contamination of the A. campestris spore cultures by the test fungi.

The results of this preliminary experiment indicated the presence of two problems: (i) When comparing the effects of fungi of different growth rates, difficulty is encountered in standardising the amount and age of mycelium present. In the preliminary experiment, cultures of all species were inoculated at the same time and grown for equal periods before A. campestris spores were exposed to them. The amount of mycelium present at the time of spore inoculation by this technique, differed from species to species. In the following experiments, however, some standardisation of the amount of mycelium present was secured by using cultures in which mycelium just covered the surface of the agar. Cultures were inspected at intervals during growth and the more quickly growing species were removed from the incubator at a suitable stage and stored at 4°C until other species had made the same amount of growth. Spore cultures were then introduced to the petri dishes and all again incubated at 24°C.
(ii) It was necessary to devise a modified method for testing the effects on germination of A. campestris spores of mycelium of freely sporing fungi.

With the first method, spores from such fungi contaminated the agar discs bearing A. campestris spores. The following technique was, therefore, adopted.

Agar discs were placed in the bases of sterile petri dishes and inoculated with spores of A. campestris. Sterile filter paper was then placed over each of these petri-dish bases and sealed to the outside by means of 'cellotape' to prevent entry of contaminants. Thus covered, the petri-dish bases containing the spore cultures were inverted over fully grown cultures of the test mycelia in the bases of other petri-dishes. Randomised sets of these germination chambers were enclosed in polythene bags, to prevent drying, and incubated.

The effects on germination of A. campestris spores of mycelium of a series of Hymenomycetes, which had been isolated by tissue culture techniques, were compared with those tested previously, using the method described in paragraph (i) above. (Expt. A.) A number of sporing fungi of other groups were tested by the modified method described in paragraph (ii) above. (Expt.B. The species used were the following:

Experiment A.	Experiment B.
A. campestris.	Penicillium notatum.
Polyporous betulinus.	Mucor hiemalis.
Merulius lacrymans.	Botrytis cinerea.
Laccaria laccata.	Cunninghamella elegans.
Hygrophorous virgineus.	
Hypholoma fasciculare.	
Russula atropurpurea.	

The results of Experiment A are recorded in table 17 of appendix and those of Experiment B in table 18. A summary of these results is given on page 73.

All the fungi in Experiment A stimulated germination of A. campestris spores. Laccaria laccata, Hypholoma fasciculare, Hygrophorous virgineus and Russula atropurpurea, in these conditions, caused even greater stimulation of germination of A. campestris spores than did mycelium of the same species. In several cases, all these fungi showed almost simultaneous germination of a high percentage of the spores, similar to that already commented on in describing the type of stimulation shown in the presence of Saccharomyces cerevisese. In Experiment B, germination was stimulated by Penicillium notatum, Mucor hiemalis and Botrytis cinerea. Strongest stimulation occurred in the presence of B. cinerea. Despite the precautions taken, however, some contamination with spores of the test species occurred. All spore cultures in the presence of Cunninghamella elegans were contaminated by spores of this species, which produced a profuse aerial mycelium and tended to grow through the filter paper barrier. Records taken from discs in which contamination by these spores was just beginning, showed no germination, but the result is obviously not conclusive.

Germination of Agaricus campestris spores in the presence of mycelium of other species of fungi.

	Species	Mean germination per 5,000 spores	Day	Experiment
Basidiomycetes	Agaricus campestris	176	4	A
	Merulius lacrymans	106	4	"
	Polyporous betulinus	241	4	"
	Laccaria laccata	381	4	"
	Hygrophorous virgineus	over 500	4	"
	Hypholoma fasciculare	over 500	4	"
	Russula atropurpurea	over 500	4	"
	Control - no mycelium	0	4	"
Ascomycetes	Botrytis cinerea	200	8	B
	Saccharomyces cereviseae	4	4	Preliminary
		2360	8	"
	Control	0	4	"
		22	8	"
Fungi imperfecti	Penicillium notatum	22	8	B
Phycomycetes	Mucor hiemalis	20	8	B
	Cunninghamella elegans	0 (contaminated)	8	B
	Control	0	8	B

(b) <u>Study of the effect of variation in the amount of the stimulating organism on the germination of A. campestris spores.</u>

The following investigation was designed to study the effects of the presence of varying amounts of an organism stimulating the germination of A. campestris. Saccharomyces cereviseae was chosen for this work so that a more precise control of the quantity of living material present could be achieved than is possible with filamentous fungi and also because of the unusually uniform type of germination already noted in the presence of this organism. The problem was studied in two experiments, the first using a limited range of yeast concentrations and the second a high concentration of yeast. Since the second experiment is really an extension of the first, results of both are summarised together below.

(i) Four sets of four malt agar plates were inoculated with S. cerevisеae at one, four, eight and twelve points respectively and incubated until the yeast colonies were each about one centimetre in diameter. Four discs of water agar were then placed on the lid of each plate and inoculated with spore suspension. Germination at intervals after the commencement of germination is recorded in table 19 of appendix.

(ii) A dense culture was prepared by adding one gram of Bakers' Yeast to 200 ccs. of sterile 5% glucose solution. Aliquots of this suspension were added to the bases of six petri dishes, each having on the lid three agar discs inoculated with spore suspension. Uninoculated solution was placed in the bases of six similar petri dishes. Germination on the tenth day is recorded in table 20 of appendix.

The results of both experiments may be summarised as follows:

No. of colonies of yeast.	Mean germination per 5,000 spores		
	12th day	14th day	18th day
1	0	0.6	16
4	8	617	3643
8	173	2949	4589
12	3635	3775	4992
concentrated suspension.	0	0	0

Without analysis, it is clear that the rate of germination increases with the amount of yeast present in the lower concentrations but that inhibition of germination is found in the presence of yeast suspension.

In the first experiment, germination was first noted on the twelfth day after inoculation, in the plates with twelve colonies, and followed later in the petri dishes containing smaller numbers of yeast colonies. In the second experiment, germination was completely absent on the tenth day in the plates containing yeast suspension, while good germination had occurred in the presence of uninoculated control solution. The observations were continued until the thirtieth day but during this time, no germination occurred in the presence of the yeast suspension.

Thus, while, in the range of concentrations studied in the first experiment, the stimulation of germination is proportional to the amount of material producing the volatile germination stimulant, the presence of a higher concentration of this material exerts an inhibitory effect on spore germination.

DISCUSSION.

The results gained in this section indicate that the stimulating influence of living mycelium on the germination of spores of *A. campestris* is considerably more widespread than was realised by Ferguson (1902) or even by Falk (1924) and Fried (1943). Further the stimulation has been shown, in each case, to be due to a volatile material. It is not yet clear whether any species of fungi are without influence or exert an inhibitory effect on germination of *A. campestris* spores. The present evidence indicates that many species from various groups of fungi produce volatile metabolites with the same or similar stimulatory effects.

The experiments with *Saccharomyces cereviseae* indicated that the amount of living material can affect the result. As far as possible in the present work, this factor has been standardised by using cultures in which mycelium covered approximately equal areas of the agar surface. This may be adequate for the Hymenomycete mycelia tested, which showed in these conditions, a surface mycelium with relatively little aerial growth, but it was not effective with the species of *Mucor*, *Botrytis* and *Cunninghamella* tested, since these produced a profuse aerial mycelium which filled the petri dish. Thus direct comparison cannot be made between germination in the presence of these species, as recorded in Experiment B, and germination in the presence of other fungi with the surface type of growth. It appeared that the stimulation of germination occurred more slowly in the presence of the above named species than is usual with Hymenomycete mycelia but it is possible that this may be an unfavourable effect of the high concentration of volatile material from the much greater volume of mycelium present in plates in which these species were growing.

II. Analysis of the stimulation effect.

The results of the foregoing sections demonstrated the activity of a volatile metabolite produced by *Agaricus campestris* and other fungi, which stimulated germination of *A. campestris* spores. Since carbon dioxide is the one volatile metabolite known to be common to all living cells, it was thought proper to examine first of all the possibility of carbon dioxide's being the substance involved in this stimulation of germination. It was appreciated that, if carbon dioxide was proved not to be the stimulant, chemical analysis of the volatile products of *A. campestris* mycelium would be required in order to find if any other substance was present and might be the stimulant. At this stage, however, there was not sufficient evidence to justify approaching chemists with the problem. The investigation was developed in three stages, the first two being carried out simultaneously.

1. Direct examination of carbon dioxide as a possible stimulant:
   (a) By removal of carbon dioxide from air bearing the stimulant.
   (b) By increasing the concentration of carbon dioxide present.
2. Examination of methods of isolating the stimulating material.
3. Chemical analysis.

When the first part of the investigation indicated that it was unlikely that carbon dioxide was the active metabolite, this work was discontinued. The investigations into methods of extraction from the media were also stopped when a satisfactory method for direct isolation of the gaseous material was developed. Mass-spectrometric analysis of this material was then carried out by Dr. Reed of the Chemistry Department, Glasgow University.

Results of all three lines are presented to show the method of approach

and the experimental problems involved; the literature relating to the stimulation phenomenon examined here and to analogous effects with other species is also reviewed.

Review of literature.

(1) Volatile metabolites produced by *Agaricus campestris*.

There are various reports of the detection of substances other than carbon dioxide in the presence of living mycelium of *A. campestris*, but no account of their effects on spore germination. There are no reports of these other volatile metabolites having been found in the presence of any of the other fungi which have stimulated the germination of *A. campestris* spores.

Mader (1943) found that a volatile metabolite from growing mycelium of *Agaricus campestris* could inhibit fructification in inadequately ventilated mushroom beds. He found that the inhibitory material was removed when the air was passed through mineral oil and on this basis concluded that it might be an unsaturated hydrocarbon. Stoller (1955) detected the presence of oxidases in the atmosphere above growing mycelium and suggested that such enzymes might oxidise any germination inhibitors present in the medium but did not report any tests to verify this. He also reported earlier work, in which he had observed "epinasty" of tomato plants placed under a bell-jar on top of a mushroom bed impregnated with mycelium and also in 3-gallon bottles of mushroom spawn. This response has been used by many previous workers (e.g. Denny, 1935) to detect the presence of unsaturated hydrocarbons, but Stoller does not comment on this.

Schisler (1957) also studied the inhibition of sporophore formation in

conditions of poor aeration and postulated the production of a "hormone" of high molecular weight and low volatility, accumulation of which favoured vegetative mycelial growth and inhibited strand and fructification formation. It is possible that the volatile substances affecting growth may also stimulate germination.

(2) Influence of carbon dioxide on spore germination.

The only record which was found of an investigation of the effect of carbon dioxide on spores of *A. campestris* was that of Falk (1924), who stated that carbon dioxide did not stimulate their germination. Other workers have reported stimulation of germination of spores of various fungi by increased concentrations of carbon dioxide.

Durrell (1925) found spores of *Basisporium gallarum* germinated better with some living plant material present in the same container and showed that the same effect was obtained on addition of carbon dioxide to the container. A similar effect with spores of *Ustilago zeae* was demonstrated by Platz et al (1925). The optimum concentration of carbon dioxide for germination, in this case 15%, gave a pH of 4.9 to 5.6 and these workers attributed the stimulation of germination to the change in pH resulting from the presence of carbon dioxide.

(3) Effects of substances other than carbon dioxide on spore germination in various fungi.

Various types of volatile material are known to affect germination of spores of other fungi. Brown (1922) reported effects of volatile materials from various sources on germination of spores of Aspergillus flavus. He found germination inhibited by the presence of damp blotting paper or filter paper and showed that this must be due to the activity of microorganisms, since sterilisation of the paper prevented this affect. He observed stimulation of germination in the presence of leaves of various species and in the presence of healthy, unbruised apples. The stimulation in the presence of apple might be due to any of the volatile substances known to emanate from ripening apples. Many studies of apple volatiles have been recorded and among the substances found have been ethylene (Gane, 1934), various esters (Power and Chestnut, 1920), some aldehydes and alcohols (Pentzer and Heinze, 1954). Brown obtained stimulation of spore germination, similar to that the presence of apples, in the presence of ethyl acetate and also the malate and citrate esters. Duggar (1901) had already studied the stimulation of germination of Aspergillus flavus by ethyl alcohol and, more slowly, by methyl alcohol.

Noble (1923) found germination of spores of Urocystis tritici stimulated by expressed sap of wheat, volatilising from a separate dish in the same container, by uninjured seedlings of certain non-susceptible hosts, and by benzaldehyde, salacylaldehyde and acetone. Stimulation by benzaldehyde was also reported by Ling (1940) for spores of Urocystis occulta, stimulation occurring with concentrations of the order of one part per 600,000.

A number of interesting observations have resulted from work on the germination of rust uredospores. Yarwood (1956) found increasing concentrati of uredospores of Uromyces phaseoli per unit volume resulted in decrease in germination percentage but in increase in germ-tube length. Thus he obtained evidence of the production by the spores of a self-inhibitor of germination. Coumarin, Potassium permanganate and 'Tween 60' greatly increased the percentage of heavily seeded spores but did not raise the germination to the level of the thinly seeded spores. Similar self-inhibition of spore germination was demonstrated by Fahim (1956) for spores of Botrytis cinerea.

Bailey (1923) reported that paraffin oil increased the germination of spores of Puccinia helianthi and suggested that this might be due to the absorption of inhibitory gaseous metabolites by the oil. Yarwood, however, found that, while germination on agar containing 1% (by volume) paraffin oil was greater than without oil, germination on agar in inverted plates with oil placed on the lids was not greater than in plates without oil, and concluded that Bailey's finding did not appear related to the stimulation phenomenon.

Allen (1955; 1957), working on inhibition of germination of Puccinia graminis var. tritici by a volatile material accumulating in the liquid on which the spores were floating, obtained a volatile fraction, which stimulated germination. This stimulating fraction also induced in aqueous solution the formation of structures resembling the appressoria and infection vesicles formed in the early stages of development of the fungus on the host. Examination of the effects of the distillate on other rust fungi, showed

clear effects, ranging from inhibition to stimulation, in all cases.

Forsyth (1955) identified the germination inhibitor produced by the uredospores of _P. graminis tritici_ as trimethylethylene. He showed that cyclohexene has similar effects. In the light of these results, it appears that Bailey may have been correct in claiming, as already noted, that substances inhibiting germination of _P. helianthi_ were absorbed by paraffin oil.

The chemical properties of the stimulating fraction from uredospores of _P. graminis var. tritici_, detected by Allen (1957), have been studied by French, Massey and Weintraub (1957). They found the stimulant neither acid nor alkaline, highly thermostable, not affected by reducing agents, but destroyed by oxidising agents. Other tests indicated the absence of glycol or amino groups, and the probable presence of an olefinic bond. Two or more active substances appeared to be present, one with a carbonyl and one with a hydroxyl function. Germination was also stimulated to some extent by ethylene, furfuryl alcohol, furfurylic acetate, 2-4-dinitrophenol and levulinic acid. These workers concluded that the stimulator operated by overcoming the endogenous germination inhibitor. In a later paper, French and Weintraub (1957), the stimulant was identified as pelargonaldehyde (n-nonanol). They reported that pelargonaldehyde stimulated germination and induced the formation of structures resembling the appressoria and vesicles formed on infection of the host by these uredospores and at concentrations corresponding closely to those at which the natural spore distillate produced all these effects. These workers suggested that pelargonaldehyde may arise by autoxidation of fat, which is known to be

present in the spore.

Although the above account relates to spores of species other than _A. campestris_, some of the effects reported may be analogous to the germination stimulation phenomenon in _A. campestris_. It is possible that one of the volatile materials, other than carbon dioxide, already detected in the presence of the mycelium may be the stimulant. Since the methods of detection used by these workers were somewhat empirical, it is probable that other, undetected, volatile metabolites were also present.

Methods.

The further study of the volatile metabolite from _A. campestris_ mycelium involved the design of apparatus in which spore germination could be studied in varied aeration conditions. Such an apparatus seemed simple to design in theory, but many problems arose in practice, which involved very many months of work for their solution. The following account of the method is therefore presented in two parts:

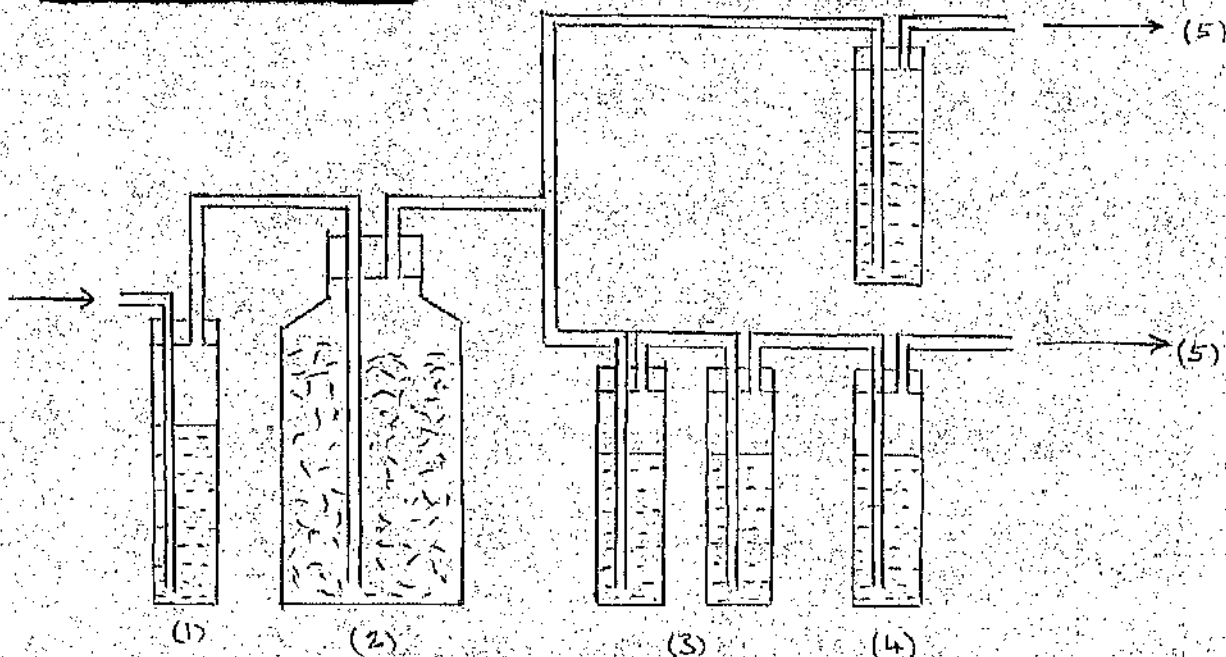
(a) Description of the apparatus finally adopted.

(b) Discussion of the problems involved in its design.

(a) The apparatus used for these tests is shown in text figure 2. Mycelium was grown in 1 litre flasks on wheat grains which had been boiled until softened, mixed with powdered chalk to maintain a suitable pH, then autoclaved. Air from a cylinder of compressed air was passed through this apparatus after any preliminary treatment involved in each experiment. In all these experiments, the solution in the carbon dioxide absorption tubes

was renewed frequently, and the carbon dioxide concentration in the airstream checked at intervals by means of the Haldane Gas Analysis apparatus.

Figure 2. Apparatus for tests of spore germination in air from cultures of mycelium.



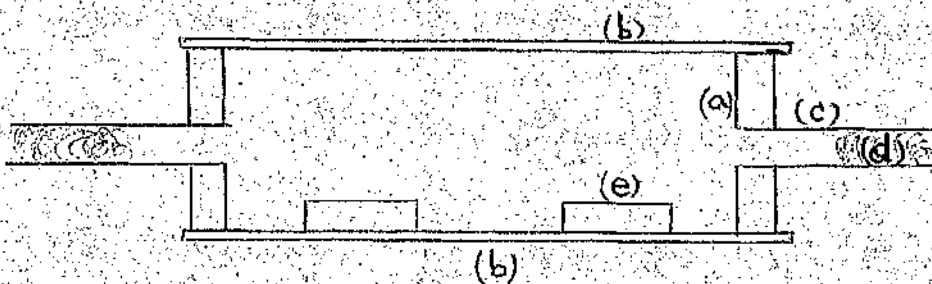
(1) Water to humidify air entering culture flask and to provide a rough check of the rate of air flow.

(2) 1 litre flask containing a four week old culture of A. campestris mycelium on autoclaved wheat grains.

(3) 40% potassium hydroxide solution for absorption of carbon dioxide.

(4) Water to humidify air which has passed through strong alkali.

(5) A series of aeration chambers. The structure of a single chamber is shown in diagram below: For description see text p. 85.



The type of germination chamber used (text figure 1) was a modification of the Van Tieghem cell. It consisted of an aluminium ring (a), two inches in diameter, half an inch in depth and one eighth of an inch wall-thickness, with glass discs (b) for lid and base. Two short lengths of copper tubing, a quarter of an inch in diameter, screwed into the wall at opposite sides (c), allowed aeration of the chambers. Half-inch cotton-wool filters (d) were placed in the side arms. The glass lids and bases were sealed to the aluminium rings by means of paraffin wax and the outside of each junction covered with a thin layer of vaseline to maintain an air-tight seal. Agar discs (e) were placed on the base of each cell and inoculated with spore suspension.

(b) Problems involved in design of apparatus.

(i) Supply of mycelium.

The mycelial cultures used in initial experiments were grown on a layer of malt agar lining the walls of large boiling tubes. Growth was very poor in these tubes, both in conditions of continuous aeration and with intermittent aeration when the tubes were closed with cotton-wool plugs between treatments. The fungus was also found to grow poorly on malt agar lining two-foot lengths of 1" diameter glass tubing.

In further experiments, air was passed through two-foot lengths of nylon tubing, two inches in diameter, packed with well grown cultures of mycelium on wheat grains. In these, also, the mycelium soon died, possibly due to an inadequate rate of aeration.

Finally, flasks containing cultures of mycelium on autoclaved, boiled

wheat grains were used in order to obtain large volumes of the gases given off by the mycelium and since the open structure of this medium should allow efficient aeration. Here also, however, the mycelium tended to die when cultures were sealed and aerated. In order to overcome this difficulty, the time during which such a culture was subjected to these experimental conditions was limited by changing the culture aerated several times during an experiment.

(ii) Choice of germination chambers.

The type of germination chambersdesired for this work was one which allowed both efficient aeration of spore cultures and convenient observation of germinating spores. The first type used consisted of boiling tubes (three quarters of an inch wide) with agar discs on the side inoculated with spore suspension, the tubes being sealed by a rubber stopper carrying a long inlet- and short outlet-tube. No germination occurred in these and it was later shown that the presence of rubber inhibits germination of these spores.

Similar tubes, without rubber stoppers, were next used, closed this time by a cotton wool plug carrying a long inlet-tube, air being allowed to escape through the plug. In these, however, rapid and unequal drying of discs took place, so that the variation in results was too great to permit any comparisons.

Fairly satisfactory results were obtained by a method similar to that already described in the section on effects of carbon dioxide concentrations on germination, using bell-jars as aeration chambers and placing in them petri dishes bearing inoculated agar discs on the lids and with the lids

supported by a wire triangle in order to allow free aeration of the spores. When the plates were removed for observation, the wire support was simply withdrawn, allowing the lid to fall into its usual position. Limitations of apparatus, however, made it difficult to secure adequate replication with this method. The Van Tieghem type of germination chamber, already described, was therefore developed and found convenient for studying spore or mycelial cultures in controlled conditions of aeration. Application of this method was greatly impeded by difficulties of finding suitable materials for sealing the aeration cells. Vaseline was found too soft at incubator temperatures to keep the cells sealed when air was passing through under slight pressure. Ester wax provided a strong glass to metal seal but no germination occurred in apparatus sealed with this wax. Plasticene was a convenient sealing agent but was also found to inhibit germination. On the basis of tests of various sealing agents, described in the following section, paraffin wax was finally chosen for sealing the cells.

(iii) Tests of materials suspected of affecting spore germination.

Methods.

Each substance to be tested was placed in the bases of a set of five petri dishes, each bearing on the lid five agar discs inoculated with spore suspension. Water was added to the bases of all the chambers including the control petri dishes to maintain uniform humidity. Germination in these conditions was compared after ten days of incubation at 24°C.

Effects of the presence of various sealing agents on germination.

Germination in the presence of equal amounts of vaseline, ester wax, paraffin wax and plasticene was compared with that in control conditions by the method given above. Results, recorded in table 21 of appendix, were as follows:

Material present	Mean germination per 5,000 spores
Paraffin wax	222
Ester wax	0
Vaseline	124
Plasticene	51
Water	177

Complete inhibition of germination occurred in the presence of ester wax and analysis of the results showed that there was a significant reduction of germination in the presence of plasticene, but not in the presence of vaseline or paraffin wax.

Effects of presence of rubber on germination.

Spore germination in the presence of autoclaved, chopped rubber was compared with that in control conditions by the method given above. Results, recorded in table 22 of appendix, were as follows:

Material present	Mean germination per 5,000 spores
Rubber	0
Water	280

The results show without analysis that germination was inhibited by volatile material from the rubber. It appears, therefore, that the absence of germination in tubes closed with rubber stoppers (cf. para.(2)) may have been due directly to the presence of rubber.

1. Study of the influence of carbon dioxide on germination of A. campestris spores.

This work was carried out with the intention of finding whether the stimulation of A. campestris spore germination by living mycelium could be due to the action of carbon dioxide evolved by the mycelium. When this primary purpose was fulfilled, further study of the effects of carbon dioxide on germination in this species was postponed.

The examination of the relationship of carbon dioxide to the problem of stimulation of germination was carried out in two parts:

(a) Study of germination in air which originally contained the germination stimulant but which had passed through carbon dioxide absorbent before reaching the spores.

(b) Study of germination in atmospheres of enriched carbon dioxide concentration.

The results of both parts are discussed together at the end of this investigation.

Experimental.

(a) A preliminary experiment was first carried out to find whether it was possible to carry the stimulatory metabolite from A. campestris in an airstream passing over the spores, for experiments in part (a), and also to find what levels of carbon dioxide were presented to the spores by such an airstream and hence which concentrations of carbon were likely to be important in part (b) of the investigation.

(i) Preliminary examination of properties of air passing from a culture of A. campestris mycelium.

Air from a cylinder, after passing through a large bottle containing mycelium of A. campestris growing on wheat grain, was passed into a bell-jar enclosing petri dishes bearing the spore cultures. A similar container was aerated with air from a similar bottle in which the mycelium had been killed by autoclaving. The carbon dioxide levels of the air from the mycelium were measured by means of the Haldane Gas Analysis apparatus.

Germination on the seventh and tenth days of the experiment is recorded in table 23 of appendix, together with the percentage of carbon dioxide present. The results may be summarised as follows:

Airstream	Day	Mean germination per 5,000 spores	Mean % CO_2
From mycelium	7	12.7	13%
	10	1%	4%
Control	7	0	0.03%
	10	0.7	0.03%

Without analysis, it was clear that spore germination was stimulated in air passing from living mycelial cultures and this air had a greatly increased concentration of carbon dioxide.

It was concluded that concentrations of carbon dioxide of the range 4% to 13%, detected here in the stimulatory airstream, might be of interest in part (b) of this study.

(ii) Effects on germination of passing air bearing the germination stimulant through carbon dioxide absorbent before it reaches the spores.

The first experiment below was carried out by a method similar to that above. One bell-jar containing spore cultures was aerated with air; the second with air which had passed through a bottle containing a culture of A. campestris mycelium, and the third with a similar airstream which had passed through solutions of 40% potassium hydroxide and N/20 baryta. Each airstream was bubbled through water before passing over the spores.

The above method did not, however, allow sufficient replication for adequate analysis. In order to obtain more complete results, therefore, this problem was studied using the apparatus described in text figure 2. Series of four cells were aerated with each airstream being tested.

The results of the experiment carried out by the first method, (Experiment 1) are recorded in table 24 of appendix and those of three experiments carried out by the second method, (Experiments 2, 3, and 4), are recorded in Table 25. The results of all these experiments are summarised in text table on page 93.

Conflicting results were obtained in these experiments. The finding of inhibition, instead of stimulation, in air from the A. campestris mycelium in experiments 2 and 3 suggested that the character of the mycelial emanations was altered in these conditions.

Effects of removal of carbon dioxide from air from cultures of mycelium.

Summary of results.

Source of air-stream.	Treatment	Experiments			
		1	2	3	4
Air	Untreated	O	++	+	++
	CO_2-free		+	O	O
Agaricus campestris	Untreated	+++	+	O	+++
	CO_2-free	+++	+	+++	O
Hygrophorous virgineus	Untreated			++++	
	CO_2-free			+++	
Saccharomyces cereviseae	Untreated				O
	CO_2-free				O

In this summary the results are recorded qualitatively, since numerical values are unsuitable for comparing several experiments.

O = no germination.
+ = very poor germination.
++ = germination.
+++ = stimulation.
++++ = strong stimulation.

It was therefore decided to extend this study to other species which had, already, been shown to stimulate germination, in the hope that these might be less affected by the experimental conditions and might be less affected by the experimental conditions and might yield analogous results. The species chosen were _Hygrophorous virgineus_, in experiment 3, and _Saccharomyces cerevisea_, in experiment 4. The former was grown on malt agar and the latter in 5% glucose solution.

The conflicting results of these experiments indicate the possibility of interaction of more than one factor in the processes stimulating germination. For example, in experiment 4, there was no germination in cultures aerated with air from which carbon dioxide had been removed, while in other experiments, removal of carbon dioxide from air from cultures of _A. campestris_ mycelium either had no apparent effect, or was associated with a slight reduction in germination or (in experiment 3) was even associated with a marked stimulation to germination. The results suggest a number of interesting lines for further examination. The object of this work, however, was simply to determine whether or not there was a direct correlation between the presence of carbon dioxide and the stimulatory effect. The results give no indication of this and the effect of increased concentrations of carbon dioxide in part (b) below supported this opinion. It was therefore decided that the immediate object of the work was attained by these experiments and that further study of the interesting accessory problems would be postponed as they were irrelevant at the moment.

(b) Effects of increased concentrations of carbon dioxide upon germination of Agaricus campestris.

Two experiments were set up for this purpose. In the first one, adequate replication was possible, but the concentrations of carbon dioxide could only be determined at the beginning and end of the experiment. In the second experiment, satisfactory controlled conditions were established, but limitations of apparatus at the time prevented it from being set up with adequate replicates for conclusive analysis. This experiment fulfilled its purpose in indicating that carbon dioxide did not stimulate germination. It showed also that a full-scale experiment could be set up on these lines but this was not carried out since the information already gained by this stage made it clear that further study in this direction was irrelevant to the aim of the present work.

(i) Eight small (500 ccs.) bell-jars, standing on glass plates and closed at the top by a glass disc, were sterilised and in each was placed a slide bearing four discs of water agar inoculated with spore suspension. The bases and covers were sealed to the bell-jars with plasticene. A calculated volume of carbon dioxide was passed into four of the bell-jars through the top opening to give a concentration of 20% carbon dioxide, and the containers again sealed. Initial and final concentrations of carbon dioxide in all the bell-jars were measured by means of the Haldane apparatus.

Germination on the eighth day, together with the carbon dioxide concentration in each container at this time, is recorded in appendix table 26, and summarised below:

% Carbon dioxide		Mean germination per 5,000 spores
Initial	Final	
20%	(0.16 - 12)%	3
0.03%	0.03%	50

Without analysis it was clear that inhibition of germination had occurred in the presence of the higher concentration of carbon dioxide. As was expected with this type of gas chamber, the level of carbon dioxide concentration fell considerably during the experiment. The results indicated, however, that even the concentrations of carbon dioxide finally recorded may inhibit germination. From this experiment, therefore, it was concluded that further studies of the effect of carbon dioxide on spore germination should be carried out in a lower range of concentrations of carbon dioxide. This was carried out by the second apparatus, in which more precisely controlled conditions could be maintained.

(ii) Germination in 1% and 0.1% carbon dioxide was compared with germination in normal air by a method similar to that of Brown (1922). Sets of three petri dishes, each having on the lid three water agar discs inoculated with spore suspension, were placed in three similar, sterile bell-jars. The lid of each petri-dish was supported by a triangle of copper wire placed on top of the base, so that the atmosphere of the bell-jar was continuous with that of the petri dishes, while the discs could be examined without contamination by merely withdrawing the wire support and allowing the lid to fall over the base into its normal position. Each bell-jar was sealed to its glass base by means of vaseline and was closed

at the top by a rubber stopper carrying a glass tube.

One bell-jar contained normal air and the atmospheres in the other two containers were adjusted to 0.1% and 1%, respectively, by the method of Kidd (1914), the pressure in the bell-jar, as indicated by a mercury manometer, being reduced by a calculated amount and carbon dioxide from a cylinder being introduced, until normal pressure was again reached. The bell-jars were incubated at 24°C, the carbon dioxide concentration being checked at intervals and adjusted when necessary. The carbon dioxide level in these containers was found to remain constant within limits of ± 0.1%.

Germination on the tenth day is recorded in table 27 of appendix, and may be summarised as follows:

% Carbon dioxide present	Mean germination per 5,000 spores
Atmospheric	333
0.1%	8
1%	6

Empirical observation indicated inhibition of germination by concentrations of carbon dioxide of 1% and 0.1%. This experiment was carried out with inadequate replication for analysis but it confirms earlier, less conclusive, experiments by finding no indication of stimulation of germination of A. campestris spores by simple increase of the atmospheric concentration of carbon dioxide.

Discussion.

From the evidence of both parts of this study, it appears that carbon dioxide is not the germination stimulant of A. campestris spores evolved by living mycelium. The conflicting results in the series of experiments carried out in part (a) indicate a complex interaction of factors which will justify analysis by more accurately controlled experiments. Some of the results observed appear to be due primarily to the response of the test mycelium to the experimental conditions. It is possible that, in the conditions prevailing in the aerated containers in these experiments, A. campestris mycelium may no longer produce the stimulant or may evolve it in concentrations unfavourable to the spores. Detailed discussion of these changes is, however, irrelevant here.

In work recorded in section (b), inhibition of germination was found consistently, even at the lowest concentrations tested. It was obviously unlikely that carbon dioxide could stimulate germination. The concentrations of carbon dioxide detected in air from A. campestris mycelium in the preliminary experiment of this section are of interest in this connection. It appears from them that concentrations of carbon dioxide which are normally inhibitory no longer exert the same effect in the presence of A. campestris. This raises the possibility of interaction between the effects of the volatile germination stimulant and carbon dioxide; a possibility which may be of interest for later investigation.

Various other lines of evidence exclude carbon dioxide as a possible germination stimulant in A. campestris. If carbon dioxide was the stimulant, it is most likely that it would affect germination through its influence on

the pH of the medium, as was found by Platz et al (1925) in their work, already mentioned, on the stimulation of germination of _Ustilago zeae_ by carbon dioxide. As has been shown, however, in an earlier part of this thesis, changes in pH of the medium, of the range which might be induced by carbon dioxide, did not increase germination to an extent at all comparable with that induced by the presence of mycelium of _Agaricus campestris_ or by medium in which it has been grown. Further, pH measurements in studies of germination on medium from agar cultures of _A. campestris_, recorded in an earlier part of this work, showed that both the culture medium, in which germination had been stimulated, and control medium, which showed no stimulation, had a pH of 5 at the time of recording of the results. The finding that the germination stimulant volatilises from medium in which mycelium of _A. campestris_ has been grown and can affect germination of spores at some distance from it, provides further evidence against carbon dioxide being the active material. The carbon dioxide concentration in such culture medium must quickly come to equilibrium with the atmospheric concentration of carbon dioxide and is unlikely to cause the stimulation of germination which is observed after one week's exposure of the spores to the presence of the solution.

The results in this section were thought to be sufficiently clear to justify the search for other stimulatory substances in the emanations from the mycelia. It was originally proposed to complete this study of the effect of carbon dioxide simultaneously with the other studies. When convincing evidence of the presence of other factors was obtained, however, this work was temporarily stopped.

2. Examination of methods of isolating the stimulating material.

(a) Distillation.

As a preliminary to the examination of the possibility of separation of the stimulant by distillation from medium in which mycelium of Agaricus campestris had been growing, the heat stability of the material was examined.

(i) Study of the effect of heat on the germination stimulant in medium from cultures of A. campestris.

This problem was examined with spores placed directly on agar culture medium and also with spores held in the presence of, but not in contact with, liquid culture medium. In both cases the effects on germination of medium removed from A. campestris cultures was compared with those of similar medium which had been kept at 100°C for 1 hour, or which had been autoclaved at 10 lbs pressure for 20 minutes, and also with the effects of control medium in which mycelium of A. campestris had not been grown.

In the first experiment, drops of spore suspension were placed on discs of each medium tested, on the lids of eight petri dishes, each of which contained two discs of each of the four media. The culture medium used in this experiment was 2% malt agar from petri dishes in which mycelium had been grown for one month on cellophane placed over the agar surface. At the end of this time the cellophane was removed aseptically and the medium melted and removed for treatment.

In the second experiment, spores were inoculated on agar discs on the lids of petri dishes and exposed to test solutions placed in the bases of the petri dishes. Aliquots of media of each treatment were placed in the

bases of sets of six petri dishes. The medium for this experiment was removed from cultures of A. campestris in 2% malt extract solution.

Germination in experiment 1 is recorded in table 28 of appendix and that in experiment 2 in table 29 of appendix. The results are summarised below:

Experiment	Medium	Treatment	Mean germination per 5,000 spores	
			7th day	
1 Spores in contact with test-medium.	Malt agar from cultures.	Melted	436	
		1 hour at 100°C	127	
		Autoclaved	210	
	Uninoculated malt agar.	Melted	2	
			6th day	11th day
2 Spores in presence of test-medium.	Malt solution from cultures.	Untreated	243	4,000
		1 hour at 100°C	0	205
		Autoclaved	0	500
	Uninoculated malt solution.	Untreated	0	242

From empirical observation of the results of the first experiment, it was clear that stimulation of germination has occurred on all media from cultures of A. campestris. Analysis of variance showed, however, that heat treatment led to a significant reduction in the amount of stimulation induced by the culture medium. In the second experiment, only spores in the presence of untreated culture medium showed stimulated germination on the sixth day but, on the 11th day, analysis of variance showed significantly

better germination in the presence of autoclaved culture medium than in the presence of control medium.

Thus, while these experiments show loss of the germination stimulant from culture medium on heat treatment, they suggest that the substance is moderately thermostable. Sufficient material remains after heat treatment to stimulate germination of spores in contact with the treated culture medium. Slower stimulation is shown by spores in the presence of, but not in contact with, autoclaved culture medium and none in the presence of medium kept at 100°C for 1 hour. The greater effect of boiling than of autoclaving in this case may, possibly, be due to the greater length of the boiling treatment, resulting in more complete removal of the stimulant.

(ii) Distillation of culture medium.

Simple distillation of samples of medium in which *A. campestris* mycelium had been growing was carried out at 35°C and at 70°C, the vapour being passed through ice-cooled receivers. No distillate was, however, obtained by this method. The vapour from the culture medium was also passed through water in similar, cooled receivers, but no stimulation of germination was shown in the presence of this water.

It was concluded that a much more efficient collection system would be required to trap the volatile material from culture medium. The material was probably too volatile to accumulate in a simple ice-cooled condenser.

(b) Ether extraction of culture medium.

25 ccs. of ether were added to a culture of A. campestris mycelium growing on 200 ccs. of liquid medium. The mixture was shaken and left for several hours and the mycelium then removed by filtration. More ether was added to the filtrate and the ether fraction separated from the residue and allowed to evaporate to dryness, then made up with water to 100 ccs. Both the ether fraction and the residue were left overnight at 56°C to allow all traces of ether to evaporate.

Aliquots of the ether extract, the residue, untreated culture medium of similar origin, and control malt solution, were placed in the bases of four sets of five petri dishes, each bearing on the lid three agar discs inoculated with A. campestris spores. Germination on the tenth day of the experiment, recorded in table 30 of appendix, showed the following results:

Solution present		Mean germination per 5,000 spores
Culture solution	Untreated	325
	Ether fraction	0
	Residue	136
Control solution		0

Stimulation of germination occurred in the presence of untreated culture medium and of the residue but the ether extract exhibited no activity. Without analysis it appeared that the treatment had resulted in only slight loss of activity by the residue.

It was concluded that the germination stimulant in medium in which mycelium has been growing is not readily soluble in ether, and is unlikely to be isolated by this method.

(c) Isolation of the stimulating material from air which has passed over A. campestris mycelium.

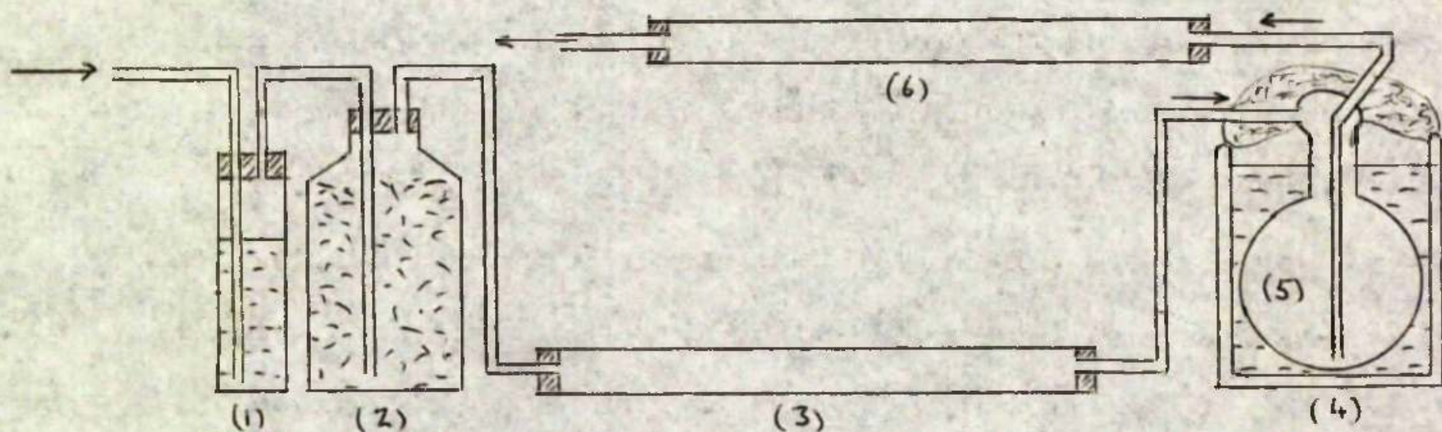
The apparatus used in this work is illustrated in text figure II. Air passing from cultures of A. campestris mycelium was dried with phosphorous pentoxide and passed through a receiver cooled in liquid air. A crystalline deposit collected in the receiver in 24 to 72 hours.

A solution of this material in distilled water was prepared by adding 50 ccs. of ice-cooled distilled water to the cooled receiver and allowing the solution to come slowly to room temperature. This procedure was adopted to allow maximum solubility of the volatile substance. 5 ml aliquots of this solution were placed in the bases of ten petri dishes, each bearing on the lid two agar discs inoculated with spore suspension. Distilled water was placed in the bases of a number of similar petri dishes. Germination after seven days, recorded in table 31 of appendix, gave the following totals:

Solution present	Total germination per 5,000 spores.
Volatile material	173
Water	0

It was clear, from empirical observation, that the material collected for analysis contained the germination stimulant.

Fig. 3. Apparatus for collection of volatile material from living cultures of Agaricus campestris mycelium.



(1) Water to humidify air stream and provide a rough check on the rate of flow

(2) Culture of A. campestris mycelium on wheat grain.

(3) Drying tube (2 feet x 1 inch) lined with phosphorous pentoxide.

(4) Dewar flask containing liquid air and covered with cotton wool to reduce evaporation.

(5) "Quickfit" collecting flask; air passes in the direction indicated. (when reverse direction was used, material tended to solidify in long tube, blocking the inlet.)

(6) Drying tube to remove water vapour from any atmospheric air sucked back to cooled receiver.

3. Chemical analysis of volatile metabolites.

(a) Volatile material from *Agaricus campestris* mycelium.

Mass spectrometric analysis of the material collected by the method described in the last section from air passing from cultures of *A. campestris* mycelium, was carried out by Dr. R. I. Reed, of the Chemistry Department, Glasgow University.

A seven-carbon olefin was found to be present. This was confirmed by analysis of four further samples, from different cultures, and the compound identified as 2:3-dimethyl-1-pentene. No other compound was detected in the material by this method of analysis.

(b) Volatile material from *Saccharomyces cereviseae*.

If the above olefin is found to be the germination stimulant evolved by *Agaricus campestris* mycelium, it is likely that other fungi which have shown this type of stimulation of *A. campestris* spores may produce the same or similar compounds. A further analysis was carried out at the same time as the above series to find whether one of these other fungi evolved such a substance. *Saccharomyces cereviseae* was chosen for this purpose, since sufficient material of active cultures of this organism was readily prepared.

Mass spectrometric analysis of material isolated by the above method from air passing from a culture of *S. cereviseae* growing in 5% glucose solution, showed that a five-carbon olefin was present. This was identified as 3-methyl-1-butene.

Discussion.

Since no substance other than 2:3-dimethyl-pentene has been found in material isolated in this way from air from A. campestris cultures, and since this material showed strong volatile stimulation of germination, it appears likely that this olefin is the stimulant.

The finding that Saccharomyces cerevisiae, which, like A. campestris, stimulates germination of A. campestris spores, also evolves an olefin, tends to support the idea that the olefin is the stimulant but suggests, also, that the reaction may be common to a variety of compounds of this class.

It is possible, but unlikely, that a trace of some other substance present in the material may have been overlooked. It is therefore necessary to test the effects of synthetic material to determine whether the above substances can stimulate germination of A. campestris spores. It has not yet been possible, however, to obtain a sample of 2:3-dimethyl-1-pentene for this purpose. A small sample of 3-methyl-1-butene has recently been obtained from British Hydrocarbons, Ltd., Grangemouth, in order to test its effects on germination. The low boiling point (25°C) of this very volatile liquid makes it difficult to handle and to maintain as suitable concentrations in the presence of the spores for a sufficient time to affect germination. Methods for testing it are being examined, but it is thought that, because of its higher boiling point (84°C), 2:3-dimethyl-1-pentene should be more easily handled in germination tests.

The identification of olefins in volatile material from A. campestris and Saccharomyces cerevisiae confirms the suggestion of Mader (1943) that an unsaturated hydrocarbon gas is evolved by mycelium of A. campestris and

the report of Gane (1935), who showed that the presence of aerobic yeast cultures inhibited the growth of etiolated seedlings, a response characteristically induced by these gases.

The possibility that the various fungi which stimulated these spores in the same way as A. campestris may also evolve olefins is supported by the identification by Fergus (1944) of "ethylene" in emanations from Penicillium digitatum. Such speculations are of interest in relation to the many previous reports of the physiological activity of olefins and of their evolution by plant organs. It is possible also that the apparently unrelated stimulation of these spores by orchid stigmatic mucilage (Kehl, 1943) could be a similar effect, since Denny (1935) demonstrated the production by "pistils" of various species of emanations which induced "leaf epinasty", a response used frequently to detect low concentrations of hydrocarbon gases.

Only one previous report of evolution of a higher olefin from plant material has been found: the spectrometric identification of trimethylethylene as the endogenous germination inhibitor of uredospores of Puccinia graminis var. tritici, (Forsyth, 1955). Although the olefins detected in plant emanations have been generally identified as ethylene, the chemical tests used were not always specific for ethylene and would have given positive results with other olefins (e.g. Young, Pratt and Biale, 1952). It is possible therefore that evolution of these substances is a more general effect than was previously realised.

Conclusion.

It has been shown in Part 2 that the stimulation to germination of <u>A. campestris</u> spores by living mycelium is due to a volatile material diffusing into the air and into the culture medium and that the effect is not confined to <u>A. campestris</u> mycelium.

Volatile material was isolated from air which had passed over <u>A. campestris</u> mycelium and its germination stimulating activity demonstrated. Since analysis of this material showed the presence of an olefin and no other substance, it was concluded that this was the germination stimulant evolved by <u>A. campestris</u> mycelium. Some consideration has been given to the implications of these results.

Part 3.

Effects of various factors on growth of Agaricus campestris mycelium.

PART 3. EFFECTS OF VARIOUS FACTORS ON GROWTH OF AGARICUS CAMPESTRIS.

1. Effects of buffer on growth in liquid medium.
2. Effects of different amounts of inoculum on growth in liquid medium.
3. Effects of malt concentration on growth in liquid medium.
4. Effects of presence of rubber on growth on solid medium.
5. Effects of aeration on growth on solid medium.
6. Effects of medium containing metabolic products from *A. campestris* mycelium on growth of mycelium of this species.

Part 3. EFFECTS OF VARIOUS FACTORS ON GROWTH OF AGARICUS CAMPESTRIS.

This part of the thesis comprises a number of investigations which arose in connection with germination studies recorded in Part II. The primary aim of this work was to find suitable methods for growing *A. campestris* mycelium in liquid medium in order to obtain quantities of culture solution containing the germination stimulant, for tests of the properties of the stimulating material and with a view to its possible extraction from this medium. At the same time some preliminary investigation was made of the unfavourable response of *A. campestris* mycelium to aeration, noted in Part II, Section III.

This work was designed primarily to assist in the examination of the germination stimulation phenomenon but some of the results obtained are of sufficient intrinsic interest to merit further study. At present, however, each investigation has been carried only to an extent useful to the germination studies which occupy the rest of this thesis.

1. *Effects of buffer on growth in liquid medium.*

Preliminary experiments showed rather poor growth of *A. campestris* mycelium in unbuffered malt solution. The following study was undertaken in order to find whether it was necessary to buffer the solutions in which mycelium of *A. campestris* was to grow and also to find the most satisfactory means of buffering solutions in the present work.

Frear, Styer and Haley (1928), in one of the few early studies on growth of mycelium of *A. campestris* in pure culture in synthetic liquid media, found growth best in cultures of pH near to 6.0. They adjusted the pH by addition of sulphuric acid and potassium hydroxide solution, but do not appear to have buffered the media. They found that cultures with the greatest growth changed the medium to a marked extent. Lambert (1938) stated that growth on compost can take place in a range of pH's from 3.4 to 9.0 but that the optimum varied with the medium. Treschow (1944), carrying out a detailed study of the effect of pH on growth in solution, showed that the mycelium was very sensitive to phosphate concentration. He used strong buffer solutions to prevent any shift in pH but found that no growth occurred at phosphate concentrations greater than 0.066 M. The effect of the phosphate varied with the pH of the medium. Treschow recorded optimum growth at a pH of 6.8, with a phosphate concentration of 0.0066 mol., but at this low concentration of buffer, the pH could not be kept constant. He states, however, that there was no considerable acid or base formation by the mycelium.

Throughout the work recorded in this section, the cultures of *A. campestris* mycelium were grown in solutions at the pH 6.8, which was

recorded as optimum by Treschow (1944).

Methods.

In this and the succeeding experiments, cultures were grown in 100 ml. conical flasks, each containing 30 ml. of culture medium, usually 2% malt extract solution. The solutions were inoculated with standard discs, ½ cm. in diameter, cut from petri dish cultures of A. campestris mycelium on 2% malt agar. The temperature of incubation of all cultures was 24°C.

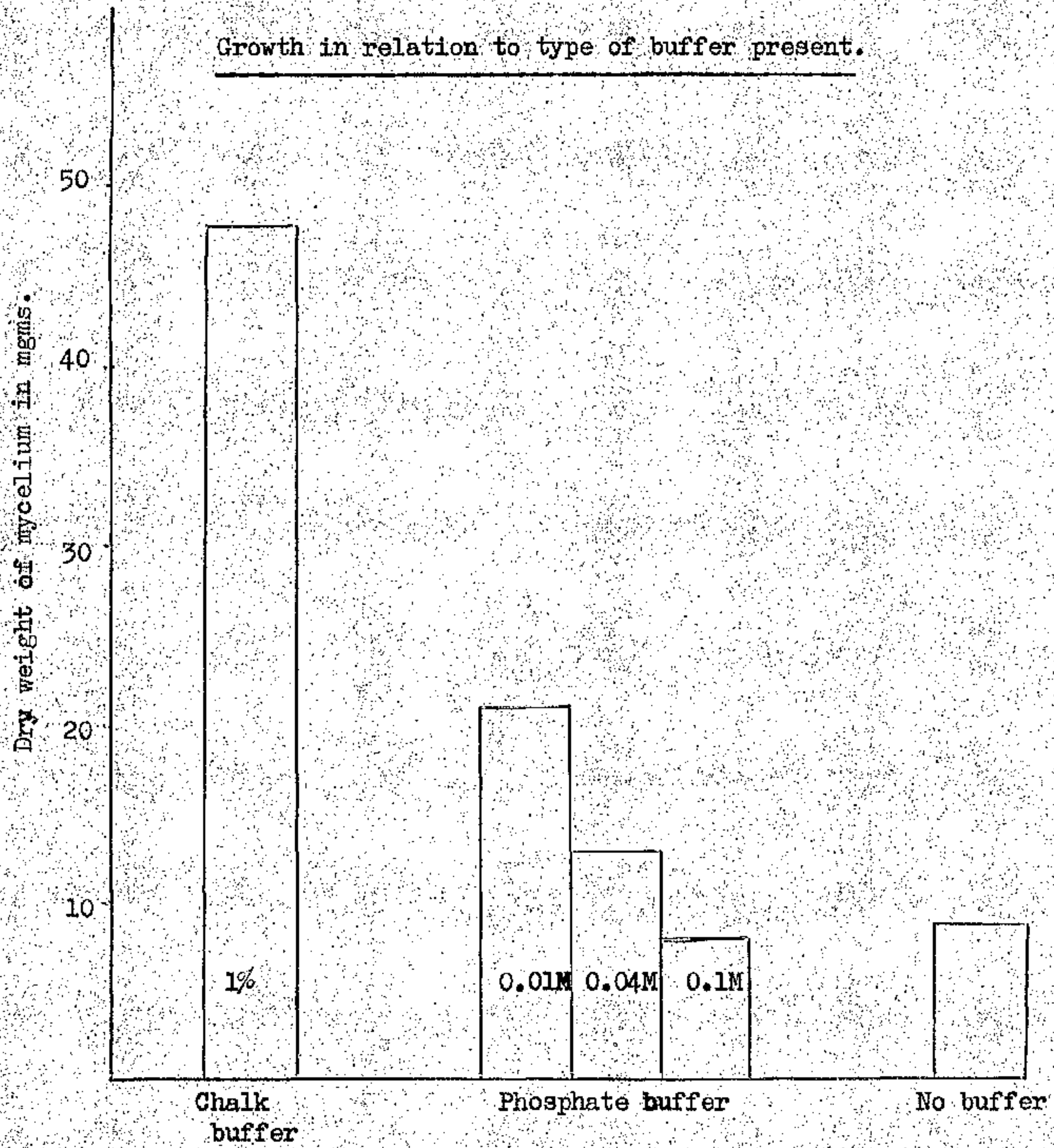
Growth was measured by the dry weight of mycelium produced in a fixed time, usually one month. This period was chosen since it allowed an easily measurable amount of growth to take place without exhaustion of the food supply or apparent staling of the medium. Dry weights were determined by the following standard procedure:
As much as possible of the culture medium was poured from the flask and the mycelium washed in several changes of water to remove traces of culture medium. The mycelium was then filtered onto weighed filter paper in a Buchner funnel. The filter paper, plus mycelium, was air dried in an oven at 95°C until constant weight was obtained and the dry weight of the mycelium recorded. Where chalk was used to buffer culture solutions, this had to be removed before the mycelium could be weighed. For this purpose, a standard volume of concentrated hydrochloric acid was added to each flask and left until all the chalk had disappeared. The mycelium was then again washed before filtering and drying.

Experimental.

Growth of mycelium was compared in 2% malt extract solutions buffered with 0.01, 0.04, and 0.1M phosphate, with 1% powdered chalk, and unbuffered.

Seven flasks containing each medium were inoculated with A. campestris mycelium and the dry weights of mycelium produced were compared after one month's growth. The results are recorded in table 32 of appendix and summarised in histogram (p. 115).

Empirical observation showed best growth in solutions buffered with 1% chalk, while unbuffered solutions gave only very poor growth. Growth varied inversely with the concentration of phosphate in solution, being extremely poor in solutions containing 0.1M phosphate. Even 0.01 M phosphate resulted in less than half as much growth as in chalk-buffered solutions.


Growth in relation to type of buffer present.
Dry weight of mycelium in mgms.
50
40
30
20
10
1%
0.01M
0.04M
0.1M
Chalk buffer
Phosphate buffer
No buffer

Discussion.

From this work it is clear that much better growth is obtained by buffering solutions with chalk than with phosphate as studied by Treschow. The results agree in general with Treschow's conclusions with respect to the effect of phosphate concentration on growth. He found, however, that the concentrations which gave optimum growth were not sufficient to maintain a constant pH.

The very much better growth found in solutions buffered with chalk than in any other conditions may show the favourable effect of the absence of inhibitory concentrations of phosphate and at the same time a suitable pH. In unbuffered solutions, any favourable effect of the absence of phosphate may be masked by an unsuitable pH. It is probable that the growth obtained in the lower concentrations of phosphate represents a balance between the favourable effects of buffering the solution and the unfavourable direct effects of the phosphate ion.

It was not the intention of this present study to carry the investigation of these factors further, but only to find a convenient method of buffering malt solutions so that satisfactory growth could be obtained for other studies. This was achieved by the finding that satisfactory growth could be obtained by use of a chalk buffer.

2. Effects of different amounts of inoculum on growth in liquid medium.

Treschow (1944), in carrying out nutritional studies on A. campestris mycelium in conditions similar to those of the present experiments, normally inoculated culture solutions with 5 mm. squares, 1 mm. in depth, cut from cultures on malt agar 1 cm. in depth. This size of inoculum remained on the surface of the medium and gave rise to a floating colony of mycelium. Treschow found that there was no difference in the dry weight of mycelium produced whether or not submerged growth occurred. He also showed that the position in the culture from which the discs were taken did not affect growth, even though the peripheral areas were younger.

Treschow found that growth increased only slightly with the size of inoculum but was not directly proportional to it. The following experiment was designed to find to what extent variation in the amount of inoculum affected growth in the present case.

Experimental.

Culture solutions in three sets of six flasks were inoculated with one, two or four, respectively, standard discs of inoculum. These were each 6 mm. in diameter and cut from a colony of mycelium on 2% malt agar, 2 mm. in depth.

Dry weights of mycelium after one month's growth are recorded in table 33 of appendix and summarised below:

No. of discs of inoculum.	Mean dry weight of mycelium. (mgms.)
1	75.4
2	73.6
4	89.6

Analysis of variance showed no significant difference in dry weight production from one disc of inoculum and that from two discs. Dry matter production in flasks with four discs of inoculum was, however, significantly better than with one or two discs. It was clear, however, that dry matter production was not directly proportional to the size of inoculum.

This result, which is similar to that of Treschow, shows less marked effect of the size of inoculum on amount of growth than might at first sight have been expected. It is likely, however, that on a high energy substrate such as malt, the "inoculum potential", discussed by Garrett (1954) in relation to strand growth in A. campestris, is not so important as on less readily available media.

3. Effects of malt concentration on growth in liquid medium.

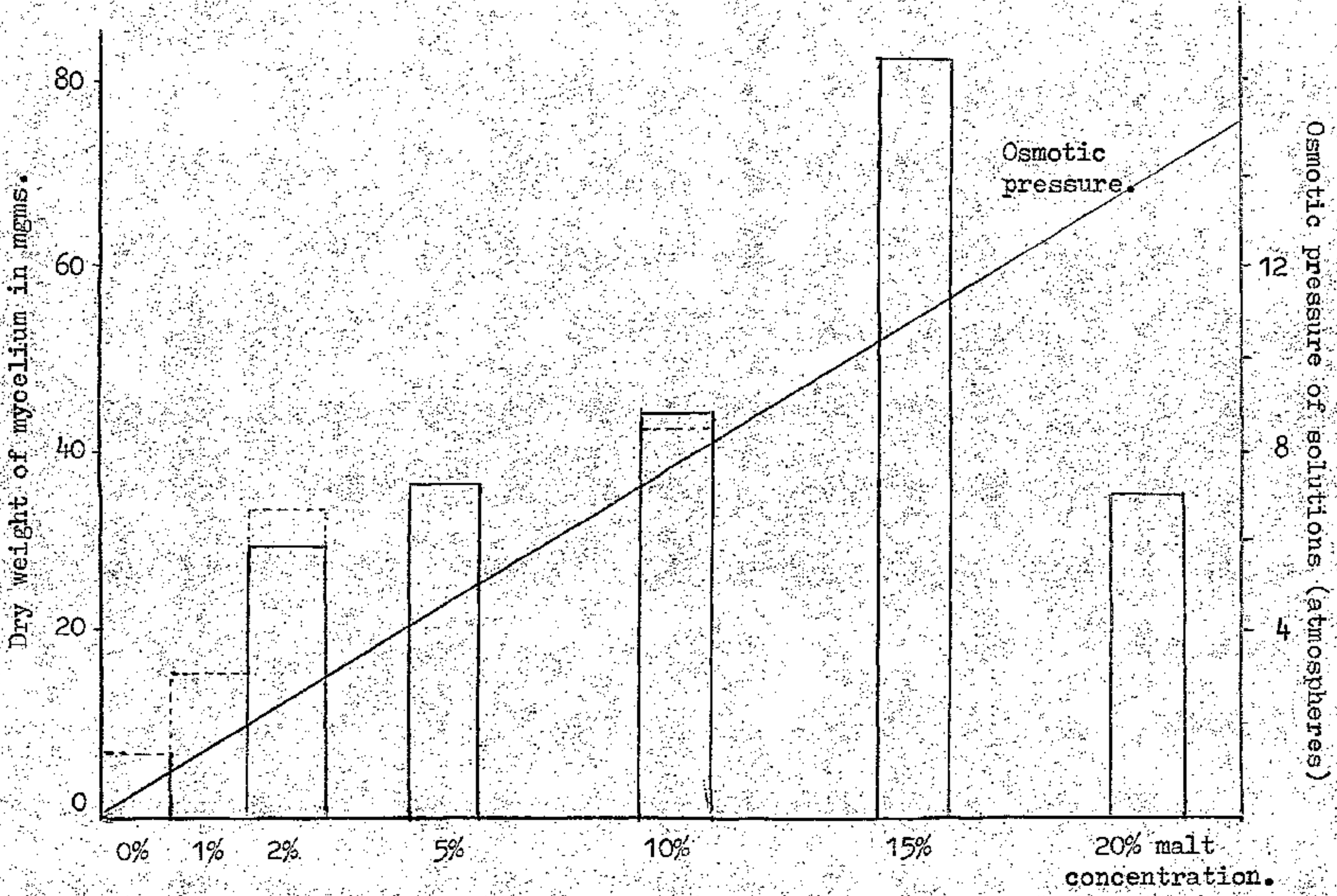
Introduction.

This investigation was carried out in order to find whether better growth of A. campestris mycelium could be obtained in medium of a higher concentration of malt than the 2% malt extract initially used for this work. All routine cultures of this fungus had been grown on 2% malt agar, which was considered to be a complete medium. This medium has been used previously by many workers. Garrett (1954) found better growth of mushroom mycelium on malt extract than on any other common media used. Previous reports do not appear to provide the information on the behaviour of mycelium of this species in higher concentrations of malt. Treschow, studying growth in glucose solution, found growth markedly inhibited in solutions containing 0.2 mol. sucrose and arrested in 0.6 mol. glucose. The optimum concentrations for growth lay between 0.02 and 0.1 mol. Below 0.01 mol., carbohydrate deficiency occurred. He recommended that the total molar concentration of a synthetic medium should not exceed 0.1 M and the concentration of inorganic salts should be kept at a low level.

Experimental.

30 ml. aliquots of each of the solutions to be tested were sterilised in 100 ml. conical flasks and each received a standard inoculum of A. campestris mycelium. All solutions were buffered with 1% chalk. Dry weights of mycelium after one month are recorded in table 34 of appendix and summarised in histogram (p.120).

Growth in relation to malt concentration in solution.



(——) = mean values from experiment (2).

(-----) = adjusted mean values from experiment (1).

Two experiments were carried out by this method:

In experiment 1, growth was compared in distilled water, 1%, 2%, 5% and 10% malt extract solution, and was found to increase with malt concentration throughout the range studied.

In experiment 2, the range of malt concentrations was extended to include 10% and 15% malt solutions. Growth was found to increase with malt concentration up to 15% malt, but at 20% malt, the amount of growth was only approximately equal to that in 5% malt solution.

Discussion.

In the second experiment, it appeared that the optimum concentration of malt for growth of A. campestris mycelium lay around the 15% level. The 20% malt solution was less favourable for growth, probably due to the unfavourable osmotic pressure of this solution. For more accurate determination of the optimum and also of the maximum concentration in which growth of this mycelium can take place, it would be necessary to compare growth in more closely graded solutions in suitable ranges of malt concentration. The information gained in the above experiments is, however, adequate for present purposes.

Determinations of the depression of the freezing point of similar solutions gave the osmotic pressures of the culture solutions, recorded in histogram. It was found that the osmotic pressure of the optimum solution, 15% malt, was approximately 9 atmospheres and that in the 20% malt solution, approximately 12 atmospheres. These are the osmotic pressures of 0.4M and 0.5M sucrose solutions, respectively. Thus, optimum growth in malt solution appeared to occur at a concentration higher than the range (0.01 to 0.1M

sucrose solution) recorded by Treschow as optimum. The reduction of growth in solutions of osmotic pressure 12 atmospheres, however, agrees broadly with Treschow's finding that a 0.06 M solution arrested growth.

4. Effects of the presence of rubber on growth on solid medium.

In part 2, Section III, where spore germination was studied in air passing from cultures of A. campestris mycelium, growth of mycelium was found to be rather poor in containers closed with rubber stoppers and aerated. Since the presence of rubber had already been shown to inhibit germination of spores of A. campestris, it appeared possible that growth of mycelium might, also, be affected by volatile substances diffusing from the rubber. The following experiment was carried out to examine this possibility.

Twenty petri dishes, each containing in the base malt agar inoculated centrally with a standard disc of inoculum, were inverted. Autoclaved chopped rubber was placed on the lid of 10 plates and these incubated together with the untreated plates. The radial distance from the edge of the inoculum disc to the margin of each colony, measured on the 3rd, 6th and 20th days of the experiment, are recorded in table 35 of appendix. Each value given is the mean of measurements in four directions at right angles. The results may be summarised as follows:

Treatment	Mean radial growth (mms)		
	3rd day	6th day	10th day
Rubber absent	4.3	9.5	28
Rubber present	4	9	30.4

Empirical observation indicated that the presence of rubber does not affect growth of mycelium in these conditions.

Thus it appears that the unfavourable effect on growth in containers sealed with rubber stoppers, unlike the effects on germination of spores in

such conditions, is probably due, not to the presence of rubber, but to the prevailing conditions of aeration.

such conditions, is probably due, not the presence of rubber, but to the

5. <u>Effects of aeration on growth of A. campestris mycelium on solid medium.</u>

The above experiment indicated that it was unlikely that the poor growth observed in part 2, Section III, in aerated containers closed with rubber stoppers was due to volatile material from the rubber. The effects of aeration on mycelium of this species was therefore investigated.

In studies of spore germination in air from cultures of <u>A. campestris</u> mycelium, it was found difficult to maintain the mycelium in a suitable condition while air was being passed through it at a slow rate. In these cases, mycelium, whether on malt agar or on wheat grain media, grew normally while the flask containing it was closed with a plug of cotton wool, but not when this was replaced with a rubber stopper carrying aeration tubes such that air entered near the bottom of the culture medium and left from the top of the container.

The appearance of these cultures changed markedly, no longer showing fluffy aerial mycelium but, instead, an adpressed habit and a considerable amount of strand formation. Spores aerated with air from such cultures did not usually show the stimulation of germination characteristically induced in the presence of the mycelium. Inocula from cultures in such condition have, however, been found still capable of normal growth when transferred to fresh medium. Although the rate of air flow through these cultures was slow, the resulting exchange of gases in the containers was probably greater than in unsealed containers, in which the gaseous interchange occurs at the relatively small surface of a large volume of culture.

Previous records do not provide much information on the aeration

requirements for ordinary mycelial growth as distinct from reproductive growth. Treschow (1955) raised the question of whether the mycelium of A. campestris can grow anaerobically. He studied the growth rate of this mycelium in tubes filled with compost, plugged with cork and sealed with paraffin wax, and found that lack of air interchange did not have an inhibitory effect on the growth of the mycelium. Treschow also mentions the interesting statement of Kostyschow (1907) that mushroom tissue does not produce alcohol in anaerobic conditions. It appears possible, therefore, that the changed behaviour of mycelium in the controlled aeration conditions described above is not due to inadequate supply of oxygen but to the effects of aeration on the concentration of some volatile metabolite present.

The results of several previous workers have indicated the production of physiologically active metabolites by A. campestris mycelium. This work concerned principally the problem of sporophore initiation in badly ventilated conditions but may relate also to the changes in growth discussed above.

Mader (1943) found that the inhibition of sporophore formation and abnormal growth of sporophores in inadequately ventilated caves was not due to the accumulation of carbon dioxide, as claimed by Lambert (1933), but to a volatile substance produced by the mycelium. He showed that normal fruiting occurred in containers, of which the atmosphere was recirculated through alkaline potassium permanganate solution and also in air recirculated through oil which, while not affecting carbon dioxide, would remove any unsaturated hydrocarbon gases. Mader therefore suggested that the volatile fructification inhibitor was an unsaturated hydrocarbon.

Stoller (1955) criticised Mader'stheory and reviewed other existing

theories, including his own, that volatile oxidising agents produced by the mycelium affected fruiting. He had detected such substances in the atmosphere above mycelium impregnated compost and suggested that the oxidising intensity of these substances could prevent the thickenings of strands of hyphae from which sporophores develop. He stated that the casing soil is an alkaline, oxygenated medium for the destruction of the volatile substances. Stoller also reported "epinasty" of tomato plants enclosed under bell-jars on beds of compost impregnated with mushroom mycelium and also in large bottles containing mushroom spawn, but he does not mention that this reaction has been used by many workers to detect the presence of unsaturated hydrocarbon gases. Thus Stoller's observations support Mader's conclusions.

Schisler (1957) investigated Stoller's theory that rapid oxidation-reduction reactions, favoured by the high pH of the casing soil, removed a volatile substance affecting sporophore initiation. He showed that mushroom beds cased with silica sand could yield normally, provided an adequate water content was maintained in the sand. He pointed out that, according to Stoller's hypothesis, such a casing material should have resulted in no yield. Schisler carried out a series of careful respiration studies on growth and fructification, on normal compost cased with soil, in aerated flasks. He found a fluctuating respiration rate. Peaks of respiration occurred during the anaestemosing of hyphae to form rhizomorphs, at the initiation of "pin-head" formation and during the expansion of the sporophore. He found a very rapid rate of airflow to be necessary for normal fruiting. In conditions of inadequate aeration, mycelium grew as a

"sheeting" over the surface of the soil but no initiation of sporophores took place. He suggested that an inhibitory substance of high molecular weight and low volatility was produced by the mycelium.

Schisler correlated his findings of stimulated respiration at the onset of reproductive growth with the findings of other workers of increased respiration induced by plant growth substances and suggested that the postulated volatile inhibitory substance may at certain concentrations function as a hormone and provide the stimulus for fruiting. He concluded that the casing layer functions as a barrier, sufficiently inhibiting the volatilisation of the postulated "hormone" to give concentrations suitable for stimulation of fruiting. He supported this hypothesis by an experiment on mushroom beds with different depths of soil, in which he found that with a thin layer of casing soil mycelium just grew over the surface of the soil without fruiting. Even uncased trays, however, were induced to fruit after repeated waterings and Schisler suggested that, here, a water layer at the surface acted as a barrier, suitably concentrating the "hormone".

The importance of aeration has been noted also in studies on sporophore production in other Hymenomycetes. Thus, Etter (1929) found that, although sporophore formation began in flasks, only the smallest could develop inside containers of normal size and that it was necessary for the sporophore initia to grow through and beyond the mouth of the flask before it could produce the characteristic type of pileus. In these cases, increased aeration, probably removing inhibiting metabolites, seems to have a formative rather than an initiating effect on fructification.

While the preceding account gives the impression that the changes in

growth habit associated with rhizomorph formation and fruiting are largely controlled by conditions of aeration, various other factors may be important in the change from normal mycelial growth. Strand formation can occur in various conditions, e.g. ageing of A. campestris cultures is usually accompanied by strand formation. Rudimentary fructifications have been found on old mushroom spawn contained in bottles, where the change in growth habit does not appear to be brought about by any change in aeration conditions. Garrett (1954) has shown that a certain "inoculum potential" of the mycelium is necessary before strand formation can occur. These other aspects will not be discussed here since only the influence of aeration on the type of growth is relevant to the present stages of this work.

Experimental.

The following experiment was carried out to find if aeration affected growth on solid medium, and also to find if the amount of mycelium present had any influence. This study was carried out in aeration chambers of the type used in germination studies described in Part 2.

A firm malt agar medium which could be handled conveniently was obtained by using 2% malt in 3% agar. Discs of this medium, 1½ inches in diameter, were cut from petri dishes containing a standard depth of medium, placed on the glass bases of the aeration chambers and each inoculated centrally with a 4 mm. disc from a culture of mycelium on malt agar. Four sets of six such chambers were prepared and received the following treatments:

(1) aerated (in parallel).

(2) control series, without aeration.

(3) non-aerated series, having a disc, one inch in diameter, taken from a

culture of mycelium of the same species, placed on the lid of each aeration chamber.

Growth after one week, as measured by the increase in radius of each colony, is recorded in table 30 of appendix. Each value given is the mean of four radii, measured at right angles. This method was adopted because of the small size of the colonies in this short experiment which was likely to give only very small differences in dry weight. In other experiments, dry weights were recorded after one month's growth.

The results of the above experiment may be summarised as follows:

Treatment		Mean radial increase (mms.)	Type of growth.
Aerated	in parallel	2.79	Adpressed with tendency to strand formation.
Non-aerated	in presence of A. campestris mycelium	5.37	Abundant aerial mycelium.
	Control	3.96	Normal aerial mycelium.

Empirical observation of the appearance of the colonies suggested the following conclusions:

(a) Better growth in the presence of mycelium of the same species than in controls.

(b) A reduction of growth in aerated conditions.

From analysis of the results, however, it appeared that these differences were not brought out by the linear measurements.

Analysis of variance showed that growth in the presence of mycelium of

the same species was significantly better than in any of the aerated cultures but was not significantly better than in the controls. Differences between control and aerated colonies were not found to be significant. The radial differences recorded, however, corresponded to marked differences in the type of growth observed.

Discussion.

The results of the above experiment appear to support the conclusions of Mader (1943) and Schisler (1957) that accumulation of a volatile metabolite encourages vegetative mycelial growth whereas removal of this material results in strand formation and a tendency to reproductive growth. Further studies of this problem would be of considerable interest.

It is tempting to conclude from the evidence of the above workers and the indications given by this experiment, that the postulated material affecting growth could be the same metabolite which has, already, been found to stimulate germination, but the information available does not justify further speculation at this stage. Some supporting evidence is, however, provided by the following experiment.

6. Effects of medium containing metabolic products from A. campestris mycelium on growth of mycelium of this species.

Introduction.

The following experiment was designed to find if the medium from A. campestris cultures, which had, in Part II, been shown to stimulate germination of spores of this species could influence also the growth of mycelium of this species.

The results of previous workers indicate, in general, unfavourable conditions for growth on medium on which mycelium of the same species has been growing. Such media normally contain staling products inhibitory to growth. Several studies have indicated chemotropic growth of mycelium away from such self-staled medium. Graves (1916), for example, studying the influence on growth of Rhizopus nigricans of medium which had been "staled" by growth of this fungus, found that hyphae turned away from the medium in which they had been growing, towards any other medium without spores or hyphae in it. He found the staling products to be thermolabile and their effect to be markedly reduced by boiling. Thus these effects were not due simply to exhaustion of nutrients or other permanent change.

The following experiment was carried out to find in what way A..campestris culture medium, which had been shown to contain the germination stimulant, affected mycelial growth.

Experimental.

20 ml. aliquots of 2% malt extract solution, buffered with 1% powdered chalk, were placed in 20, 100 ml. conical flasks. 10 ml of medium from one month old cultures of A. campestris in similar medium was added to each of ten flasks and 10 ml of uninoculated 2% malt solution, similarly buffered, was added to each of the remainder. Standard inocula of A. campestris mycelium were placed in each flask.

Dry weights of mycelium after one month's growth are recorded in table 37 of appendix and summarised below:

Solution added	Mean dry weight of mycelium (mgms.)
Medium from cultures of A. campestris.	93
Uninoculated control medium.	62

Analysis of variance showed that the difference in growth between these two conditions was greater than the 0.01 level of significance. It was concluded therefore that medium in which mycelium of A. campestris has been growing stimulated growth of mycelium of the same species.

Discussion.

It appears from the above experiment that mycelium of A. campestris must bring about some change in the medium in which it is growing, which makes the medium more suitable for growth. This is a marked contrast to the self staling effects shown by many fungi. The effect cannot be due merely to a change in pH of the medium due to the growth of mycelium, since excess chalk was present as buffer throughout the growing period. It is possible that the effect could be due to a change in nutrient status, such as a conversion of complex substances to more readily available forms. In the light of earlier work, however, it is likely that the stimulation of growth may be due to a specific metabolite secreted by the mycelium. Medium in which mycelium of A. campestris has been growing has, already, been shown to contain a volatile metabolite which stimulated germination of spores of this species, and it is reasonable to suppose that such a substance may affect growth of mycelium in some way.

This can be regarded only as a preliminary hypothesis at this stage of the work. Some support for it has, however, been found in the preceding experiment.

Conclusion.

The earlier sections of this part of the thesis concerned simply the establishment of suitable conditions for growth of mycelial cultures. The last two sections, however, indicated a favourable effect of a volatile material from A. campestris mycelium on growth of this species and marked stimulation of hyphal growth by a metabolite, probably the same one, present in the medium from cultures of this mycelium.

These results appear to relate to earlier reports of volatile metabolite of A. campestris and to the investigations recorded in part 2.

GENERAL CONCLUSION.

General Conclusion.

The investigations of part 2 of this thesis lead to the hypothesis that the mycelial germination stimulant of A. campestris spores is an olefin. It is likely also that the endogenous germination determining factor of these spores, postulated in part 1 is the same material or similar. The work in part 3 points to the suggestion that the volatile growth factor detected by Mader (1943), Stoller (1955) and Schisler (1957), and thought by Mader to be an unsaturated hydrocarbon, may be the same metabolite responsible for the effects on spore germination.

These findings explain many of the discrepancies of previous work and may relate to various aspects of plant physiology. Many effects of unsaturated hydrocarbon gases on plant organs have been noted during the past sixty years and in more recent times there have been various reports of evolution of such gases from plant tissues in concentrations too low to be detected conveniently by chemical means. These gases characteristically exert their effects on living tissues at very low concentrations and their presence is usually overlooked. Crocker (1948), however, concludes that all respiring tissues may evolve such substances.

It will obviously be of interest to examine the possible mechanism of the effect of olefins on the metabolism of A. campestris. At this stage, speculation can only be based on previous studies with higher plants and these tend to indicate a respiratory effect. A comprehensive review of literature relating to this subject has been prepared but is not presented here since not strictly relevant to the present stage of the work.

SUMMARY.

STUDIES RELATING TO SPORE GERMINATION IN AGARICUS CAMPESTRIS L. ex Fr.

Summary

Part 1. EFFECTS OF PHYSICAL FACTORS ON GERMINATION OF SPORES OF AGARICUS CAMPESTRIS.

I. Germination of Agaricus campestris spores in relation to the condition of the medium.

1. The effect of nutrient on germination was studied by comparing germination on agar medium containing different concentrations of malt extract, ranging from 0 to 10%. The optimum concentration for germination was 2% malt extract, although germination did occur at all the levels tested.

2. An investigation of the effects of the hydrogen ion concentration of the medium on germination confirmed and extended previous records. Germination occurred on medium of pH values from 4 to 7, the optimum being at pH 5 and the slowest germination at pH 7. No germination occurred in media of pH's 3, 8 or 9.

3. In studies designed to find the most suitable conditions for studies of spore germination, better germination occurred on solid agar medium than in small drops of liquid medium.

The rate of germination of spores in suspension in various liquid media was very slow and only low levels of germination were attained in those conditions. Germination was not improved by aeration, addition of nutrient, increased oxygen concentration nor by increased concentrations of spores within the range studied.

Pretreatment of spores by submergence in water for varying periods

before inoculation on solid medium did not appear to affect the length of time required for germination on solid medium.

II. Germination in relation to spore concentration.

In work recorded above, liquid medium was found unsuitable for examining the effectsof spore concentration, because of the low level of germination in these conditions. This problem was therefore examined by the use of solid medium.

The rate of germination was found to increase with the concentration of spores present but, although germination began earlier at the highest concentrations tested, subsequent observations showed that the total percentage germination was reduced at the greatest density of spores.

III. Germination in relation to age of spore.

Germination of spores of ages from one month to ten years was compared. Spores of all ages were found viable but the time required for germination increased gradually with age. This long viability considerably exceeds the longest periods previously claimed for these spores.

Part 2. EFFECTS OF BIOLOGICAL FACTORS ON GERMINATION.

I. Effects on germination of A. campestris spores of mycelium of the same species.

1. This work has confirmed previous authors' reports of the stimulation of germination by the presence of living hyphae of the same species in direct contact with the medium in which the spores are present. It has extended these qualitative observations by showing that germination is stimulated when medium bearing the spores is held in the presence of, but not directly in contact with the living hyphae.

2. A previous report of stimulation of germination of spores on medium on which A. campestris mycelium has been growing has been confirmed. Germination has also been found to be stimulated when spores are held in the presence of, but not in contact with such culture medium.

Thus, it has been shown that the stimulating factor is a volatile metabolite which diffuses from the mycelium into the air and into the medium on which the mycelium is growing, and which volatilises from this culture medium.

3. Living mycelium of the following species of fungi was shown to stimulate germination of A. campestris spores separated from it by air: Merulius lacrymans, Polyporous betulinus, Hygrophorous virgineus, Hypholoma fascicularе, Russula atropurpurea, Laccaria laccata, Saccharomyces cereviseae, Penicillium notatum, Botrytis cinerea, Mucor hiemalis. No species tested in these conditions was found to exert an inhibitory effect or to be without effect on these spores.

(b) The effects on germination of the presence of increased amounts of the stimulating organism were studied using Saccharomyces cerevisaee, the amount of which could be controlled more precisely than appeared possible with a filamentous fungus. Germination increased with the amount of yeast present, in the lower ranges, but was inhibited in the presence of a high concentration

II. Analysis of the stimulation effect.

Three lines of approach to this problem were followed:

1. Direct examination of carbon dioxide as a possible stimulant,

   (a) By removal of carbon dioxide from air containing the germination stimulant;

   (b) By studying the germination of spores in normal air containing increased concentrations of carbon dioxide.

2. Examination of methods of isolating the stimulatory material.

3. Chemical analysis of the stimulant.

1. Study of the influence of carbon dioxide on the germination of A. campestris spores.

(a) Germination in normal air, and in air which had passed through cultures of A. campestris, was compared with germination when these air streams had passed through carbon dioxide absorbent. Similar experiments were carried out with Hygrophorous virgineus and Saccharomyces cereviseae as stimulating organisms, since the condition of the A. campestris cultures used deteriorated in these experimental conditions and it was hoped that these other species might be less unfavourably affected. The conflicting results obtained give no indication of a direct correlation between the

presence of carbon dioxide and the stimulatory effect.

(b) Spore germination was inhibited in atmospheres containing increased amounts of carbon dioxide, even at a concentration as low as 0.1% carbon dioxide. It was, therefore, concluded that carbon dioxide is not the stimulant.

In the development of suitable methods for the work recorded above, several problems arose in connection with the response of the spores and of aerated mycelium to experimental conditions. Investigation of some of these factors indicated that germination was inhibited in the presence of rubber and in the presence of ester wax and of plasticene.

The observation of an apparent deterioration in the condition of cultures of A. campestris mycelium aerated in these experiments led to a preliminary study of growth in relation to aeration, recorded in Part 3.

2. Examination of methods of isolating the stimulating material.

(a) The effects of heat treatment on the germination stimulant present in medium from A. campestris cultures were studied as a preliminary to examining the possibility of separating the stimulant from such medium by distillation. The active material was found to be fairly thermostable, although the amount of stimulant present was reduced after heat treatment. It was not, however, found possible to obtain a detectable amount of the stimulant from the medium by distillation and it was concluded that the active material was too volatile to be collected by the condensing system used.

(b) The possibility of extraction of the germination stimulant from culture solution with ether was examined. The active material was found insoluble in ether and remained in the residue after ether extraction.

(c) Samples of volatile material produced by A. campestris mycelium were collected by passing air from A. campestris cultures into a receiver cooled in liquid air. Marked stimulation of germination of A. campestris spores was found in the presence of an aqueous solution of this material.

3. Isolation and identification of the stimulant.

(a) Samples of the volatile stimulant from A. campestris cultures collected in a receiver cooled in liquid air were analysed by Dr. Reed of the Chemistry Department, Glasgow University using the mass spectrometer. A seven-carbon olefin was found present and was identified as 2:3-dimethyl-1-pentene.

(b) Analysis of a similar sample collected from air passing from a culture of Saccharomyces cerevisease, demonstrated the presence of a five-carbon olefin, 3-methyl-1-butene.

Since no substance other than 2:3-dimethyl-1-pentene has been found in material isolated in this way from air from A. campestris mycelium, and since this material has been shown to contain the stimulant, it is concluded that this olefin is probably the germination stimulant produced by A. campestris mycelium.

Part 3. EFFECTS OF VARIOUS FACTORS ON GROWTH OF AGARICUS CAMPESTRIS.

This part of the thesis records the results of a variety of minor investigations which were associated with the planning of work recorded in previous sections.

1. In order to find a satisfactory method of maintaining a pH favourable to A. campestris, growth was compared in solutions buffered with various levels of phosphate, in solutions buffered with powdered chalk and in unbuffered solutions.

Growth was very poor in unbuffered solutions and was inhibited in all but the lowest concentrations of phosphate. Best growth occurred in solutions buffered with powdered chalk, where the pH remained close to the optimum for mycelial growth and the inhibitory phosphate was absent.

2. The effect of size of inoculum used was examined to find to what extent the rate of growth could be increased by the use of larger inocula. It was found that growth increased slightly with the amount of inoculum but not, however, in direct proportion to its size.

3. The effect of the concentration of nutrient present in culture media was examined by comparing growth in malt solutions of different concentrations. Optimum growth occurred in 15% malt solution of osmotic pressure 9 atmospheres. Somewhat poorer growth was found in 20% malt solution.

4. In studies of germination (part 2, Section II), in air from cultures of A. campestris mycelium, difficulties arose through the attenuated growth observed in aerated bottles sealed with rubber stoppers. Since it had already been found that the presence of rubber inhibited germination of

A. campestris spores, the effect of rubber on growth of mycelium was tested. It was found, however, that the presence of rubber did not affect growth.

5. Since the above mentioned change in the condition of mycelial cultures in aerated containers could not be attributed to a direct inhibitory effect of rubber, a preliminary examination of the response of *A. campestris* mycelium to aeration was made.

Growth on solid medium in aeration chambers of the type developed in Part 2, was compared in aerated and non-aerated conditions and in the presence of mycelium of the same species. The appearance of the colonies after one week's growth suggested better growth in unaerated than in aerated conditions, and also some stimulation of growth in the presence of added mycelium. Analysis of records of radial increase of colonies did not show these differences to be significant, but linear measurements do not appear to give an adequate estimate of growth in this experiment. The method was adopted because of the relatively small amount of growth during the short period of this experiment. Growth in the presence of mycelium was significantly better than in aerated cultures, the former producing aerial mycelium and the latter only an adpressed type of growth.

6. In Part 2, Section I, it was shown that a volatile metabolite, secreted into medium in which mycelium of *A. campestris* had been growing, stimulated spore germination. An experiment was carried out here to find whether this active metabolite might also affect mycelial growth. It was found that growth of *A. campestris* mycelium was stimulated when similar medium taken from older cultures of *A. campestris* mycelium was added to the culture solution.

The findings of these last two experiments may relate to the reports by previous workers of the production by *A. campestris* mycelium of a volatile substance which inhibits strand formation and reproductive growth and favours vegetative mycelial growth if allowed to accumulate.

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APPENDIX.

Table 1. Effects of salt concentration on germination.

Germination per 5,000 spores.

	Plate	Salt concentration (%)						
		0	2	3	4	5	7	10
10th Day.	1	0	1	0	0	0	0	0
	2	0	0	0	0	0	1	0
	3	0	2	2	0	0	0	0
	4	0	5	0	0	0	0	0
	5	0	0	0	0	0	0	0
	6	0	10	0	0	0	0	0
	7	2	0	0	0	0	0	0
13th Day	1	5	100	200	5	0	0	0
	2	0	0	0	0	5	0	0
	3	0	400	120	0	200	0	0
	4	0	20	0	0	0	200	0
	5	0	200	100	0	0	0	0
	6	0	500	0	1	0	0	0
	7	0	2	0	0	0	0	0
16th Day.	1	4000	>500	>500	>500	5000	5000	5000
	2	1000	1000	0	>500	5000	5000	5000
	3	5000	>500	>500	2000	>500	5000	5000
	4	2000	>500	5000	2000	100	>500	500
	5	2000	>500	>500	4000	1000	1000	400
	6	5000	>500	>500	5000	1000	5000	-
	7	0	>500	2000	100	500	500	>500

Table 2. Comparison of germination on malt extract agar and on plain agar, both of pH 6.

Germination per 5,000 spores
(based on counts of 15,000 spores)

Plate	Disc	2% malt agar	Water agar
1	1	2	1.5
	2	1	1.5
	3	1.25	2
	4	1.5	1.5
	5	2.5	.55
2	1	22.5	12.5
	2	10	5
	3	20	8.5
	4	8	7.5
	5	3.5	2.5
3	1	8.5	4
	2	12	8
	3	12.5	4.5
	4	6	5
	5	6.5	5
4	1	7	7
	2	28	11
	3	16.5	3
	4	17	10.5
	5	14.5	6.5

Table 3. Germination of spores of [illegible] in relation to the pH of the medium.

Germination per 5,000 spores.

Plate	Day	pH 3	4	5	6	7	8	9
1	7	0	0	60	5	0	0	0
	10	0	70	120	10	0	0	0
	14	0	>500	>500	60	20	0	0
	20	0	>500	>500	>500	>500	0	0
2	7	0	0	50	15	0	0	0
	10	0	50	100	50	0	0	0
	14	0	>500	>500	>500	90	0	0
	20	0	>500	>500	>500	>500	0	0
3	7	0	0	30	10	0	0	0
	10	0	0	150	20	0	0	0
	14	0	50	>500	>500	10	0	0
	20	0	>500	>500	>500	>500	0	0
4	7	0	0	50	20	0	0	0
	10	0	0	110	100	0	0	0
	14	0	>500	>500	>500	40	0	0
	20	0	>500	>500	>500	500	0	0
5	7	0	0	50	30	0	0	0
	10	0	0	150	50	0	0	0
	14	0	60	>500	>500	-	0	0
	20	0	>500	>500	>500	-	0	0
6	7	0	0	100	90	30	0	0
	10	0	60	140	170	50	0	0
	14	0	>500	>500	>500	150	0	0
	20	0	>500	>500	>500	>500	0	0
7	7	0	0	60	50	0	0	0
	10	0	90	150	120	0	0	0
	14	0	>500	>500	>500	20	0	0
	20	0	>500	>500	>500	>500	0	0

<u>Table 4. Effect of aeration on spore germination in liquid medium.</u>

Sample	Germination per 5,000 spores (means of 5 samples)	
	Aerated	Non-aerated
1	0.4	0
2	0	0.6
3	2.15	0.2
4	1.15	0.05
5	2.5	0.75
6	0	2
7	0	0.24
8	0	0.44
9	0	1.22
10	0	0.72

Table 5. Effect of oxygen concentration on germination of spores in suspension.

Germination per 5,000 spores - (means of 5 samples)

% oxygen in airstream	(5 - 10)%	20%	(30 - 35)%	100%
Corresponding O_2 level in solution.		0.6%	1%	2%
Tube				
1	22.0	0.4	0.6	692.0
2	1.6	0.4	0.2	1.0
3	1.0	141.0	89.0	0.6
4	0.6	1.4	0.4	0.4
5	6.0	0.2	2.0	2.0

Table 6. Effects of presence of nutrient on germination in aerated suspension.

Germination per 5,000 spores (means of five samples)

Tube	Water	1% malt
1	0.25	1.5
2	1.65	0.5
3	0.0	0
4	0.75	0.15
5	0.65	1.0

Table 7. Effects of spore concentration on germination of spores in aerated suspension.

Concentration of Suspension.	Tube	Germination per 5000 spores
60,000 spores per cu. mm.	1 2 3	0.05 0.05 0.025
1/10 dilution	1 2 3	0.525 0.65 0
1/20 dilution	1 2 3	0.1 0 0

Table 8. Germination of spores on solid medium after pretreatment by submergence in water.

		Germination per 5,000 spores.									
Days in suspension		0		1		2		4		10	
Days on solid medium		7	8	7	8	7	8	7	8	7	8
Plate	**Disc**										
1	1	20	50	10	55	13	45	0	12	3	37
	2	3	20	0	9	6	32	12	31	0	25
	3	10	110	1	7	20	50	8	30	8	30
	4	15	65	2	16	19	43	6	27	8	30
	5	7	120	4	20	21	48	18	40	6	22
2	1	2	5	35	100	6	50	1	29	20	100
	2	7	6	10	40	5	52	3	20	23	100
	3	6	9	5	32	5	25	2	17	17	60
	4	0	14	5	19	6	42	2	12	15	50
	5	1	12	10	35	9	60	3	25	25	60
3	1	8	30	1	23	8	20	6	40	12	90
	2	3	12	10	25	4	43	5	30	10	50
	3	1	27	10	75	7	56	20	100	5	100
	4	3	20	20	30	2	25	3	46	3	50
	5	3	35	10	120	6	36	8	37	5	23
4	1	1	33	14	90	9	40	9	27	19	45
	2	2	62	9	50	6	27	10	34	30	50
	3	1	80	40	80	5	30	1	15	33	20
	4	2	20	20	90	8	59	3	36	40	50
	5	3	33	17	50	13	60	2	27	23	100
5	1	5	25	4	55	5	28	3	21	14	42
	2	4	28	22	100	3	17	3	31	8	40
	3	3	60	7	35	4	42	3	22	15	100
	4	25	50	1	30	2	20	3	20	12	30
	5	2	45	11	80	3	12	6	34	20	65

Table 9. Comparison of germination on solid medium with germination in distilled water.

Plate	Disc	Germination per 5,000 spores	
		2% agar	Water
1	1	250	150
	2	550	150
	3	200	50
	4	300	100
	5	5	50
2	1	300	100
	2	100	25
	3	40	50
	4	125	0
	5	75	5
3	1	175	150
	2	175	9
	3	175	5
	4	150	50
	5	125	125

Table 10. Germination of A. concentric spores in relation to the the concentration of spores.

Germination per 5,000 spores

Spores per field		50,000	10,000	5,000	1,000	500	100	50
Plate	Day							
I	5	4	6	1	0	0	0	0
	6	2	3	5	4	1	0	0
	7	4	15	5	4	0	5	0
	8	7	35	18	30	2	0	0
II	5	5	3	5	3	1	0	0
	6	3	12	1	10	1	0	0
	7	0	6	4	9	8	0	0
	8	10	20	35	30	9	5	0
III	5	3	2	1	0	0	0	0
	6	1	2	2	1	2	0	0
	7	5	7	0	10	10	5	5
	8	10	30	30	40	25	20	5
IV	5	3	5	3	0	0	0	0
	6	2	3	1	10	4	0	0
	7	5	12	7	0	1	0	0
	8	10	20	17	7	1	5	0
V	5	5	2	1	1	0	0	0
	6	5	3	3	2	1	0	0
	7	7	6	0	10	10	5	3
	8							
VI	5	1	3	4	1	0	0	0
	6	5	3	2	3	6	5	0
	7	0	50	10	20	25	10	0
VII	5	1	4	2	6	0	4	0
	6	5	0	2	5	10	1	0
	7	0	10	6	15	22	5	3

Table 11. Germination of A. campestris spores in relation to age of spore.

Germination per 5,000 spores.

Age of spores		10 Years	7½ yrs.	5½ yrs.	4½ yrs.	3½ yrs.	2½ yrs.	1 year	7 months	2 months	1 m
Plate	day										
1	9	0	0	0	0	0	50	15	20	400	
	11	0	0	0	0	0	500	200	300	)500	
	15	200	200	250	100	200	)500	)500	)500	)500	)
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)
2	9	0	0	0	0	0	7	0	15	500	
	11	0	0	0	0	0	250	4	1000	)500	)
	15	0	15	0	0	20	)500	)500	)500	)500	)
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)
3	9	0	0	0	0	0	45	20	50	400	
	11	0	0	0	0	0	400	300	400	)500	
	15	10	300	100	200	7	)500	)500	)500	)500	)
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)
4	9	0	0	0	0	0	10	7	0	100	
	11	0	0	0	0	0	300	15	5	500	
	15	0	5	)500	2	100	)500	400	400	)500	)
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)
5	9	0	0	0	0	0	5	20	1	300	
	11	0	0	0	0	0	30	90	50	)500	
	15	50	0	0	0	0	)500	)500	)500	)500	
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)
6	9	0	0	0	0	0	200	3	150	300	
	11	0	0	0	0	0	)500	15	400	)500	
	15	300	400	0	0	)500	)500	)500	)500	)500	)[illegible]
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)[illegible]
7	9	0	0	0	0	0	0	0	80	400	[illegible]
	11	0	0	0	0	0	0	5	15	)500	)[illegible]
	15	200	300	0	0	0	5	15	)500	)500	[illegible]
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)[illegible]
8	9	0	0	0	0	0	70	100	30	)500	
	11	0	0	0	0	0	500	400	300	)500	[illegible]
	15	10	500	300	100	200	)500	)500	400	)500	[illegible]
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)[illegible]
9	9	0	0	0	0	0	0	20	15	)500	1[illegible]
	11	0	0	0	0	0	50	)500	250	)500	[illegible]
	15	400	10	500	400	500	)500	)500	)500	)500	)[illegible]
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)[illegible]
10	9	0	0	0	0	0	80	0	0	400	
	11	0	0	0	0	0	200	25	30	)500	1[illegible]
	15	)500	300	200	500	0	)500	500	)500	)500	)[illegible]
	30	)500	)500	)500	)500	)500	)500	)500	)500	)500	)[illegible]

Table 12.

Effect of presence of Agaricus campestris mycelium on the germination of spores of the same species.

% germination on 6th day (based on counts of 1000 spores)

Plate	disc.	Mycelium present.	Mycelium absent.
I	1	50	0
	2	50	0
	3	10	0
	4	20	0
	5	15	0
II	1	over 50	0·1
	2	over 50	0
	3	50	0·2
	4	50	0
	5	60	0
III	1	5	0
	2	5	0
	3	20	0
	4	10	0
	5	5	0·1
IV	1	10	0·1
	2	20	0
	3	10	0
	4	5	0
	5	5	0
V	1	15	0·1
	2	20	0·7
	3	20	1·5
	4	20	0·5
	5	15	0·5

The differences between treatments are clearly significant.

Table 13. Germination on sterile solid medium extracted from A. [illegible] cultures.

Germination per 5,000 spores.

Plate	Disc	Culture medium		Control medium	
		6th day	10th day	6th day	10th day
1	1	20	over 500	0	10
	2	60	" "	0	40
	3	70	" "	0	60
	4	50	" "	0	50
	5	30	" "	0	90
2	1	60	" "	0	10
	2	70	" "	0	50
	3	25	" "	0	0
	4	40	" "	0	5
	5	60	" "	0	100
3	1	20	" "	0	0
	2	50	" "	0	50
	3	20	" "	0	100
	4	40	" "	0	0
	5	30	" "	0	40
4	1	110	" "	0	0
	2	90	" "	0	100
	3	50	" "	0	5
	4	40	" "	0	70
	5	70	" "	0	2
5	1	50	" "	0	0
	2	70	" "	0	0
	3	50	" "	0	0
	4	60	" "	0	0
	5	60	" "	0	0

Table 14.

Effect of addition of culture medium to spore suspension.

Germination per 5000 spores on 6th day after inoculation.

Plate	Drop	Culture solution	Control solution.
I	1	850	0
	2	10	0
	3	250	0
II	1	600	45
	2	700	10
	3	1200	6
III	1	400	4
	2	400	0
	3	500	5
IV	1	370	2
	2	240	7
	3	400	4
V	1	1200	8
	2	800	10
	3	750	20

Table 15.

Effect of presence of culture medium on germination.

Germination per 5000 spores on 6th day after inoculation.

Plate	disc	Culture solution	Control solution.
I	1	25	0
	2	15	0
	3	20	0
II	1	10	0
	2	32	0
	3	15	0
III	1	18	0
	2	35	0
	3	40	0
IV	1	8	0
	2	10	0
	3	9	0
V	1	12	0
	2	10	0
	3	6	2

Table 16. Effect of various other species of fungi on germination of spores of *Agaricus campestris*.

Species present		Germination per 5,000 spores.																							
		Agaricus campestris				*Merulius lacrymans*				*Polyporous betulinus*				*Penicillium*				*Saccharomyces cerevisiae*				Control			
No. of days after inoculation.		4	6	8	14	4	6	8	14	4	6	8	14	4	6	8	14	4	6	8	14	4	6	8	14
Plate	Disc																								
	1	11	470	>500	>500	5	20	40	>500	0	0	0	0	0	0	0	0	1	3	-	-	0	0	10	>500
	2	10	650	>500	>500	2	25	120	>500	0	0	0	5	0	0	0	0	0	0	4	2000	0	1	40	>500
I	3	13	650	>500	>500	2	8	-	-	1	0	0	0	0	0	0	0	0	17	1000	4000	0	0	0	>500
	4	8	800	>500	>500	3	10	-	-	1	1	0	0	0	0	0	0	2	30	700	4000	0	0	0	>500
	5	29	500	>500	>500	4	20	100	>500	0	0	0	0	0	0	0	1	3	27	600	4000	0	1	20	>500
	1	80	>500	>500	>500	4	80	150	>500	0	3	8	25	0	0	0	1	30	1000	4500	5000	1	0	50	>500
	2	53	>500	>500	>500	8	70	-	-	0	0	0	0	0	0	0	11	15	2000	4700	5000	0	-	-	-
II	3	60	>500	>500	>500	6	75	500	>500	0	0	0	0	0	0	0	0	3	2000	3700	5000	0	1	5	>500
	4	75	>500	>500	>500	2	50	100	>500	0	5	6	8	0	0	0	6	22	2000	-	-	0	0	20	>500
	5	70	>500	>500	>500	4	80	-	-	1	7	5	20	0	0	0	5	10	3000	5000	5000	0	1	80	>500
	1	12	>500	>500	>500	2	5	100	>500	3	4	-	-	0	0	0	2	0	175	4500	5000	0	11	50	>500
	2	23	580	>500	>500	1	2	70	>500	3	30	-	-	0	0	0	0	0	180	-	-	0	2	3	>500
III	3	25	500	>500	>500	1	12	120	>500	6	40	-	-	0	1	0	1	3	130	3000	5000	0	1	20	>500
	4	35	600	>500	>500	1	6	60	>500	3	30	70	>500	0	0	0	0	-	-	-	-	0	1	20	>500
	5	18	500	>500	>500	3	6	110	>500	8	250	500	>500	0	0	0	5	2	176	3500	5000	0	0	5	>500
	1	8	280	>500	>500	4	12	50	>500	0	0	0	>500	0	0	0	0	0	8	-	-	0	0	0	200
	2	3	600	>500	>500	1	20	120	>500	0	0	1	>500	0	0	0	0	0	5	-	-	0	0	10	>500
IV	3	6	450	>500	>500	1	10	70	>500	0	0	0	150	0	1	0	0	0	6	55	4000	0	0	-	-
	4	5	130	>500	>500	2	17	110	>500	0	0	0	200	0	0	0	0	0	4	88	3000	0	0	30	>500
	5	4	630	>500	>500	3	16	120	>500	0	0	0	100	0	0	0	0	0	3	75	-	0	0	6	>500
	1	30	>500	>500	>500	2	30	90	>500	0	0	0	>500	0	0	0	0	0	80	200	-	0	0	35	>500
	2	35	>500	>500	>500	2	25	60	>500	0	0	1	>500	0	0	0	0	5	70	200	-	0	0	10	>500
V	3	51	>500	>500	>500	0	15	70	>500	0	0	0	150	0	0	0	0	0	50	400	-	0	0	10	>500
	4	45	>500	>500	>500	1	16	100	>500	0	0	0	100	0	0	0	0	0	125	4000	5000	0	0	-	-
	5	20	>500	>500	>500	1	14	60	>500	0	0	0	200	0	0	0	0	0	100	3000	5000	0	3	70	>500

" _ " indicates contamination of discs.

Table 17. Effects of presence of various Basidiomycetes on germination of A. campestris spores.

Germination per 5000 spores - 4th day after inoculation.

Plate	Disc	Agaricus campestris	Merulius lacrymans	Polyporous betulinus	Laccaria laccata	Hygrophorous virgineus	Hypholoma fasciculare	Russula atropurpurea	control
I	1	50	45	8	>500	250	>500	>500	0
	2	10	30	6	>500	>500	>500	>500	0
	3	30	30	20	>500	>500	>500	>500	0
	4	20	22	2	>500	>500	>500	>500	0
II	1	75	16	2	>500	>500	>500	>500	0
	2	38	28	1	>500	>500	>500	>500	0
	3	70	33	4	>500	>500	>500	>500	0
	4	60	24	1	>500	>500	>500	>500	0
III	1	320	30	52	500	>500	>500	>500	0
	2	224	44	48	575	>500	>500	>500	0
	3	130	40	40	440	>500	>500	>500	0
	4	325	20	70	650	>500	>500	>500	0
IV	1	200	350	10	165	>500	230	>500	0
	2	240	400	30	200	>500	200	>500	0
	3	450	175	5	290	>500	250	>500	0
	4	400	300	40	250	>500	>500	>500	0
V	1	50	60	20	130	250	>500	46	0
	2	90	180	10	300	400	>500	14	0
	3	230	150	20	50	500	>500	30	0
	4	>500	148	12	63	500	>500	23	0

Table 10.

Effects of various sporing fungi on germination of Agaricus campestris spores.

Experiment C.

Germination per 5,000 spores. – 5th day.

Species Plate	Disc	Controls	Penicillium notatum.	Mucor hiemalis	Botrytis cinerea	Cunninghamella elegans.
1	1	0	2	0	350	0
	2	0	4	1	100	0
	3	0	0	0	200	–
2	1	0	2	80	–	0
	2	0	0	50	0	0
	3	0	0	20	0	0
3	1	0	20	2	78	0
	2	0	10	0	–	–
	3	0	20	2	80	–
4	1	0	200	0	500	0
	2	0	50	0	400	0
	3	0	5	–	200	0
5	1	0	2	60	30	–
	2	0	10	20	200	–
	3	0	0	10	400	–

("–", indicates contamination of discs.)

Table 19. Germination in the presence of different amounts of Saccharomyces cerevisiae.

Germination per 5,000 spores.

		Number of Yeast colonies present in plates											
		1			4			8			12		
Days after inoculation		12	14	18	12	14	18	12	14	18	12	14	18
Plate	Disc												
1	1	0	0	0	8	140	4500	4	240	4500	4000	5000	5000
	2	0	0	0	4	100	4000	5	300	4800	4000	5000	5000
	3	-	-	-	25	3000	5000	-	-	-	4800	5000	5000
2	1	0	0	30	9	50	2000	180	4000	5000	45	65	5000
	2	0	0	3	-	-	-	-	-	-	50	100	4900
	3	0	7	50	-	-	-	240	1000	2000	20	50	5000
3	1	0	0	12	20	1000	4000	-	-	-	4900	5000	5000
	2	0	0	44	-	-	-	150	3500	5000	5000	5000	5000
	3	0	0	30	-	-	-	370	4000	5000	5000	5000	5000
4	1	0	0	12	0	15	2000	150	4000	5000	4500	5000	5000
	2	0	0	0	0	-	-	300	4500	5000	4800	5000	5000
	3	0	0	0	0	17	4000	160	5000	5000	4900	5000	5000

Table 20. Germination in the presence of high concentrations of *Saccharomyces cerevisiae*.

Germination per 5,000 spores – 10th Day.

Plate	Disc	Yeast present	Yeast absent
1	1	0	200
	2	0	100
	3	0	50
2	1	0	250
	2	0	200
	3	0	2
3	1	0	400
	2	0	350
	3	0	200
4	1	0	0
	2	0	200
	3	0	300
5	1	0	50
	2	0	100
	3	0	400
6	1	0	200
	2	0	250
	3	0	500

Table 21. Effects of the presence of various sealing agents on germination.

Germination per 5,000 spores on 10th day.

Material present		Paraffin wax	Motor wax	Vaseline	Plasticene	Water
Plate	Disc					
1	1	200	0	200	200	50
	2	400	0	100	0	300
	3	200	-	10	200	20
	4	200	0	5	-	30
	5	500	0	300	100	10
2	1	50	0	20	0	200
	2	200	0	300	0	50
	3	-	0	200	100	100
	4	100	0	50	50	100
	5	400	0	10	5	50
3	1	200	0	5	1	200
	2	100	0	60	-	200
	3	200	0	2	1	300
	4	250	0	50	20	200
	5	300	0	200	0	2
4	1	500	0	110	0	500
	2	150	0	20	0	5
	3	250	0	40	0	10
	4	300	0	200	50	150
	5	100	-	200	0	500
5	1	50	0	0	10	300
	2	15	0	200	5	250
	3	2	0	-	300	200
	4	200	0	100	100	-
	5	500	0	150	8	500

Table 22. Germination in the presence of Rubber.

Germination per 5,000 spores on 10th Day.

Plate	Disc	Rubber present	Rubber absent
	1	0	over 500
	2	0	" "
1	3	0	" "
	4	0	" "
	5	0	" "
	1	0	" "
	2	0	" "
2	3	0	" "
	4	0	" "
	5	0	" "
	1	0	" "
	2	1	0
3	3	0	0
	4	0	0
	5	0	100
	1	0	10
	2	0	0
4	3	0	0
	4	0	5
	5	0	2

Table 23. Germination in air passing from a culture of Agaricus campestris.

Germination per 5,000 spores

		Air from mycelium		Control	
Day		7	10	7	10
% CO_2		13%	4%	0.03%	0.03%
Plate	Disc				
1	1	10	300	0	0
	2	30	500	0	0
	3	15	200	0	0
	4	1	5	0	1
	5	20	200	0	0
	6	0	50	0	0
2	1	50	400	0	0
	2	0	50	0	0
	3	5	150	0	0
	4	20	100	0	10
	5	-	-	0	0
	6	20	200	0	0
3	1	0	3	0	0
	2	0	10	0	1
	3	2	200	0	0
	4	1	200	0	0
	5	3	100	0	0
	6	40	500	0	0

[illegible] on germination of removal of carbon dioxide from AIR from cultures.

Table 24. (Experiment 1)

Germination per 5,000 spores.

Plate	Disc	Air. untreated	A. campestris air untreated	A. campestris air CO_2-free
1	1	0	200	0
	2	0	12	100
	3	40	0	200
	4	0	00	10
	5	0	20	120
	6	0	50	21
2	1	0	10	20
	2	0	100	10
	3	0	20	2
	4	0	-	20
	5	1	200	5
	6	50	150	150
3	1	50	1	100
	2	20	100	50
	3	20	0	100
	4	10	0	0
	5	0	20	50
	6	0	10	10

Table 25. (Experiment 2) Germination per 5,000 spores.

Cell	Disc	Air untreated		Air CO_2-free		A. campestris air untreated		A. campestris air CO_2 - free	
	day	16	21	16	21	16	21	16	21
1	1	500	500	0	500	0	0	0	500
	2	400	500	10	500	1	0	0	500
	3	500	500	10	500	0	0	0	400
2	1	100	500	0	0	0	0	0	500
	2	150	500	0	0	100	500	100	200
	3	100	500	40	500	0	0	0	10
3	1	0	500	00	500	0	0	0	500
	2	70	500	-	-	2	500	2	500
	3	500	500	50	500	0	0	0	0
4	1	200	500	0	10	0	10	0	0
	2	500	500	0	100	0	200	0	10
	3	500	500	0	200	0	500	0	0

Table 2[illegible] (continued)

(Experiment 3)

Germination per 5,000 spores.

Cell	Disc	Air				A.campestris air				H. virgineus air.			
		untreated		$-CO_2$		untreated		$-CO_2$		untreated		$-CO_2$	
		Day 20	25	20	25	20	25	20	25	20	25	20	25
1	1	0	3	0	0	0	0	0	200	4000	4000	400	500
	2	0	25	0	0	0	0	0	12	4000	4000	300	500
	3	0	5	0	0	0	0	0	25	4000	4000	15	500
2	1	0	0	0	0	3	0	400	500	-	-	-	-
	2	0	0	0	0	0	0	0	450	-	-	-	-
	3	0	1	0	0	0	0	0	15	-	-	500	500
3	1	0	0	0	0	-	-	0	25	2000	4000	2	0
	2	0	0	0	0	-	-	0	20	-	-	1	0
	3	0	1	0	1	-	-	0	100	2000	4000	0	0
4	1	0	0	0	1	-	-	500	500	3000	4000	0	0
	2	0	0	0	0	-	-	0	20	2000	4000	0	0
	3	0	0	0	0	-	-	0	0	2000	4000	0	0

(Experiment 4)

Germination per 5,000 spores on 10th day.

Cell	Disc	Air		A.campestris Air		S.cerevisceae air	
		untreated	CO_2-free	untreated	$-CO_2$	untreated	$-CO_2$
1	1	200	0	500	0	0	0
	2	0	0	400	0	0	0
	3	100	0	500	0	0	0
2	1	30	0	500	0	0	0
	2	200	0	300	3	0	0
	3	0	0	500	0	0	0
3	1	400	0	100	0	0	0
	2	500	0	500	0	0	0
	3	200	0	200	0	0	0
4	1	300	0	500	0	0	0
	2	200	0	200	0	0	0
	3	400	0	400	0	0	0

Table 26.

Effects of increased concentrations of carbon dioxide.

Germination per 5,000 spores - 8th day.

Chamber	Disc	20% CO_2	Air	CO_2 concentration at end of expt.
I	1 2 3 4	0 1 4 0	120 70 60 40	12%
II	1 2 3 4	4 0 0 6	90 50 70 100	0.2%
III	1 2 3 4	3 0 2 0	80 120 110 80	0.2%
IV	1 2 3 4	0 0 0 40	- 5 2 10	0.16%

Table 27.

Germination in carbon dioxide enriched atmospheres.

Germination per 5,000 spores on 10th day.

Plate	Disc	Air	0.1% CO_2	1% CO_2
I	1	500	0	0
	2	100	0	0
	3	500	0	50
II	1	500	0	0
	2	200	0	0
	3	100	20	0
III	1	500	0	0
	2	500	0	0
	3	100	50	0

Table 28

Effect of heat on the germination stimulant in agar from cultures of Agaricus campestris.

Germination per 5,000 spores - one week after inoculation.					
Plate	Disc.	Culture medium			Control medium.
		melted	boiled (1 hour)	autoclaved	
I	1	500	0	400	0
	2	400	5	100	0
II	1	500	20	10	0
	2	500	200	500	20
III	1	500	300	250	0
	2	500	100	200	0
IV	1	400	300	500	0
	2	150	350	250	0
V	1	300	0	5	0
	2	300	20	2	0
VI	1	500	0	300	0
	2	500	200	400	0
VII	1	500	200	40	0
	2	500	300	400	0
VIII	1	500	30	0	0
	2	350	5	10	10

Table 29

Effects of heat on the volatile germination stimulant from culture medium.

Germination per 5,000 spores.

		Culture solution.						Control solution.	
		untreated		boiled		autoclaved			
Day		6	11	6	11	6	11	6	11
Plate	**Disc**								
1	1	200	4000	0	0	0	1000	0	500
	2	250	4000	0	0	0	500	0	500
	3	220	4000	0	200	0	1000	0	200
2	1	200	4000	0	500	0	500	0	500
	2	140	4000	0	500	0	500	0	500
	3	300	4000	0	200	0	500	0	500
3	1	350	4000	0	500	0	500	0	300
	2	50	4000	0	500	0	500	0	10
	3	130	4000	0	400	0	500	0	100
4	1	70	4000	0	0	0	500	0	200
	2	90	4000	0	50	0	150	0	0
	3	500	4000	0	0	0	400	0	500
5	1	170	4000	0	200	0	500	0	500
	2	300	4000	0	150	0	500	0	200
	3	400	4000	0	300	0	300	0	40
6	1	500	4000	0	0	0	200	0	0
	2	300	4000	0	0	0	500	0	0
	3	200	4000	0	0	0	500	0	0

Table 20

To find whether the stimulant is ether soluble.

Germination per 5,000 spores - 10th Day.

Plate	Disc.	Culture solution			Controls
		untreated	Ether fraction	Residue	
1	1	70	0	50	0
	2	250	0	0	0
	3	500	0	300	0
2	1	500	0	300	0
	2	400	0	0	0
	3	200	0	0	0
3	1	500	0	-	0
	2	300	0	-	0
	3	500	0	-	0
4	1	0	0	200	0
	2	-	0	50	0
	3	-	0	-	0
5	1	500	0	400	0
	2	500	0	200	0
	3	50	0	0	0

(" - " indicates contamination of agar discs)

(no value is recorded for the third disc in plate 4 containing the residue, since this disc fell from the lid into the solution.)

Table 34.

Effect of presence of material isolated from air from cultures of A. campestris mycelium.

Germination per 5,000 spores on the 7th day.

Plate	Disc	Solution of volatile material.	Water.
1	1	16	0
	2	9	0
2	1	8	0
	2	10	0
3	1	6	0
	2	15	0
4	1	4	0
	2	5	0
5	1	7	0
	2	12	0
6	1	22	0
	2	14	0
7	1	10	0
	2	7	0
8	1	5	0
	2	6	0
9	1	15	0
	2	16	0
10	1	5	0
	2	3	0

Table 31.

Growth in relation to type of buffer.

Flask	Dry weights of mycelium (mgms.)				
	Buffer Present				
	None	Phosphate			1% $CaCO_3$
		0.01M	0.04M	0.1M	
1	5	15	35	18	5
2	8	22	11	3	43
3	4	22	6	8	45
4	11	31	14	10	55
5	4	23	12	9	40
6	14	11	6	5	42
7	18	22	8	3	56

Table 32.

Effects of different amounts of inoculum on growth.

Flask	Dry weights of mycelium (mgms.)		
	Number of discs of inoculum		
	1	2	4
1	65	81	76
2	80	76	80
3	55	73	115
4	84	66	79
5	74	80	95
6	64	65	81
7	60	60	71
8	77	75	121
9	63	71	78
10	112	91	96

Growth in malt extract solutions of different concentrations.

Table 34. **Experiment 1.**

malt Conc.	Growth after one month in m.m.				
	0	1%	2%	5%	10%
Flask					
1	9	55	49	84	104
2	7	48	61	83	74
3	17	65	65	59	64
4	6	48	38	61	67
5	0	60	53	76	94
6	8	10	106	42	40
7	0	38	64	51	72

Experiment 2.

Malt Conc.	Growth after one month in m.m.					
	0	2%	5%	10%	15%	20%
Flask						
1	3	10	12	24	69	30
2	2	32	41	9	65	39
3	14	48	33	57	70	34
4	16	24	57	67	89	49
5	1	34	38	56	110	51

Table 35

Growth in the presence of rubber.

Radial growth in mm. (means of 4 radii).

	Rubber absent			Rubber present		
Day	3	8	20	3	8	20
Plate						
1	3.5	11	29.5	3.25	7.25	27.25
2	4	8.5	26.25	4	9.75	32.75
3	4.25	9.5	31.25	4.5	9.5	34.75
4	4.75	10.5	28.5	4.75	11	30.75
5	3.75	10.[illegible]	26.75	3.5	8.75	27
6	3.75	8.25	26.25	4.5	9.25	32.25
7	4.25	9.5	27.5	4	7.75	–
8	3.25	8	27.75	4	10.25	–
9	5	10.25	–	3.25	8.25	28
10	–	–	–	–	–	–

("–" indicates the presence of contaminants.)

Table 36.

Growth in one week in mm.

Chamber	Aerated	Non-aerated	
	Parallel	Control	+ mycelium
1	2.75	1.75	6.25
2	2.5	4.75	5.25
3	3.25	3.75	5.5
4	2.75	4.5	3.75
5	2.5	3.75	6
6	3.0	4.75	5.5

Table 37.

Effects of medium containing metabolic products from A. campestris mycelium on growth of mycelium of this species.

Flask	Dry weights of mycelium in mgms.	
	Malt solution from A. campestris cultures.	Uninoculated malt solution.
1	102	64
2	104	51
3	94	66
4	110	122
5	135	54
6	130	57
7	68	51
8	108	67
9	97	70
10	59	50
11	42	53
12	44	56