FACTORS AFFECTING THE GERMINATION OF THE BASIDIOSPORES OF CALVATIA GIGANTEA

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ABSTRACT

FACTORS AFFECTING THE GERMINATION OF THE BASIDIOSPORES OF *CALVATIA GIGANTEA*

by Ronald Wayne Wilson

The purpose of this investigation was to study the germination of the basidiospores of *Calvatia gigantea*. At the start of this investigation, these spores could only be germinated in malt extract agar in the presence of a culture of the yeast, *Rhodotorula mucilaginosa* var. *sanguinea*; thus, the major aspect of the study was to elucidate the action of the yeast in stimulating the spores to germinate.

The technique consisted of dispensing the spores into molten medium, and after solidification, the yeast was added at four points on the surface of the medium. Approximately 30 days were required before germination occurred. The maximum amount of germination, depending on the sporophore used, was between two hundred and six hundred spores per million.

Gibberellic acid and furfural had no effect on germination. Dinitrophenol had no effect at concentrations of 5.0×10^{-6} and lower; germination was inhibited at higher

concentrations. Light was slightly inhibitory to germination. The spores germinated over a wide range of pH values, but 4.3-5.0 was optimum.

A basal salt medium, to which various carbohydrate and nitrogen sources were added, supported a reduced amount of germination even though the R. mucilaginosa was absent. Although many carbohydrates supported germination, maltose, soluble starch, and sucrose were best. The best nitrogen source was KNO_3; amino acids and ammonium salts did not support germination unless the yeast was present. The addition of complex media to the basal medium was inhibitory unless the yeast was also added. Potato-dextrose agar supported germination nearly as well as malt extract.

The yeast stimulated germination of the spores over a distance of 15 cm. Furthermore, the yeast was capable of producing its action on the spores even though separated from them by a dialysis membrane. The possibility that the yeast produces a volatile stimulator was ruled out.

Various extracts of the yeast and filtrates of spent media were not capable of stimulating germination. Paper chromatographic studies comparing media with and without the yeast failed to show chemical differences due to the yeast.

The spores remained dormant until 23-30 days after the yeast was added. Furthermore, the stimulatory action of the yeast appeared to be continuous after 5 days of growth.

Several species of *Rhodotorula* and an unidentified bacterium were qualitatively similar to the *R. mucilaginosa* in stimulating germination.

FACTORS AFFECTING THE GERMINATION OF THE BASIDIOSPORES OF *CALVATIA GIGANTEA*

By

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To my parents

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INTRODUCTION

The discovery of the tumor-inhibiting substance(s), calvacin, in the giant puffball, *Calvatia gigantea*[1] (Persoon) Lloyd, by Lucas *et al*. (1958) launched an extensive study into the physiology of this organism. One facet of this study was the determination of variations in calvacin production by different strains which were derived from germinated basidiospores. Bulmer, Beneke, and Stevens (1962) showed that different strains did indeed vary with respect to calvacin production. However, in order to germinate the basidiospores of *C. gigantea*, Bulmer and Beneke (1960, 1961, and 1962) found it necessary to use a living co-culture of the yeast, *Rhodotorula mucilaginosa* var. *sanguinea* (Jorgensen) Harrison. This was a modification of a technique used by Fries (1941, and 1943).

The purpose of this investigation was to study in detail the factors involved in the germination of the basidiospores of *C. gigantea*. The major aspect was to

[1]Synonyms for *Calvatia gigantea* are: *Lycoperdon giganteum*, Batsch ex Pers., *Bovista gigantea*, (Batsch ex Pers.) Nees, *Lycoperdon bovista*, Fr., *Calvatia maxima* (Schaeff) Morgan, *Calvatia bovista*, Macbride, *Calvatia primitiva*, Lloyd.

elucidate the nature of the action of the R. mucilaginosa in stimulating the germination of the puffball basidiospores.

LITERATURE REVIEW

An excellent review of the scant literature on the germination of the basidiospores found in the series Gasteromycetes was presented by Bulmer (1960), and Bulmer and Beneke (1961). These two papers contained most of the pertinent facts known up to that time on the germination of the basidiospores of organisms in the Gasteromycetes. Significantly, reports on the germination of the basidiospores of _C. gigantea_ were absent. Thus, it appears that the factors known about the germination of these spores are published by the above authors (1960, 1961, 1962).

The first successful attempt these authors had at germinating the basidiospores of _C. gigantea_ employed liquid barley extract medium. This method required 7-9 weeks before colonies derived from germinated spores became visible, and then, only 1 spore in 20 million was found to germinate. Their next and most important finding was based on a technique introduced by Fries. Fries (1941) was successful in germinating the basidiospores of several genera of Gasteromycetes by the following method: (1) basidiospores were seeded onto the surface of malt extract agar, and (2) a yeast, "Hefe X", which is now believed to be _Rhodotorula_ mucilaginosa was inoculated at 8-10 points on the surface of the agar. Bulmer and Beneke (1961) modified this technique in the following manner: the spores were first seeded into malt extract agar onto which a top layer of the same medium was added, and secondly, the yeast, Rhodotorula mucilaginosa var. sanguinea (Jorgensen) Harrison (=R.m.) was added at 4 equidistant points on the surface of the top layer (see Materials and Methods section of this text for greater detail). Using this method, Bulmer and Beneke (1960, and 1961) increased germination to approximately three hundred per million.

The following are some of the most important findings of Bulmer and Beneke (1960, 1961, and 1962). The presence of the yeast was required in studying all of the variables. Chloromycetin at a concentration of 6 parts per million was found optimal for eliminating bacterial contamination without adversely affecting the germination percentages. The optimum temperature for germination was found to be between 24 and 26 C. The concentration of spores also affected germination. The percentage of spores germinating from different sporophores was different. Attempts to replace the growing yeast with the vitamins, biotin, thiamine, pyridoxine, inositol, and riboflavin were unsuccessful. The presence of _C. gigantea_ co-cultures did not substitute for the yeast. Information on the highly involved relationship between storage temperatures and intervals was also presented. In addition, numerous spore treatments and various media were reported ineffective in stimulating germination.

Bulmer and Beneke (1964) reported on the germination of the basidiospores from several species of puffballs in the general _Lycoperdon_ and _Scleroderma_. They found that these spores were affected by the yeast in the same manner as those of _C. gigantea_. Bulmer (1964) also reported successful germination of basidiospores of 42 additional species from 11 genera of Gasteromycetes using the yeast co-culture technique. He noted that these basidiospores were similar to those of _C. gigantea_ in that the percentage of germination was extremely low, and that an incubation period of several weeks was required before colonies became visible.

Dowding and Bulmer (1964) made cytological studies of _C. gigantea_ and 4 species of _Lycoperdons_. They found the basidiospore-forming mechanism in the puffballs studied to be similar to that already studied in mushrooms. Furthermore, no abnormalities occurred in the mechanism which might explain the low percentage of germination.

Duggar (1901) investigated the germination of the basidiospores from several _Boletus_ species and two species of _Coprinus_. He failed to germinate any of the spores on several media. He was, however, successful in germinating the basidiospores of _Coprinus micaceus_ by using a bean decoction. Ferguson (1902) reported that although the basidiospores of _Agaricus campestris_ germinated on many media, consistently high percentages of germination were obtained only if a living co-culture of _A. campestris_ was present.

Swartz (1928) and Kaufmann (1934) reported several unsuccessful attempts at germinating the basidiospores of several species of the puffballs in the genera _Lycoperdon_, _Bovistella_, and _Calvatia_.

Brown (1946) published a review article on biostimulators of various germination processes that occur in nature. He cited several examples of pollens, seeds, and fungal spores which require the association of some other living organism for germination. He concluded that the activators are probably metabolites of the associated organism, and that several modes of action are possible and probable.

Lange (1948 demonstrated that the basidiospores of _Coprinus_ species were stimulated by the growing germ tubes of other germinating spores, or by the growing mycelium of the same species. He also reported that the spores germinated at a rate of 0.01-1.0 percent on horse dung agar. Heat treatments of the spores had little or no effect on germination.

Kneebone (1950) investigated the germination of the basidiospores of approximately 175 species in the sub-class Hymenomycetes. The following are some of his conclusions and generalities. Those fungi with mycorrhizal relationships were ones whose spores were hardest to germinate. The amount of germination was low (usually less than 1 percent) even though most of the spores were viable as indicated by tests with 2,3,5, triphenyltetrazolium chloride. Light had little or no effect on germination. Some of the spores were stimulated by extracts of various organic substrata. There was, however, no correlation between the extracts which stimulated germination and the substrata on which the sporophores grew. He also reported that there was no evidence that furfural stimulated germination, and furthermore he observed that heat shocking, if anything, was detrimental to germination[1]. The presence of living yeast co-cultures

[1]Sussman (1953) showed that furfural was very effective in stimulating the germination of Neurospora ascospores;

had no effect on germination. He felt that nutrition was not a critical factor, but that permeability was very important in the germination process. Kneebone's conclusions were that spore metabolism was very slow, and that a critical enzyme or precursor was required to initiate the process. He seems to be of the opinion that germination may be stimulated by several different mechanisms since the factors found to be effective were so diverse.

Mandels (1955), while studying the germination of Myrothecium verrucaria spores, observed that growth ceased shortly after germ tube emergence, and that a lag phase existed for some time. The presence of yeast extract, contaminating bacteria and fungi, or biotin eliminated this lag period. Several lines of evidence were cited which indicated that the factor responsible for eliminating this lag period was biotin. Since this organism is autotrophic for biotin, it was suggested that possible alterations in permeability shortly after germination allowed biotin to diffuse out of the cells; growth only ensuing after it was established in a suitable concentration. This paper offered a philosophical

heat shocking was also important in stimulating the germination of these spores.

point concerning the distinction between germination (germ tube emergence) and early growth of the mycelium.

Good and Spanis (1958) studied several factors involved in the germination of the basidiospores of Fomes igniarus var. populinus. The spores were found to germinate between pH 3 and 7, but pH 4 was optimum. Of the contaminants that invaded the plates, none was found to stimulate germination. They reported that 8 percent malt extract was optimum for germination, although a few spores did germinate in a basal medium plus glucose or maltose. The spores also germinated well in extracts made from wounded portions of certain trees. Chromatographic studies revealed that small amounts of carbohydrates were present in these extracts. Judging from the fact that glucose and maltose supported germination, but did not augment germination when added to malt or tree extracts, they concluded that some other factor(s) must be present in the extracts which stimulated germination. Extracts from aged wounds promoted more germination than those from fresh wounds; they attributed this to alterations in the substratum by saprophytes.

Hafiz and Niederpruem (1963), Ratts et al. (1964), and Niederpruem (1964) have presented papers concerned with the metabolism and metabolic machinery of the germinating basidiospores of _Schizophyllum_ _commune_. The first paper in this series pointed out that the spores did not germinate in Petri plates which contained KOH in center wells. Thus, there was an indication that CO_2 might be required for germination. The second paper in this series listed many enzymes of the Krebs cycle and the respiratory chain which were identified in cell-free extracts of _S_. _commune_ basidiospores. The last paper presented results from cytochemical and manometric studies. The cytochemical studies indicated the presence of stored carbohydrates and the absence of stored lipids in the basidiospores. A respiratory quotient of near one suggested that the spores metabolized carbohydrates. Several carbon sources were found to markedly increase the endogenous respiration. The use of several metabolic inhibitors indicated the presence of a functional cytochrome system in these spores.

Two excellent review articles on the generalities of fungus spore germination were prepared by Gottlieb (1950, and 1964). For general information on the physiology of spore germination, the writer suggests: Cochrane (1958), Foster (1949), Lilly and Barnett (1951), and Gray (1959).

Additional knowledge concerning the physiology of C. gigantea has been gained from studies directed mainly at calvacin production and is cited below. Roland *et al.* (1960) reported on the antitumor activity of calvacin against 24 different types of tumors. Information was also presented on screening problems involved with "scaling up" the calvacin production. Sedlmayr (1960), and Sedlmayr, Beneke, and Stevens (1961 a,b) studied certain aspects of the growth requirements of *C. gigantea*. The emphasis was on carbohydrate utilization and vitamin requirements. Many carbohydrates were utilized, and all strains required thiamine. Cook (1960) showed that the variations in calvacin production by the same strains differed depending on the carbohydrate present in the growth medium. Beneke (1963), in his presidential address to the Mycological Society of America, summarized all the work done to that time on the various aspects of the *C. gigantea* studies. He also reported for the first time the presence of the dolipore-parenthesome type of septum in *C. gigantea*.

MATERIALS AND METHODS

Collection and Storage of Sporophores

The sporophores used for these experiments were collected, allowed to dry completely at room temperature, placed individually into plastic bags, and finally stored in cardboard boxes at room temperature. Collection dates and locations of the puffballs can be found in the Appendix of this manuscript. The identification numbers for the sporophores used for each experiment will be cited with the data. Taxonomic characteristics and locations for finding the giant puffball, *Calvatia gigantea*, can be found in A. H. Smith's book (1951).

Media Used

All of the dehydrated media used was Difco brand, and was prepared according to the directions on the bottles. The medium giving the highest amount of germination, and hence used for the control was Difco Malt Extract to which 20 grams per liter of agar were added. The basal medium used was essentially that of Lindeberg's (1941). The composition of the basal medium was as follows:

KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g

$FeCl_3$(sol. 1/500)	0.5 ml
$MnCl_2$(sol. 0.1 M)	0.5 ml
$CaCl_2$(sol. 0.1 M)	5.0 ml
$ZnSO_4$(sol. 1/500)	0.5 ml
Distilled H_2O	994.0 ml

To the basal medium, various carbohydrates and nitrogen sources were added. When used, chloromycetin at a concentration of 6 parts per million was added prior to autoclaving.

Apparatus and Sterilization Procedures

Waring blendors were used to prepare the spore suspensions, and the concentrations of spores in the suspensions were determined with an A-O haemacytometer. Pipettes were used to dispense the spores. Standard Pyrex Petri plates were used for most experiments, although some Falcon brand disposable plates were employed.

Glassware was sterilized by dry air at 165-180 C for 3 hours. Most media were sterilized by autoclaving at 121 C, 15 psi for 15 minutes. Details on the sterilization of special preparations will be discussed later for the purpose of clarity.

Basic Procedure Followed

The basic procedure followed for these studies was a modification of Fries' technique (1941, and 1943) as described by Bulmer (1960) and Bulmer and Beneke (1961).

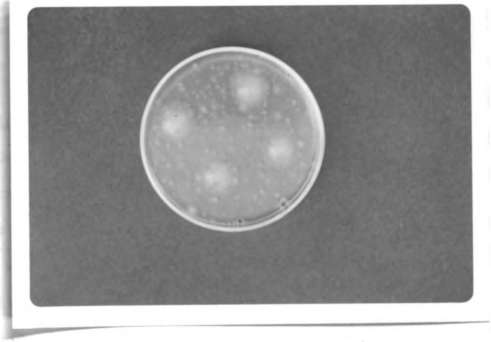
To prepare the inoculum, the basidiospores were first removed from the sporophore with sterile forceps a few mm below the peridium to avoid any contaminating agents present on the surface. A spore mass, approximately the size of a large pea, was then homogenized in a sterile blendor which contained 200 ml of sterile water for about 20 seconds. The spore concentration was determined after homogenization. The desired number of spores was pipetted into large test tubes which contained 25 ml of sterile molten medium, and then the contents poured into sterile Petri plates (entire process will be called "seeding"). At this point, the plates will be called "1 Layer" plates. In some cases, a second layer of medium was added after the first had solidified; such plates will be referred to as "2 Layer" plates. The next step was to inoculate the Rhodotorula mucilaginosa (R.m.) at 4 points on the surface of the agar. Finally, the plates were sealed with rubber gaskets. Plastic plates were sealed in polyethylene bags. The plates were incubated in an inverted position in a constant temperature room at 25 C. This room was kept dark except when entered for the removal of materials. A plate with the R.m. and germinating spores is shown in Plate I.

Special techniques and modifications to the basic procedure will be cited in the text for purposes of clarity and brevity.

Determination of Results

The amount of germination was determined by counting the number of macroscopically visible colonies only, and spores germinating under the R.m. colonies were not counted. All results were calculated on the basis of the number of spores germinating per million. All data given are averages made from counts made of not less than seven plates. All experiments were repeated at least once.

PLATE I



A standard 4-point control plate after 35 days incubation. The four pink colonies are the Rhodotorula mucilaginosa, and the many small colonies are mycelium of C. gigantea which were derived from germinated basidiospores.

RESULTS AND OBSERVATIONS

Physical and Chemical Stimulators of Germination

The purpose for using many of the following treatments known to be effective in the germination of other spores was twofold: (1) to attempt to increase the rate and/or amount of germination, and (2) to attempt to replace the growing yeast by physical or chemical stimulators of germination.

Physical Treatments and Incubation Variables

One of the first experiments was to determine how heat treatment would affect germination. To accomplish this, a spore suspension was prepared, and aliquots of this suspension were removed and heated for 10 minutes at 45, 50, 55, 60, 65 and 70 C. Approximately 75 percent of the spores treated at 50 C and 100 percent of those treated at 55 C or higher were inactivated. There was no effect on germination by treatment at 45 C. Plates without the R.m. showed no germination.

To study the effects of light, a number of control plates were kept in a constant temperature room under the conditions described before. The seeded plates which were exposed to light were left at room temperature and exposed to the fluorescent lights of the laboratory and the diffuse sun light which entered through the windows. Other plates were incubated beside the light exposed plates, however, they were placed in light-proof metal boxes.

The results shown in Table I suggest that light was slightly inhibitory to germination. Although there was no difference in the amount of growth or color of the R.m. colonies, the possibility still exists that the metabolism of the yeast was affected by the light.

Table I

Effect of Light on Germination

	Germination/Million #1601	#1608	Diameter of R.m.
Incubated in Light	242	219	18±2 mm
Incubated in Dark	270	248	18±2 mm
Control	277	234	18±2 mm

Medium used: 1.0% malt extract; 1 layer of medium

To determine if the size or nature of the Petri plates used was an important variable, the following experiments were conducted. Pyrex plates (15x100 mm, and 30x100 mm), and Falcon brand plastic plates (15x100) were seeded and inoculated in the usual manner. There was no difference in the amount of germination due to the types of plates with the two sporophores tested.

It is well known that heat, especially at autoclaving temperature, will affect media and subsequently the parameters being measured. To determine if the means of sterilizing the media had an effect on germination, several sterilization methods were employed.

Seitz filtered media were prepared by adding 12.5 ml of filtered media to 12.5 ml of sterile molten (48 C) 4 percent water-agar, and seeded. The 2 percent media which were autoclaved for 15, 30, and 45 minutes were cooled to 42 C, and seeded. After autoclaving for 15 minutes, the agar in some of the tubes was allowed to solidify, and then steamed for 10 minutes to re-melt the agar.

The results showed that malt extract which was autoclaved and steamed supported approximately 20 percent less germination than if Seitz filtered. Germination on the defined media showed no variation due to the method of sterilizing. However, due to the greater amount of time required and the greater chance for contamination encountered by the filtration method, it was decided to sterilize all other media by autoclaving. Sporophores #1601 and #1609 were used.

Since it is possible that a germination inhibitor was present in the spores or spore wall, the spores were washed (leached) five times with sterile distilled water. The results using malt extract showed that, even after washing, the presence of the yeast was necessary before germination would occur. When the defined medium was used, washed spores showed germination percentages similar to those that had not been washed (whether the R.m. was present or not).

Tween 80, a wetting agent, at concentrations of 0.25, 0.50, 1.0, and 2.0 percent had no effect on germination.

To determine if the hydrogen ion concentration affected germination, the pH was adjusted with HCl and NaOH. A NaCl control was also run to detect any effect due to the additional sodium or chloride ions.

The findings shown in Table II demonstrate that the spores germinated over a wide range of pH values, however, there was an optimum between pH values of 4.3 and 5.0.

Effects of Chemical Stimulators

The basidiospores from sporophores #1601 and #1609 were treated with the following concentrations of furfural; 1.0, 0.5, 0.1, 0.01, 0.001 and 0.0001 M. Some of the spores were treated by soaking them for periods of 24 hours, or for 7 days in the furfural solutions. The spores were then seeded into malt extract which had the above concentrations of furfural in the medium.

Table II

Effect of pH on Germination

pH Prior to Autoclaving	pH at the Time of Counting	Germination/Million #1601	#1605	Diameter of R.m.
3.5	-	-	-	-
4.0	3.7	187	124	19±2 mm
4.5 (control)	4.3	306	222	"
5.0	4.6	304	209	"
5.5	5.0	311	230	"
6.0	5.1	297	223	"
6.4	5.4	259	196	"
7.0	5.6	189	102	"
7.7	6.2	107	98	"
4.5 (NaCl)	4.3	312	206	"

Medium used: 1.0% malt extract; 1 layer of medium

Seitz-filtered gibberellic acid solutions were added aseptically to molten malt extract agar to give the following concentrations; 1/200, 1/400, 1/1,600, 1/3,200, 1/6,400, and 1/12,800 (w/v). Two sporophores were used.

Dinitrophenol was incorporated into the various media at concentrations of $1x10^{-4}$, $5x10^{-5}$, $1x10^{-5}$, $5x10^{-6}$, and $1x10^{-6}$ M. The media used were malt extract, and the basal medium plus glucose and KNO_3. Two sporophores were used.

Furfural and gibberellic acid had no effect on germination in either rate or amount, nor did they replace the growing R.m. Dinitrophenol at concentrations of $1x10^{-4}$, $5x10^{-5}$, and $1x10^{-5}$ inhibited germination completely, while the lower concentrations had no effect on the rate or amount of germination.

None of these agents had a visible effect on the growth of the yeast.

Nutritional Aspects of Germination

The literature on fungus spore germination contains no simple key to the nutritional requirements for this process. The reason is that different fungus spores germinate over an entire spectrum of nutritional conditions, ranging from distilled water to complex infusions and extracts of natural products. Since the basidiospores of *C. gigantea* had previously been germinated only in extracts, it was impossible to know what these media supplied. In addition, the presence of the growing yeast complicated the picture even more. Hence, it was thought that if a defined medium could be found which would support germination, a step would be made in resolving the question of whether simple nutrition alone was the key to the process or whether the malt extract contained a factor(s) which was stimulatory or necessary for germination. Fortunately, the first basal medium used supported the germination of a small number of spores. The ingredients of the basal medium are given in the Materials and Methods section.

Effects of Various Carbon Sources on Germination

Table III relates the findings of a preliminary investigation in which several carbohydrates were added to the basal medium. Although the R.m. growth was indicated with (+), it can be approximated from other data that each (+) would represent 2-3 mm. These results showed that the basidiospores of *C. gigantea* could be germinated without the yeast being present. Higher germination values were recorded, however, when the R.m. was present. The carbohydrates supporting the greatest amount of germination were maltose, soluble starch, and sucrose. Although the pH varied with different carbohydrates, there was no change in the pH due to the presence of the growing yeast. Citric acid, glucose-1-PO_4, K-acetate, and glycerol were also tested as possible carbon sources, but none supported germination.

These findings prompted the evaluation of the effects of various carbohydrates on different sporophores. Furthermore, it could simultaneously be determined if the germination rate was different on the synthetic medium.

Table III

Preliminary Investigation of Carbohydrates Utilized During Germination

Carbohydrate	Germination/10^6 R.m.	Germination/10^6 No R.m.	pH R.m.	pH No R.m.	R.m. Growth
Arabinose	0.0	0.0	4.2	4.2	3+
Xylose	0.0	0.0	4.0	4.0	0
Glucose	0.0	0.0	4.1	4.1	3+
Fructose	0.0	0.0	4.5	4.5	3+
Galactose	0.0	0.0	4.2	4.2	2+
Rhamnose	0.3	0.0	4.7	4.7	1+
Sorbose	0.0	0.0	4.0	4.3	1+
Cellobiose	4.0	0.0	4.5	4.5	3+
Lactose	2.0	0.0	4.6	4.6	1+
Maltose	39.0	13.0	4.4	4.4	3+
Sucrose	15.0	7.0	4.4	4.2	3+
Threhelose	4.1	0.0	4.5	4.5	3+
Inulin	0.0	0.0	4.6	4.6	3+
Dextrin	4.3	5.0	4.7	4.7	2+
Glycogen	1.7	0.0	4.7	4.7	2+
Sol. Starch	19.0	14.0	4.5	4.5	2+
No CHO	0.0	0.0	4.9	4.9	0
Malt Extract	394.0	0.0	4.3	4.3	10+

Medium used: basal plus 2.0% carbohydrates and 0.5% NH_4 tartrate; 2 layers of medium; sporophore #1601

There were differences in the rates of germination when various sporophores were tested, as the results in Table IV show. Likewise, it appears that germination was higher and began earlier if the yeast was present. Data not presented showed that galactose supported the germination of approximately 10 spores per million when the yeast was present and sporophores #1605 and #1608 were used. Dextrin supported about the same amount of germination as galactose with #1608. Fructose did not support germination of any spores. The amount of R.m. growth for these three sugars was similar to that given for the other sugars in Table IV.

Table IV

Variations of Carbohydrates Utilized During Germination by Sporophores #1601, #1605, and #1608

Carbohydrate and Sporophore	Days after Seeding	Germination/10^6 R.m.	Germination/10^6 No R.m.	Diameter of R.m. Colony (mm)
Glucose				
#1601	30	0.0	0.0	
	35	1.9	0.0	
	45	3.2	3.2	
	55	3.4	3.4	7±2
#1605	30	1.6	0.0	
	35	3.0	0.2	
	45	9.0	0.2	
	44	10.0	0.4	7±2
#1608	30	1.3	0.0	
	35	1.5	0.0	
	45	1.5	0.0	
	55	1.7	0.0	7±2
Sucrose				
#1601	30	6.2	2.4	
	35	12.0	9.0	
	45	17.0	12.0	
	55	19.0	13.0	7±2
#1605	30	3.0	0.2	
	35	12.0	0.8	
	45	15.0	0.9	
	55	16.0	0.9	7±2

Table IV (Continued)

Carbohydrate and Sporophore	Days after Seeding	Germination/10^6 R.m.	Germination/10^6 No R.m.	Diameter of R.m. Colony (mm)
#1608	30	25.0	2.4	
	35	41.0	5.0	
	45	50.0	7.0	
	55	51.0	7.0	7±2
Maltose				
#1601	30	21.0	0.0	
	35	44.0	1.9	
	45	51.0	12.0	
	55	53.0	18.0	7±2
#1605	30	2.3	0.0	
	35	8.0	0.0	
	45	23.0	6.1	
	55	26.0	8.0	7±2
#1608	30	0.8	0.0	
	35	7.0	0.3	
	45	18.0	0.4	
	55	19.0	0.4	7±2
Sol. Starch				
#1601	30	4.0	2.2	
	35	11.0	7.0	
	45	15.0	10.0	
	55	16.0	12.0	7±2
#1605	30	2.1	1.0	
	35	9.0	4.0	
	45	20.0	7.9	
	55	21.0	13.0	7±2
#1608	30	27.0	18.0	
	35	33.0	19.0	
	45	46.0	27.0	
	55	48.0	34.0	7±2

Table IV (Continued)

Carbohydrate and Sporophore	Days after Seeding	Germination/10^6 R.m.	Germination/10^6 No R.m.	Diameter of R.m. Colony (mm)
Glycogen				
#1601	30	0.0	0.0	
	35	0.4	0.0	
	45	2.1	0.6	
	55	4.0	1.1	7±2
#1605	30	2.2	0.3	
	35	11.0	1.7	
	45	18.0	5.0	
	55	22.0	5.0	7±2
#1608	30	6.5	0.7	
	35	8.0	1.7	
	45	11.0	4.4	
	55	12.0	5.0	7±2
Malt Extract				
#1601	40	55.0	0.0	
	35	146.0	0.0	
	45	256.0	0.0	
	55	261.0	0.0	20±2
#1605	30	89.0	0.0	
	35	205.0	0.0	
	45	297.0	0.0	
	55	308.0	0.0	20±2
#1608	30	67.0	0.0	
	35	145.0	0.0	
	45	233.0	0.0	
	35	236.0	0.0	20±2

Medium used: basal plus 1.0% carbohydrates and 0.5% KNO_3; 1.0% malt extract; 1 layer of medium; pH values were similar to those in Table III

Seven concentrations from 0.25 percent to 2.0 percent of glucose, soluble starch, and sucrose revealed that the maximum germination occurred between 0.75 and 1.25 percent. Using similar concentrations of malt extract, it was found that 1.0 percent of this medium gave the maximum amount of germination; germination decreased sharply between 3 and 6 percent. Germination was approximately eight-fold greater at 1.0 percent than at 6 percent. Two sporophores were used.

<u>Germination Versus Growth on Various Media</u>

Since it could be argued that the observed germination on a given carbohydrate was only a matter of selecting those strains which could grow on that particular carbohydrate, the following was done. Small portions of a single spore colony on a given carbohydrate were transferred to plates containing the same basal ingredients but different carbohydrates. The growth was measured every two days for a three week period. The carbohydrates used for the germination media and transfer media were: glucose, maltose, soluble starch, and sucrose.

The results showed that, disregarding the slight differences in the amount of mycelium used to make the transfers, all transfers grew well and at the same rate regardless of the carbohydrate used for germinating the spores. This would indicate, at least for the colonies and sugars used, that the spores contain the metabolic machinery to utilize the carbon sources equally.

Since all of the carbohydrates tested contained glucose, it might be argued that differences in osmotic pressure were producing an effect. This possibility should be considered in the light of two other experiments. Chromatographic studies (page 57) revealed that sucrose was hydrolized to glucose and fructose soon after the R.m. was added. Hence, the sucrose medium would have had the same osmotic pressure as the medium containing an equal weight of glucose. The previous studies which showed that fructose did not support germination; hence, there should have been no additive effect.

Effects of Various Nitrogen Sources on Germination

The information in Table V demonstrates that differences existed between the amount of germination and the nitrogen sources present in the medium. It appears that KNO_3 was the best nitrogen source. Furthermore, there is a subtle suggestion that ammonium salts were inhibitory unless the

Table V

Investigations on Nitrogen Sources Utilized During Germination

Carbohydrate and Nitrogen	Germ./10^6 R.m.	Germ./10^6 No R.m.	pH R.m.	pH No R.m.	R.m. Growth
Glucose					
KNO_3	4.5	0.7	4.2	4.4	3+
NH_4Cl	0.0	0.0	4.4	4.5	3+
NH_4NO_3	0.0	0.0	4.3	4.1	3+
$(NH_4)_2SO_4$	0.0	0.0	4.0	4.0	3+
NH_4Tartrate	0.0	0.0	4.1	4.1	3+
$Mg(NO_3)_2$	6.0	4.0	-	4.1	3+
no Nitrogen	3.2	0.0	4.3	4.3	2+
Sucrose					
KNO_3	11.0	4.0	4.0	4.1	3+
NH_4Cl	0.0	0.0	4.0	4.0	2+
$(NH_4)_2SO_4$	0.0	0.0	4.1	4.1	3+
$Mg(NO_3)_2$	11.0	6.0	4.1	4.1	3+
NH_4Tartrate	17.0	0.0	4.3	4.3	3+
NH_4NO_3	0.0	0.0	4.0	-	2+
no Nitrogen	4.0	1.1	4.3	4.3	2+
Maltose					
KNO_3	47.0	20.0	4.4	4.5	3+
NH_4Cl	2.0	0.0	4.1	4.1	2+
NH_4NO_3	0.0	0.0	4.4	4.4	3+
$(NH_4)_2SO_4$	0.0	0.0	4.3	4.3	3+
NH_4Tartrate	43.0	18.0	4.5	4.5	3+
no Nitrogen	12.0	4.6	4.5	4.5	2+
Sol. Starch					
KNO_3	18.0	16.0	4.5	4.5	2+
NH_4Cl	3.1	0.0	4.6	4.5	2+
NH_4NO_3	4.0	1.2	4.5	4.5	3+
$(NH_4)_2SO_4$	0.0	0.0	4.4	4.5	2+
$Mg(NO_3)_2$	6.0	6.0	4.5	4.5	2+
NH_4Tartrate	4.2	0.0	4.6	4.6	3+
Malt Extract (control)	264.0	0.0	4.3	4.3	10+

Medium used: basal plus 2.0% carbohydrates and 0.5% nitrogen sources; 1 layer of medium; sporophore #1601 yeast was present. Similar results were obtained with 2 other sporophores.

The amino acids, cysteine, histidine, glutamine, glycine, alanine, arginine, and casamino acids were also used as nitrogen sources. Germination was only observed when maltose was the sugar and glycine or alanine were the nitrogen sources, and the yeast was present. The yeast growth was 7-8 mm with the amino acids and 12 mm with the casamino acids.

The fact that some spores germinated in both experiments without a nitrogen source corroborates the work of Knaysi (1945). He found that the endospores of _Bacillus subtilis_ did not require an exogenous nitrogen source to germinate. Judging from these data, it suggests that one possible action of the yeast is to remove inhibitors from the incubating medium.

Effects of Supplementing the Basal Medium

Malt extract and peptone were added to the basal medium to determine: (1) if, in the absence of the R.m., chemically undefined media were inhibitory to germination, and (2) if the amount of R.m. growth, which is less on the defined medium, could be a limiting factor.

The data in Table VI demonstrate that, when malt extract was added to the basal media, germination was

Table VI

Effects of Supplementing the Basal Medium

Medium	Germination per Million						Diameter of R.m. Colony (mm)
	#1601		#1605		#1609		
	R.m.	no R.m.	R.m.	no R.m.	R.m.	no R.m.	
Glucose							
no N	8.1	1.5	12.0	4.0	5.1	0.8	7±2
KNO_3	5.2	0.9	18.0	8.1	10.0	8.6	8±2
Peptone	0.0	0.0	0.0	0.0	0.0	0.0	13±2
Malt Ex.	147.0	0.0	160.0	0.0	129.0	0.0	15±2
Maltose							
no N	21.0	7.3	21.0	14.0	19.0	9.6	7±2
KNO_3	64.0	38.0	21.0	11.0	73.0	23.0	8±2
Peptone	3.0	0.0	0.0	0.0	0.0	0.0	13±2
Malt Ex.	166.0	0.0	187.0	0.0	116.0	0.0	15±2
Sucrose							
no N	6.3	4.2	14.0	6.7	11.0	0.0	7±2
KNO_3	12.0	9.8	31.0	21.0	23.0	10.0	8±2
Peptone	1.1	0.0	0.0	0.0	0.0	0.0	13±2
Malt Ex.	151.0	0.0	149.0	0.0	114.0	0.0	15±2
S. Starch.							
no N	10.0	11.0	15.0	8.0	12.0	5.5	7±2
KNO_3	24.0	18.0	23.0	19.0	19.0	14.0	8±2
Peptone	0.0	0.0	0.0	0.0	0.0	0.0	13±2
Malt Ex.	154.0	0.0	166.0	0.0	121.0	0.0	15±2
Malt Ex. control	269.0	0.0	330.0	0.0	178.0	0.0	19±2

Medium used: basal plus 1.0% carbohydrates and 0.5% KNO_3, peptone, malt extract; 1.0% malt extract for controls; 1 layer of medium inhibited if the R.m. was absent. If the R.m. was present, the addition of malt extract increased both the amount of germination and the growth of the R.m. The peptone, although it supported good growth of the R.m., inhibited germination in all cases but two (3.0 and 1.1 germinations per million) even when the R.m. was present.

To examine these questions in greater detail, various percentages of malt extract and carbohydrates were mixed. The results are shown in Table VII. Two points can be observed here; 0.1 percent malt extract was inhibitory in half the cases when added to plates that did not have the yeast, and completely inhibitory when 0.3 percent or greater was used, and furthermore, the amount of yeast growth and subsequently the amount of germination became a function of the percentage of malt extract.

Effects of Thiamine

Since the amount of yeast growth was reduced on the basal medium, it was thought that by increasing its growth, germination might also be increased. Thus, thiamine was added to the medium. The results showed that the R.m. growth was increased (7 mm-17 mm) due to the added thiamine, however there was no increase in the amount of germination.

Table VII

Effects of Various Percentages of Carbohydrates and Malt Extract

%Glucose	%Malt	"X"	Germination/Million #1601 R.m.	#1601 No R.m.	#1605 R.m.	#1605 No R.m.	Dia. of R.m.
1.0	0.0		2.1	0.4	11.0	4.1	7±2
0.9	0.1		28.0	0.0	54.0	0.2	10±2
0.7	0.3		81.0	0.0	94.0	0.0	13±2
0.5	0.5		134.0	0.0	197.0	0.0	14±2
0.3	0.7		173.0	0.0	252.0	0.0	15±2
0.1	0.9		248.0	0.0	298.0	0.0	17±2
0.0	1.0		264.0	0.0	316.0	0.0	19±2
%Sucrose	%Malt	"X"					
1.0	0.0		14.0	4.0	16.0	3.4	7±2
0.9	0.1		32.0	0.0	56.0	0.0	10±2
0.7	0.3		78 0	0.0	87.0	0.0	13±2
0.5	0.5		139.0	0.0	201.0	0.0	14±2
0.3	0.7		180.0	0.0	270.0	0.0	15±2
0.1	0.9		241.0	0.0	300.0	0.0	17±2
0.0	1.0		264.0	0.0	316.0	0.0	19±2
%Sol Star.	%Malt	"X"					
1.0	0.0		17.0	14.0	26.0	28.0	7±2
0.9	0.1		45.0	0.1	94.0	1.2	10±2
0.7	0.3		93.0	0.0	122.0	0.0	14±2
0.5	0.5		143.0	0.0	187.0	0.0	15±2
0.3	0.7		178.0	0.0	286.0	0.0	16±2
0.1	0.9		252.0	0.0	309.0	0.0	18±2
0.0	1.0		264.0	0.0	316.0	0.0	19±2
	%Malt	"X"					
	0.1		24.0	0.0	57.0	0.0	10±2
	0.3		87.0	0.0	105.0	0.0	14±2
	0.5		153.0	0.0	191.0	0.0	15±2
	0.7		171.0	0.0	265.0	0.0	16±2
	0.9		257.0	0.0	314.0	0.0	19±2

Medium used: basal plus indicated amounts of carbohydrates and malt extract, and 0.5% KNO_3; 1 layer of medium

When thiamine was added to the malt extract medium there was no effect. Sporophores #1601 and #1608 were used. This suggests that increased diametric growth of the yeast is not the sole factor in increasing germination.

Miscellaneous Media used To Study Germination

The basidiospores from sporophores #1601, #1605, and #1609 were used for the following experiments. The basal medium, to which 5 concentrations from 0.5 to 10.0 percent coconut milk had been added supported a very few germinations when the R.m. was present. Sabourauds-glucose agar did not support germination, however, the amount of R.m. growth was greater than on the malt extract. Corn meal agar to which 0.5 percent yeast extract was added failed to support germination. The R.m. had the same diametric growth as the malt extract controls, but was a pale yellow. Czapek's agar supported no germination and the R.m. grew poorly.

Potato-dextrose agar gave results nearly comparable to those obtained with the malt extract controls. There was no germination without the R.m., and with the R.m., the germination was 75-85 percent of that on the malt extract controls. The growth of the R.m. was not quite as great as with the malt extract, and the colonies had a watery appearance.

Malt extract agar did not support germination. At this point, the writer calls attention to the fact that Difco Malt Extract Agar is not, as a glance at the Appendix will show, merely Difco Malt Extract to which agar has been added.

Manipulation of the Incubation Conditions and Characterization of the Action Provided by the R.m.

One Layer Versus Two Layers of Medium

Bulmer (1961, and 1962) suggested that the function of the added layer of medium above the seeded layer might be to impose semi-anaerobic conditions on the incubating spores. The results in Table VIII show that the amount of germination was essentially the same whether the spores were seeded into the bottom, top, or mixed into both layers. Furthermore the data suggest that the top layer of malt extract serves to provide for an increased amount of yeast growth and subsequently more germination. This is further supported by experiments with the basal medium (Table VIII). This information shows that, with 1 or 2 layers of basal medium, the R.m. growth was the same, and hence there was no major difference in the amount of germination.

This evidence suggests that the action of the yeast is to provide a stimulator rather than remove an inhibitor from the malt extract . The reasoning is that the added malt extract would also add more inhibitor and thus cancel the effect of the increased amount of yeast growth.

Table VIII

Effect of the Number of Layers of Medium

Number of Layers	Germination per Million #1601	#1608	#1611	Diameter of R.m.
Malt "X"				
1 Layer	234	204	318	19±2
2 Layers (spores in bottom layer)	306	277	471	25±2
2 Layers (spores mixed in both layers)	341	265	456	25±2
2 Layers (spores in top layer)	312	261	478	25±2
Basal Medium plus:				
Glucose				
1 Layer	2.1	1.7	7.6	8±2
2 Layers	2.3	1.6	7.9	8±2
Maltose				
1 Layer	37	33	-	8±2
2 Layers	38	31	-	8±2
Sol. Starch				
1 Layer	13	47	22	8±2
2 Layers	13	42	21	8±2

Medium used: basal plus 1.0% carbohydrate and 0.5% KNO_3; 1.0% malt extract

Effects of Petri Plate Orientation

Bulmer (1960) reported that the amount of germination was reduced when the plates were incubated in an inverted position as compared to those incubated upright. The knowledge gained from Table IX explains this observation on the basis that a greater amount of yeast growth occurs on upright plates.

Table IX

Effect of Petri Dish Orientation

Orientation of the Plates	Germination/Million #1601	Germination/Million #1609	Diameter of R.m.
Plates Inverted	226	186	20±2 mm
Plates Upright	291	262	27±2

Medium used: 1.0% malt extract; 1 layer of medium

Effects of the Incubating Atmosphere on Germination

Experiments were designed to determine if the R.m. could stimulate germination if it was isolated from direct contact with the seeded medium. The first was to determine if a volatile stimulator was produced. To do this, tops of 50x55 mm Petri plates were placed as "wells" inside 30x100 mm plates, and sterilized. Sterile malt extract agar was poured into the wells and the yeast inocculated. Finally, seeded medium was added to the area outside the wells. The plates were incubated in both inverted and upright positions. Controls with the wells seeded were used as well as 4-point plates. In none of the trials was germination observed outside the wells. Three sporophores were used.

To further study the effects of the atmosphere above the incubating spores, various trapping substances were placed in center wells, and the area surrounding the wells were seeded and inoculated as usual. After sterilization, 4 ml of the following solutions were added to the wells: H_2O, conc. H_2SO_4, 2N KOH, K_2MnO_4, and mineral oil. The results showed that there was no effect due to the presence of the various trapping substances.

The final attempt to study the possible effects of the incubating atmosphere was done by exchanging the air in the plates. The air was exchanged by opening the plates while in the upright and inverted positions. The plates were opened on the 3, 5, 8, 10, 13, 15, 18, 21, and 25th days after seeding and inoculating. The results failed to show differences between the plates that had been opened and the controls.

Effects of the Number of R.m. Colonies

Since previous data suggested that the amount of germination was a function of the R.m. growth, it was questioned what the effect of different numbers of R.m. colonies would be. The results (Table X) show that, although the total yeast growth is approximately the same, germination increased with the number of colonies added. This data indicates that possibly the amount of diffusion is important, or that the postulated stimulator breaks down as it diffuses outward from the colony. The results were determined after 55 days of incubation.

Table X

Effect of the Number of R.m. Colonies

Number of Colonies	Total Area of R.m. Colonies	Germination/10^6
1 (1-point)	1024 mm^2	59
2 (2-point)	1230 mm^2	123
4 (4-point)	1092 mm^2	259

Medium used: 1.0% malt extract; 1 layer or medium; sporophore #1601

Diffusibility of the Action Provided by the Yeast

To determine the effect of distance over which the action of the yeast was effective in stimulating germination, the following experiment was done. Large prescription bottles were seeded, the medium allowed to solidify (bottles were placed flat-side down), and the R.m. inoculated at the open end.

The results (Figure I) bring to light two points; first, germination occurred in the greatest percentage near the yeast colony and fell-off sharply as the spores became more remote to the colony, and second, the action of the yeast took place over a distance of at least 15 cm. One observation which is not shown by the data is that the spores more remote from the yeast were sometimes the first to germinate; this might be accounted for by differences in growth rate rather than germination rate.

In experiments where the amount of medium in the top layer was varied, it was found that more time was required for germination when the top layer was increased in depth.

To further study the diffusion of the action provided by the R.m., the apparatus in Plate II was chosen. The basis for using this apparatus was that a stimulator should diffuse over the "bridges" and stimulate germination in the seeded medium. On the other hand, if the yeast acted by removing an inhibitor, all of the inhibitor might not diffuse over the bridges to be neutralized by the R.m.

FIGURE I

Effect of Distance from R.m. Colony on Germination

#1601

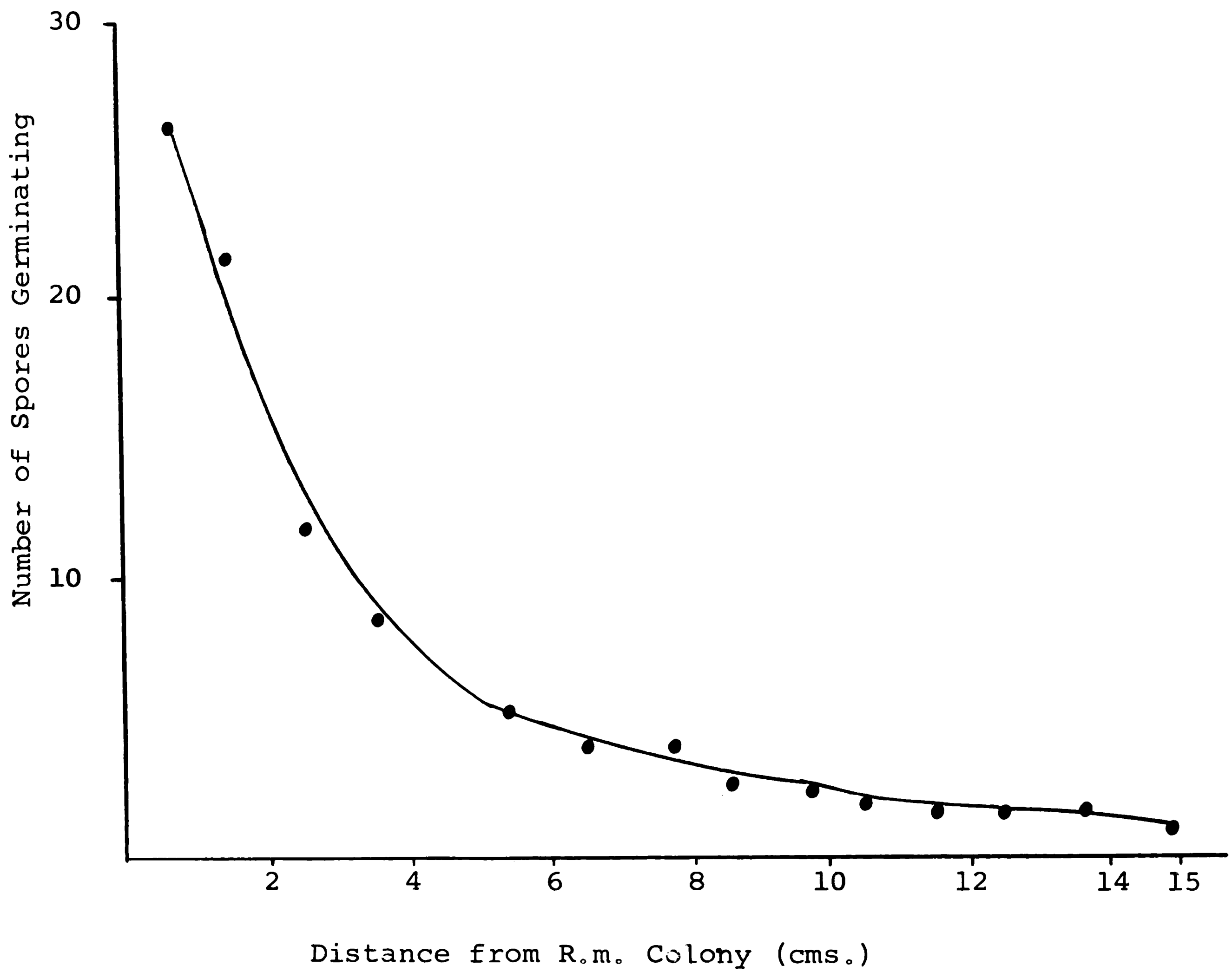
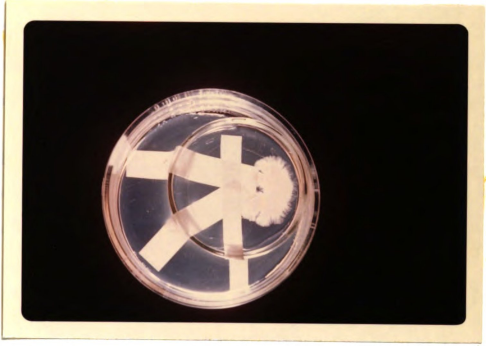


PLATE II



Experimental apparatus for "agar bridge" tests. Inner plate (well) contains malt extract agar and R.m. colony. The outer plate has malt extract agar which is seeded with basidiospores of C. gigantea. The well is connected to the outer plate by paper bridged which are covered with agar.

The bridges were made of filter paper, cotton string, and glass strips. The plates were assembled and the bridges inserted prior to autoclaving. Next, the center wells were filled with malt extract agar, and the outer plates seeded. The final step was to add molten water-agar to the bridges with a pipette; several layers were added so that the agar was 1-2 mm thick. No germination was found to occur with the two sporophores tested.

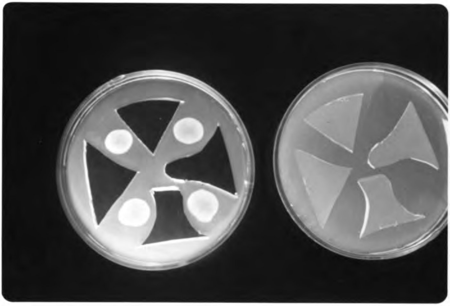
Transfer of the Postulated Stimulator via Agar Blocks

The basis of this experiment was much the same as that of the classical auxin experiments in which the auxin was allowed to diffuse into agar blocks from a cut-off cole-optile tip. This block in turn was transferred to the stump of a decapitiated coleopile and the elongation response observed. Thus, if a compound is produced by the R.m. which diffuses into the ambient medium, it should follow that this compound would be present in the medium. Proceeding on this assumption, one might suppose that by removing blocks of the agar near the yeast colony and transferring these blocks to previously seeded plates, the compound would diffuse from the blocks into the seeded plates; then the germination response would occur.

To determine if this proposal was true, a series of Petri plates was seeded. At the same time, a second series was seeded and inoculated with the R.m. The third series was seeded and inoculated. The following procedure was then carried out after 5, 10, 15, 20, 25, and 30 days of incubation. Blocks were cut from the seeded and inoculated plates; similar blocks were cut from plates that had been inoculated only. These blocks were then transferred to the plates which had been seeded. An illustration of the experimental arrangement is shown in Plate III. The plates were held for 8-10 weeks after the blocks had been transferred.

The results showed that none of the basidiospores in the plates receiving the agar blocks germinated. The agar blocks from the series of plates which had been both seeded and inoculated did have a few colonies. These colonies were present only in those blocks which had been incubated for 20, 25, and 30 day periods. This latter observation supports the results of Bulmer (1960). He found that growing mycelium of C. gigantea did not enhance germination.

PLATE III



An illustration of the attempt to transfer the postulated stimulator from an R.m. inoculated plate (left) by agar blocks to a plate seeded with _C. gigantea_ basidiospores (right).

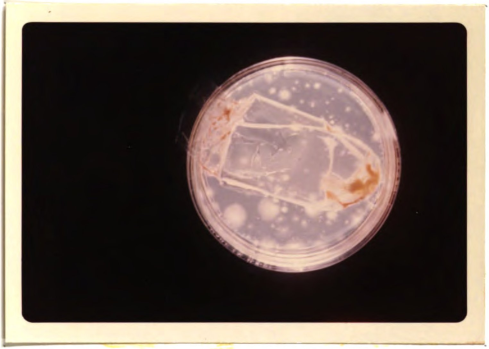
Effects of Separating the R.m. from the Spores with a Dialysis Membrane

Studies were undertaken to ascertain if the stimulatory action of the yeast on the spores would take place if a dialysis membrane was inserted between them. To accomplish this, the experimental arrangement shown in Plate IV was used. Although germination did occur, it was observed that it was retarded approximately 2 weeks in comparison with 4-point control plates. To show that the yeast had not escaped, the bags were removed and streaked onto malt extract agar plates. Smears were also made of the seeded medium which had been in contact with the bags. In both cases the smears were negative; indicating that the yeast had not penetrated the bags. Sporophores #1601, #1605, and #1609 were used.

To quantify any differences in the amount of germination due to the presence of the dialysis membrane, the experimental apparatus shown in Plate V was employed.

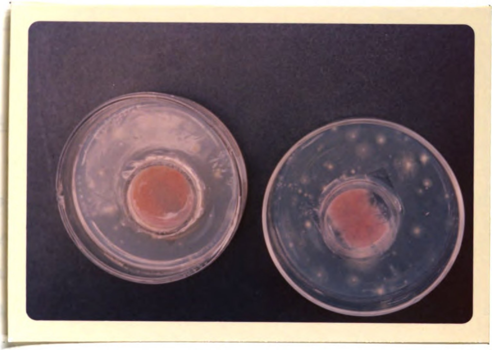
The results in Table XI show that the membrane decreased both the rate and the total number of spores germinating. Smears showed that no yeast had escaped through the membrane.

PLATE IV



Colonies derived from germinated basidiospores of C. gigantea after 9 weeks of incubation. The R.m. was retained within a dialysis membrane.

PLATE V



A comparison of the amount of germination when a dialysis membrane was used to separate the basidiospores of C. *gigantea* from the yeast. The plate on the left has a membrane inserted between the yeast and the spores. The plate on the right is a control without the membrane.

Table XI

Quantitative Results Using Dialysis Membrane to Separate the R.m. from the Incubating Spores

Days after Seeding and Inoculating	Germination per Million #1601		#1603	
Membrane:	present	absent	present	absent
30	0	87	0	93
60	78	252	84	279
90	132	273	143	291

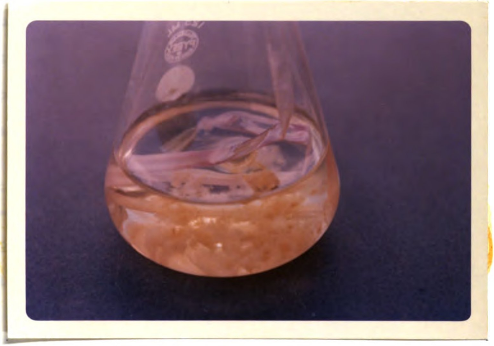
Medium used: 1.0% malt extract; 1 layer of medium with chlormycetin added

Germination in Liquid Malt Extract via Dialysis Membrane

The procedure followed for these experiments was much the same as in the preceeding experiments. However, smaller dialysis tubing and 125 ml Erlenmyer flasks which contained 50 ml of malt extract broth were used (Plate VI). The results were obtained by two methods. One method was to examine the broth microscopically for the presence of germ tubes. Aliquots were also centrifuged to concentrate the spores. The other method was to pipette aliquots of the broth onto malt extract agar (plate-out), and wait for the appearance of visible colonies.

Microscopic examinations for germinating spores were negative for the first nine weeks of incubation; after this, germ tubes began to emerge. The plated-out aliquots did not show colonies until the 11th week (suspensions had been

PLATE VI



Colonies of mycelium derived from germinated basidiospores of C. gigantea in malt extract broth. The R.m. is retained within the dialysis membrane. The picture was taken 17 weeks after the spores and yeast were inoculated.

plated-out on the 9th week). This points out that 10-14 days are required for the germlings to grow to visible colonies. It should be pointed out that the yeast within the membrane was pale pink until about 4 weeks after inoculation before it obtained the normal color.

Quantitative results showed that approximately 40 percent as many spores germinated in this manner as did the 4-point controls. Sporophores #1601 and #1608 were used, and chloromycetin was added to the broth.

Attempts To Find Active Fractions of the Malt Extract or the R.m.

Filtrates of Malt Extract in which the R.m. Grew

Although one would expect a great deal of difference in the metabolism of an organism growing under different conditions, it was thought that perhaps the postulated stimulator could be produced in broth medium, or possibly that an inhibitor was removed or converted. To test this hypothesis, 2 percent malt extract was placed in flasks, and the flasks inoculated with the R.m. These flasks were allowed to incubate for periods of 10, 20, and 30 days. Prior to seeding, the yeast cells were removed by either Seitz or Millipore filtration and the broth collected.

To 12.5 ml of molten 4 percent water agar, 12-13 ml of this broth was added. The seeding was done in the usual manner. Chloromycetin was added to the water agar. This experiment was tried several times, and 6 different sporophores were used. No germination was ever found to occur.

The effects of Spent Agar Medium

Malt extract agar plates were poured and inoculated with the R.m. They were allowed to incubate for 10, 20, 30, 40, 50, and 60 days. Next, the yeast colonies along with the medium below them were cut out, re-melted, and seeded. The remaining agar in the plates was treated in the same manner. No germination occurred on plates which were not inoculated with the R.m. Plates with the R.m. contained a reduced amount of germination, but the diameter of the R.m. colonies was equally reduced. These findings indicate that the action of the yeast is more than a simple starvation of the incubation medium.

Attempts to Extract an Active Fraction of the R.m.

The purpose of these experiments was to determine if a cell-free preparation could be obtained which would replace the growing R.m. The R.m. cells were acquired from different growing conditions, and the extracts were prepared in different ways.

The cell-free extracts were prepared by breaking the cells in chilled Waring blendors which contained glass beads and 60 ml of homogenizing medium. The extracts were sterilized by Seitz and Millipore filtration, and aliquots of 1, 2, 3, and 4 ml were added to 25 ml of sterile molten malt extract agar.

The individual extracts were prepared as follows: extract #1 was prepared from cells which had been grown on malt extract agar slants for 23 days (10 slants were used), extract #2 was prepared from yeast that was removed from 10 4-point plates (5 weeks old), extract #3 was identical to #2 except that the R.m. was 8 weeks old, extract #5 was the same as #2 except that the yeast was boiled 10 minutes prior to homogenizing, extract #6 was prepared by homogenizing the cells in 1 percent malt extract instead of distilled water (cells were 5 weeks old), and extract #7 was made by blending the agar medium, spores, germlings, and yeast from 10 4-point plates which were 5 weeks old. The results were all negative.

Chemical Analysis of the Incubation Media

Since it appeared obvious from previous experiments that the yeast exerted an effect on the basidiospores via the agar medium, it was thought that perhaps differences in the composition between media with and without the R.m. could be found. The hope was that a unique compound could be found and eluted which, when added to incubation medium, would produce the same effect as the growing yeast.

To study some of the possible changes brought about by the yeast, paper chromatographic and spectrophotometric methods were used. The procedure followed for the chromatography was to remove plugs (8 mm in diameter) of medium from plates with and without the R.m.; the medium was then allowed to diffuse from the plugs into the paper. The solvent used was n-butanol: acetic acid: water (4-1-5 v/v). Several reagents were then used to determine if compounds appeared or disappeared due to the presence of the R.m. The reagents used were: (1) ammonical silver nitrate (Williams et al.) and periodate-benzidine (Litwack) for the detection of carbohydrates, (2) a modified ninhydrin reagent (Litwack) for ninhydrin positive compounds, (3) a molybdic acid reagent (Block) for detecting phosphate esters, and (4) potassium permanganate for aliphatic acids (Block).

The results of analyzing the defined medium showed only one difference that was a result of the R.m. The difference was that, when sucrose was the carbon source, it was hydrolyzed to glucose and fructose. A timed study showed that the hydrolysis began on the third day of incubation and was complete by the twelfth. Attempts to analyze the malt extract medium were unsuccessful due to the large number of compounds present.

Attempts to analyze media spectrophotometrically were accomplished by preparing extracts of media with and without the R.m. The extracts were concentrated by lyophilization. The results showed no apparent increase or decrease in optical density in either the UV or visible regions due to the R.m.

Time-Rate Studies on Germination

To determine what effects prolonged incubation without the yeast would have on the spores; and if the presence of the yeast was necessary for an extended period of time, the following experiments were done.

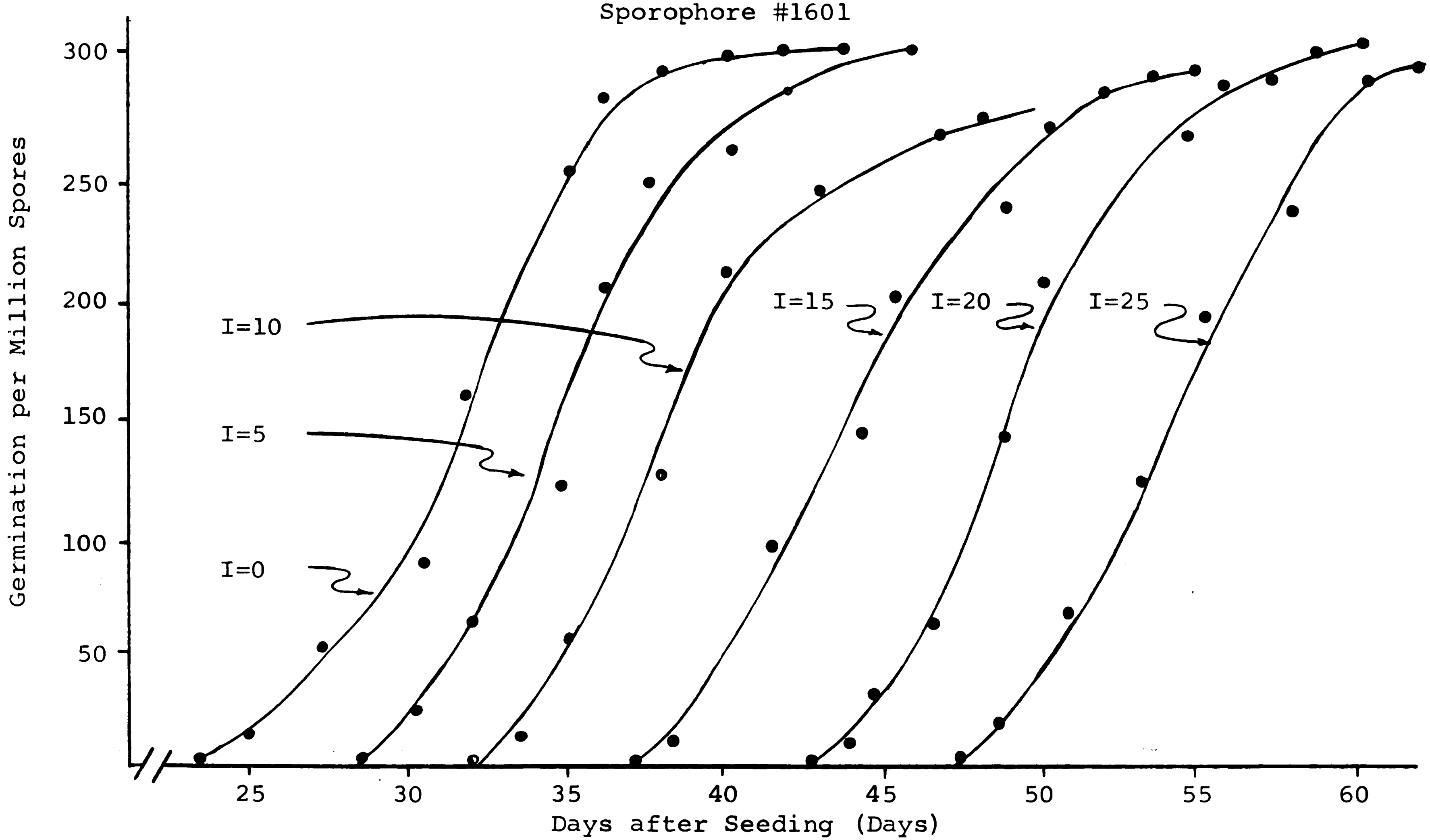
Effects of Adding the R.m. at Various Time Intervals

The first experiment to clarify these points was done by seeding plates at time zero. The yeast was then inoculated on to aliquots of the plates at time zero and at 5 day intervals thereafter. The results are shown in Figure II.

These findings point out that 23-25 days of incubation were required after the R.m. was inoculated before colonies appeared; and that most of the colonies appeared between the 28th and 35th day after the inoculation of the R.m. It also appears that the maximum amount of germination was not affected by prolonged incubation without the R.m. Furthermore, since the lines are essentially parallel, it would seem that there is no differential in the rate of germination due to the prolonged incubation without the yeast. Judging from these figures it appears that the spores will remain dormant in the medium until the yeast is added. Similar results were obtained with #1608.

FIGURE II

Effects of the Time of Addition of the R.m. on Germination
Medium: 2 Layers, 1% Malt Extract
Sporophore #1601



I = time of inoculating the R.m. to the seeded plates.

Effects of Removing the R.m. at Various Time Intervals

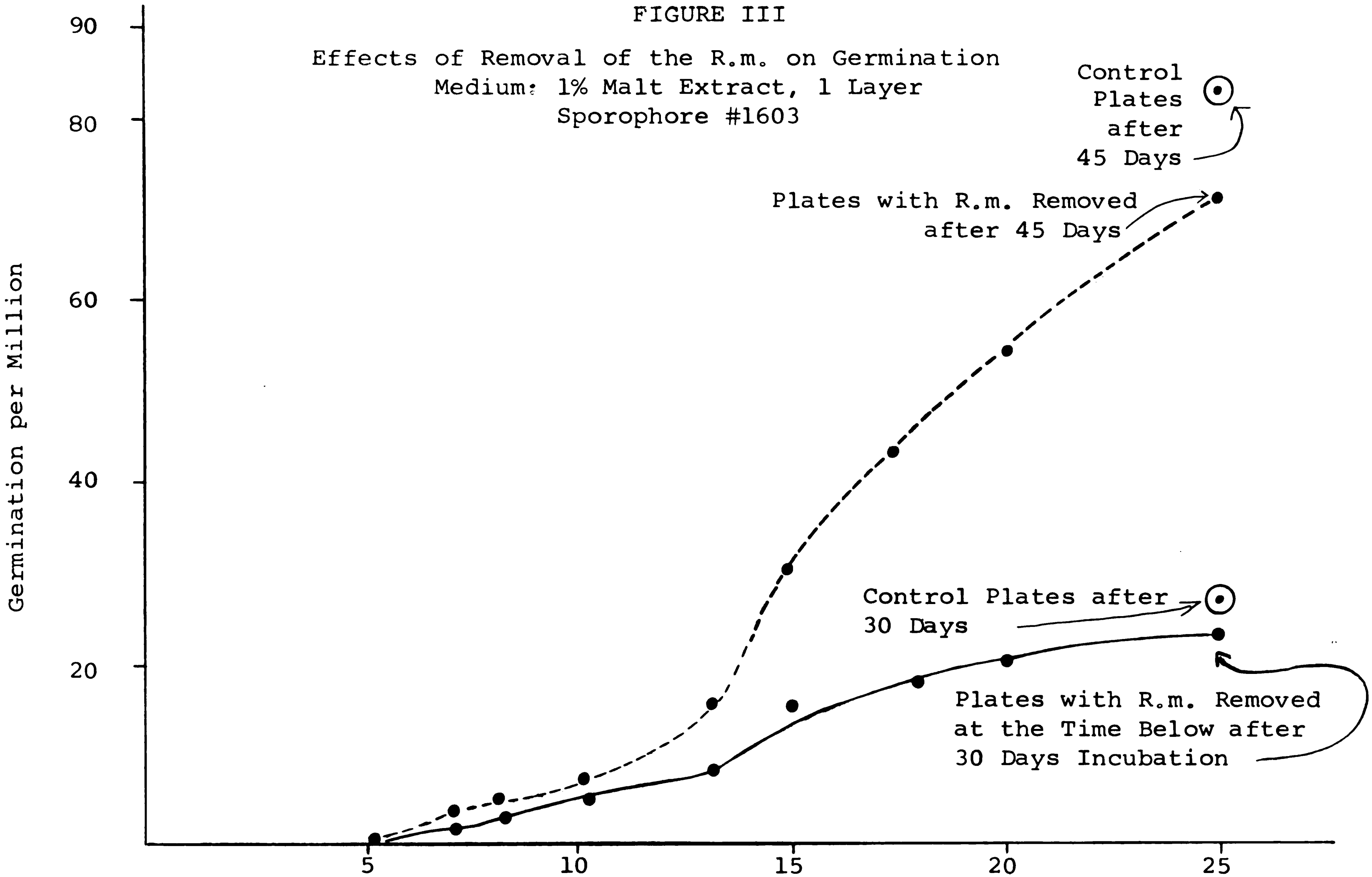
This experiment was carried out in the following manner: (1) all the plates were seeded and inoculated (1-pint) at time zero, and (2) on various succeeding days, aliquots of the plates were opened and the yeast colonies removed. The results are given in Figure III.

It is obvious that the yeast must be present for at least 6-7 days before the spores are affected. Furthermore, there is some action due to the R.m. between the 25th and 30th day of incubation; this can be realized if one subtracts the amount of germination in plates from which the yeast was removed on the 25th day from the controls. The data also suggest that the action of the yeast in evoking germination occurs mostly between the 13th and 20th day of incubation.

Since the rate of R.m. growth would bear on the above experiments, the rate of the R.m. growth was also determined (Figure IV). The results demonstrate that growth the first 5 days represents nearly half of the total growth, and the growth was nearly complete by the 15th day.

FIGURE III

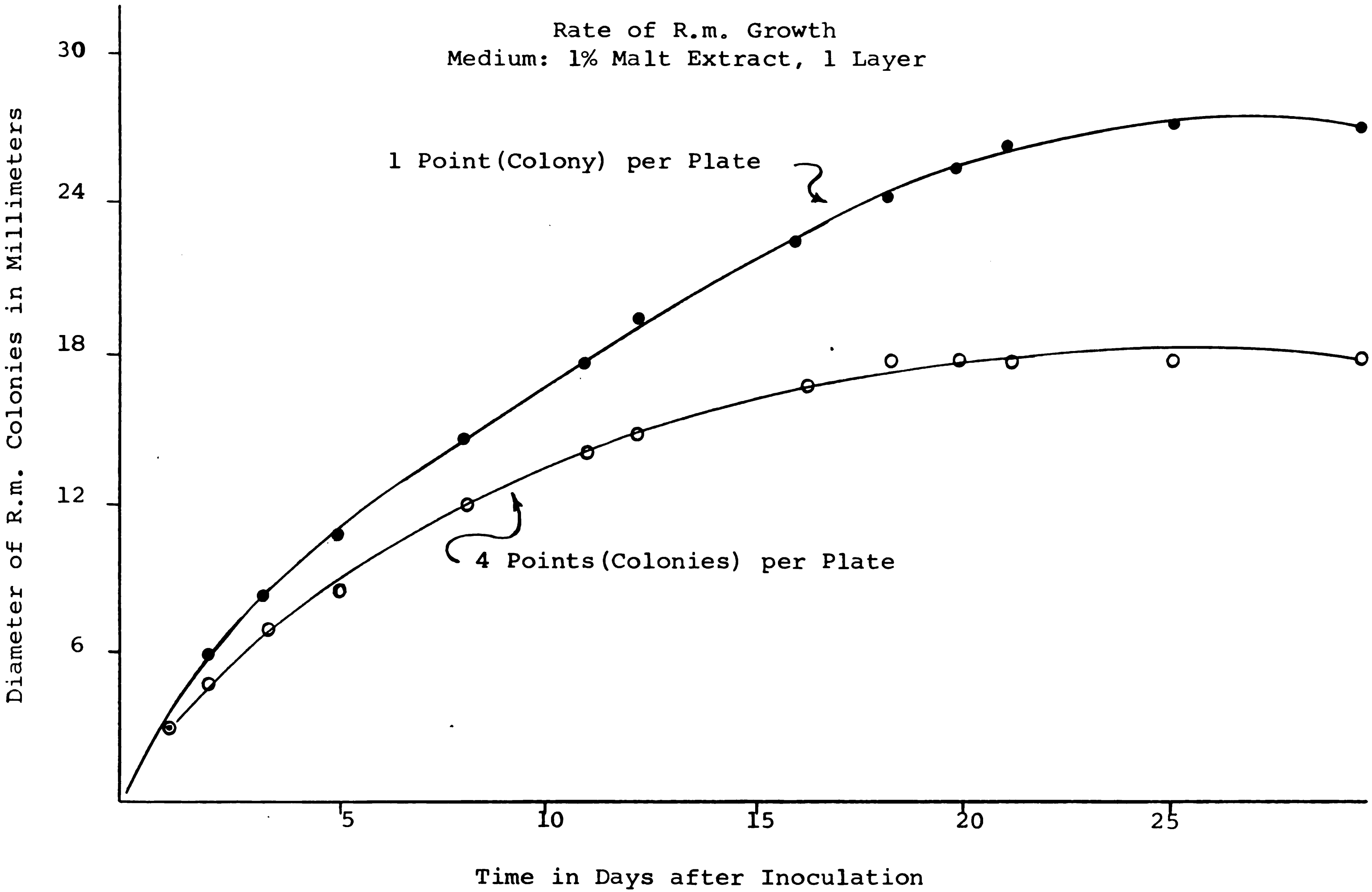
Effects of Removal of the R.m. on Germination
Medium: 1% Malt Extract, 1 Layer
Sporophore #1603



Time (Days) after Seeding Spores and Inoculating the R.m. that the Yeast Colonies were Removed from the Plates

FIGURE IV

Rate of R.m. Growth
Medium: 1% Malt Extract, 1 Layer



Effects of Different Species of Yeast and Bacteria

There is an obvious question that could be asked about the relationship between the yeast and the germination process: is the R.m. unique in stimulating the germination of the basidiospores? Tables VIII and IX show that other species of _Rhodotorula_ are effective in stimulating germination. Furthermore, the different species affect the germination of spores from various sporophores differently. For example, _R. rubra_, and _R. aurantiaca_ were more stimulatory to the germination of spores from #1609 than was the _R. mucilaginosa_. In addition, it is clear that when different carbohydrates were substituted into the basal medium, the amount of germination with a given yeast varied. _Saccharomyces cereviciae_ (colorless yeast) failed to stimulate germination of spores from 3 sporophores.

One observation in these experiments was that the _R. mucilaginosa_, _R. rubra_, and _R. aurantiaca_ were all deeply pigmented, while the others had less color. Possibly, one significant fact is that these three species appeared to stimulate the greatest amount of germination.

Effects of Bacterial Co-Cultures

An accidental deep yellow gram-negative bacterial contaminant also stimulated the spores to germinate, but as Table X indicates, the bacterium did not stimulate germination as well as the R.m. The counts were made after 5 weeks of incubation. However, when the plates were checked 13 weeks later it was found that the number of germinations had increased approximately six-fold. Bulmer (personal communication) reported that the bacterium which he found to stimulate the germination of the *C. gigantea* basidiospores was also bright yellow. Again, there may be significance to the fact that the stimulating organisms were pigmented.

Bacilus subtilis, *Pseudomonas aeruginosa*, and *Serratia marcescens* did not stimulate the germination of any spores from #1601, #1605, and #1608. The latter two pigment producing organisms were green and red, respectively.

Of the many other fungi and bacteria which invaded the writer's experiments, none was found to stimulate germination.

Table XII

Effects of Using Different Species of Rhodotorula

Rhodotorula species	Germination per Million #1601	#1603	#1609
R. mucilaginosa	379	349	97
R. aurantiaca	246	198	148
R. rubra	98	212	148
R. minuta	21	186	161
R. pallida	23	19	0(7)*
R. flava	31	24	0(23)*
no yeast	0	0	0

Medium used: 2.0% malt extract; 2 layer of medium;
*counts made 2 weeks later

Table XIII

Effects of Different Species of Rhodotorula
and Different Carbohydrates

Rhodotorula species	Sucrose	Glucose	Maltose	S.S.	Malt "x"
R. mucilaginosa	18	6	34	26	269
R. rubra	4	5	15	17	89
R. aurantiaca	-	8	-	21	146
R. minuta	11	3	10	16	18
R. pallida	11	1	12	21	24
R. flava	10	3	13	25	24
no yeast	4	3	14	18	000

Medium used: basal plus 1.0% carbohydrates and
0.5% KNO_3; 1.0% malt extract; 1 layer of medium;
sporophore #1605

Table XIV

Effects of a Yellow Bacterium

Type of Plate	Germination per Million #1601	#1608	Diameter of R.m. Colony (mm)
1-Point Plate			
R. mucilaginosa	35	25	28±2
Yellow Bacterium	7	10	12±2
4-Point Plate			
R. mucilaginosa	277	234	17±2
Yellow Bacterium	17	22	8±2

Medium used: 1.0% malt extract; 1 layer of medium

DISCUSSION

The purpose of this investigation was to further elaborate on the germination of the basidiospores of *Calvatia gigantea*, especially as the process is affected by the yeast, *Rhodotorula mucilaginosa*. It was also hoped that the information found here would be applicable to the germination of basidiospores from other species of Gasteromycetes which Bulmer and Beneke (1960, 1961, 1962, 1964a,b) have shown to be affected by yeast.

One of the major facets of this study was to establish the mode(s) of action of the R.m. The several modes of action considered were: (1) the R.m. produced a metabolite which activated the germination machinery, (2) the R.m. served to remove or convert an inhibitor that was of spore origin or present in the medium, or (3) the R.m. altered the medium in a physico-chemical manner.

The approaches employed in this study were: to determine if chemical and/or physical treatments would affect germination, to study the nutritional aspects of the germinating spores, and to elaborate on the effects of the environment on the incubating spores. Attempts were also made to isolate a stimulatory compound from the yeast, and the media were examined for differences due to the presence of the R.m. Time-rate studies were made, and other species of yeast and bacteria were considered for their effects on germination.

In the interpretation of the results, one must consider every variable not only as it might affect the basidiospores but also as it might affect the R.m. It should also be reiterated that germination was measured by the presence of macroscopically visible colonies. Hence, the variables studied can not be confined only to the process of germ tube emergence, a parameter often used to study fungus spore germination, but rather, one must consider the effect of the variables on the growth of the hyphae derived from the germinated spores. Furthermore, some spores could have germinated but were masked by the mycelium arising from earlier germinating spores.

When it was found that a reduced number of spores would germinate on a chemically defined medium without the R.m. co-culture, the possibility of the R.m. acting solely by removing an inhibitor of spore origin was ruled out; however, the amount of germination was higher if the yeast was present. The amount of R.m. growth was also reduced on the defined medium and could be increased with the addition of thiamine, however, the thiamine had no effect on germination. It was found that in general ammonium salts and amino acids did not support germination unless the R.m. was present. Thus there is a suggestion that the action of the R.m. is to remove, or to convert reduced nitrogen sources to a usable form.

The addition of peptone to the defined medium inhibited germination even though the yeast was present. Malt extract, when added to the basal medium, inhibited germination if the yeast was absent, but enhanced both germination and yeast growth on the R.m. inoculated plates. This latter evidence indicated that an inhibitor is present in the malt extract and that the R.m. removes or converts the inhibitory substance; it does not, however, give evidence for or against the possibility that the R.m. produced a stimulator. It is also possible that the malt extract contains a germination or growth factor which becomes operative after the inhibitor is removed.

Other types of media failed to support germination while in some cases, supported good yeast growth. This finding suggested that the presence of a large amount of yeast growth. Plates incubated in the upright position showed more germination than those in inverted plates because of the greater amount of yeast growth in the former.

The experiments in which agar-covered bridges connected the growing R.m. and the seeded medium provided possible clues for resolving the stimulator-inhibitor dilemma. The information was indirect in nature since the yeast did not stimulate germination via the bridges. The first possible solution is that the R.m. failed to remove the inhibitor from the seeded medium because all the inhibitor could not diffuse over the bridges (most likely). While, if a stimulator had been produced, it should have passed over the bridges. It is also possible that the stimulator was inactivated while passing over the bridge, but this is unlikely for reasons to be discussed later. Similarly, there are several possible explanations why the action of the yeast was not transmitted via agar blocks from yeast inoculated plates to seeded plates. One is that the R.m. acted to remove an inhibitor from the medium, enough of the stimulator may not have been transferred, or the stimulator was unstable. Another possibility is that the blocks were not added at the correct time, however, the time rate studies indicated that the action of the yeast was continuous. Since the R.m. was effective in stimulating germination over a distance of 15 cm, it is suggestive that if a stimulator is produced it is stable enough to diffuse that far.

That a volatile stimulator was not produced by the R.m. was demonstrated by the lack of germination when the yeast was isolated from direct contact with the spores. Opening the plates periodically during incubation had no effect on germination, nor did the presence of trapping substances placed in center wells. This indicated the air surrounding the seeded spores is of little importance.

Experiments demonstrating that the R.m. was active in enhancing germination through a dialysis membrane did not resolve the mode of action of the yeast. The amount and rate of germination, however, was reduced by inserting the membrane. This suggests that the molecule(s) involved was large enough to be hindered as it passed through the membrane.

The use of different species of yeast and bacteria revealed several facts; the R. mucilaginosa was not unique in its effect on the germination of C. gigantea basidiospores, and the organisms which enhanced germination were pigmented. Furthermore, there was a correlation between the degree of pigmentation and the amount of germination. There was, however, a longer time period required before germination occurred when the yellow bacterium and some of the yeasts were used. The interpretation of these facts present two views. First, all of the organisms affecting germination did so by producing a common metabolite, possibly a by-product of pigment formation. Second, they all removed the substance(s) responsible for inhibiting germination. It is the writer's opinion that there probably are many organisms in the microbial world which also act on basidiospores of the Gasteromycetes.

To demonstrate unequivocally that the yeast acted via producing a stimulator, one would have to isolate a fraction of the yeast or of the medium which would act in the prescribed manner; in the experiments done, this was not achieved. Likewise, attempts to analyze the incubation media, which had and had not been inoculated with the R.m. failed to show chemical differences due to the yeast.

SUMMARY

1. The germination of the basidiospores of *Calvatia gigantea* was studied; emphasis was placed on the elaboration of the stimulatory role of the yeast, *Rhodotorula mucilaginosa*, on the process.
2. A basal salt medium, to which various carbon and nitrogen sources were added, was found to support a reduced amount of germination even in the absence of the yeast. Maltose, soluble starch, and sucrose were the best carbon sources, and KNO_3 was the best nitrogen source. Ammonium salts and amino acids did not support germination. Malt extract inhibited germination if the R.m. was not present.
3. Potato-dextrose agar supported germination nearly as well as malt extract agar.
4. The addition of excess malt extract agar increased the amount of germination by increasing the amount of R.m. growth. Inverted Petri plates had more germination than those incubated upright; the increase was due to an increased amount of R.m. growth.
5. Germination occurred at a distance of 15 cm away from the R.m., but the amount was inversely proportional to the distance from the yeast colony.

6. There was no evidence that a volatile stimulator was produced by the R.m. The air above the spores was apparently unimportant in the germination process.
7. Various extracts of the yeast and filtrates of the spent medium did not stimulate germination.
8. The spores remained dormant in the medium for 23-30 days after the R.m. was added. The action of the R.m. in stimulating germination appeared to be continuous after inoculation onto the seeded plates.
9. The action of the yeast took place when it was separated from the spores by a dialysis membrane.
10. Chemical analysis failed to show differences between medium that had been inoculated with the R.m. and the same medium that had not been inoculated.
11. Several species of Rhodotorula and an unidentified bacterium stimulated germination.
12. Although there were quantitative differences in the amounts of germination of spores from various sporophores, the qualitative action of the yeast, and the other variables affected germination similarly.

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APPENDIX

Background Information on the Sporophores
Used for This Investigation

Code Number	Date of Harvest	Location of Harvest
1601, 1602 1603, 1604 1605, 1606	September, 1961	Southwest corner of the M. S. U. Golf Course
1608	June, 1963	E. Lansing, Mich.
1609	September, 1961	Ada, Michigan
1611	October, 1961	Grand Rapids, Mich.

Background Information on the *Rhodotorula*
Species Used for This Investigation*

Genus and Species	Code Number
Rhodotorula aurantiaca	NRRL Y-1581
R. flava	NRRL Y-1585
R. glutinis	NRRL Y-2502
R. minuta	NRRL Y-1589
R. mucilaginosa	NRRL Y-174
R. pallida	NRRL Y-339
R. rubra	NRRL Y-1592

*Received from: ARS Culture Collection
Northern Utilization Research and Development
Peoria, Illinois

Ingredients of Media

Difco Malt Extract Agar	Maltose	1.27%
	Dextrin	0.027%
	Glycerol	0.023%
	Peptone	0.008%
	Agar	1.500%
Difco Malt Extract	A dehydrated infusion of barley which contains:	
	Diastase	
	Dextrin	
	Dextrose	
	Protein bodies and salts from barley	