A Study of the Reaction of *Aspergillus terreus* and *Trichoderma viride* to a Textile Rotproofing agent used on Jute.

by

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A Thesis submitted to the University of St. Andrews for the degree of Master of Science.

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DECLARATION.

I hereby declare that the following thesis is based on the record of work done by me, that the thesis is my own composition and that it has not previously been presented for a Higher Degree.

The research was carried out in the British Jute Trade Research Association, Dundee, under the direction of Professors J.H. Burnett and J.A. Macdonald.

CERTIFICATE.

I certify that Margaret I. Wilson, B.Sc., has spent four terms of Research Work under my direction and that she has fulfilled the conditions of Ordinance 51 (St. Andrews), and that she is qualified to submit the accompanying thesis in application for the Degree of Master of Science.

J A Macdonald

CAREER.

I graduated with Honours in Botany at the University of St. Andrews in 1958.

I was then appointed Research Scientist with the British Jute Trade Research Association in Dundee.

ACKNOWLEDGEMENTS.

I wish to acknowledge the continued help of Professor J.H. Burnett and Professor J.A. Macdonald who supervised this work, and also to thank Dr. Stout and the Council of the British Jute Trade Research Association for allowing this research to be carried out.

CONTENTS.

INTRODUCTION.

Jute hessian has many varied uses. It may often be exposed to conditions of moisture and temperature well suited to micro-organism growth and, as a result, the fibres soon become degraded. In a temperature of $28^{o} \pm 2^{o}C$ and a relative humidity of about 100% jute becomes completely degraded by micro-organism attack within fourteen days. This compares favourably with cotton and flax which degrade even more quickly.

Under normal conditions in this country this represents several months, but in order to increase the life of the fibres the cloth is usually treated chemically with various types of rotproofing agents.

There are many of these rotproofing agents used commercially today, but on critical examination there are only a few which can be considered really effective. Of these, two are used with regularity - copper naphthenate and pentachlorophenol laurate.

Copper naphthenate is a very good rotproofer, but is green in colour, has a waxy feel and a rather unpleasant smell. It cannot be used in contact with rubber as the latter is corroded by the copper present.

Pentachlorophenol laurate is colourless, odourless, and

stands up to microbiological attack reasonably well. It does not leach out of the cloth to any great extent but the cloth treated with this is degraded to the same degree as untreated material when exposed to sunlight. All rot-proofed cloth is tested for its efficiency against sunlight because a proofing treatment which provided protection against both microbiological and actinic degradation would be of value, since jute is often exposed to outside conditions, as well as those best suited for microbiological attack. - Ashcroft, Bell and Wilson (1960).

Pentachlorophenol laurate can be mixed with the oil emulsion at the batching stage of jute processing and this is of importance because it saves time and expense of cloth treatment at a later stage. It is also thought that by using this method of application the ultimate cells in the jute fibre may have a greater chance of being impregnated with the proofer than if treatment was done on the completed hessian.

Even with these proofing treatments the cloth is eventually attacked in places, or as a whole, by micro-organisms - fungi and bacteria.

It was therefore thought that it would be interesting to determine the reaction of certain cellulose destroying

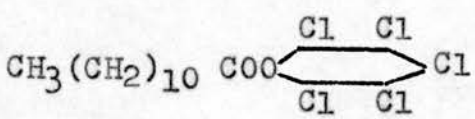
fungi to a rotproofer which is in general use.

It is well known (Siu, 1951) that it is the cellulose in the fibres that the micro-organisms attack and there is present in jute 64.4% cellulose and 12% hemicellulose.

The two cellulose destroying fungi examined in this project are Aspergillus terreus and Trichoderma viride, both of which have been seen growing profusely on cloth subjected to rotting tests carried out in the British Jute Trade Research Association.

Both fungi were isolated from the soil during routine investigation. They were grown on agars which appeared to be best suited to them. Aspergillus terreus was grown on Czapek Dox (A_1) and Trichoderma viride on malt extract agar (A_2).

To this agar was added various quantities of pentachlorophenol laurate, or as it is generally known, L.P.C.P., and hereafter it will be referred to as L.P.C.P. The formula is as follows:



The rate of growth, colour and morphology of the fungi were noted throughout all these experiments.

The required percentage L.P.C.P. on the cloth to give adequate protection from microbiological attack is considered to be 1 - 1.7% (British Standards 2087 Booklet 1954).

It was desirable to investigate if it was possible to "train" the fungus to grow on increasing amounts of rotproofer in the agar, beginning with very low concentrations, and then increasing the dose in a step-wise manner to see if a strain resistant to the rotproofer could be developed.

The types of experiments which were carried out are as follows:-

(a) Gradient plate test.

There was a gradient of increasing concentration of L.P.C.P. across the petri plate. Inoculations of conidial suspensions were made either at the low concentration side, or over the whole plate.

(b) Solid medium growth tests.

Essentially these consisted of a series of media differing in concentration of added L.P.C.P. The tests were done in two ways. Either (1) conidial suspensions were inoculated on all plates, and samples picked off for further testing, or (2) initially conidial suspensions were

plated on to low concentration plates, and the colonies that grew transferred by "replica" plating to the next highest concentration and so on.

(c) Spore germination tests.

These were done either by using cellophane discs on agar, or by coating slides with a nutritive mixture. The nutritive mixture sometimes had L.P.C.P. added to it and by this means the percentage germination could be estimated.

Lacto phenol cotton blue (A3) was used as a stain throughout, except when the cytological examination was made.

Observations were done with a Watson's microscope using x 43 high power and x 97 oil immersion objectives and a x 10 eye piece. For spore measurement and cytology a Cooke microscope was used with a x 40 high power, a x 95 oil immersion and a x 10 eyepiece.

Kohler illumination (Barer, 1953) gave the best lighting and a ground glass filter was placed between the point-o-light lamp and the microscope condenser for all observations. During photography this filter was removed.

For routine petri dish examination a Baker stereoscopic microscope, with x 20 eyepieces was used.

When photographs were taken Kodak Rapid Process Panchromatic plates P.300 were used; upon most occasions 1 second exposure was best, but this did vary depending upon the subject and the power of magnification.

MATERIALS AND METHODS.

Cloth Proofing.

Pentachlorophenol laurate (L.P.C.P.) is produced in several forms, two of which - the solvent soluble and the emulsion forms - have been applied in these tests. The solvent soluble form was used for treating cloth in the laboratory since the cloth becomes wet very easily with solvent solution and this improves impregnation of the L.P.C.P. The emulsion form was used in all the agar media, the necessary quantities being pipetted into the test tubes before autoclaving began.

Cloth Rotting Tests.

Any cloth used in these tests was a 10½ oz. jute hessian which was stainless - i.e. contains little oil, and is the type used for carpet and linoleum backing.

Some cloth rotting was carried out using the flat plate (Ashcroft & Bell, 1958-59) and soil burial (Siu, 1951) methods of testing. The flat plate method originated in Jute Research and consists of glass plates 12"x 25" upon which a paste of soil/horse manure and water, six inches in width, is placed. On this soil mixture, in turn, are placed treated cloth strips 3" wide. The glass plates are

stacked in a trough of water and a perspex hood covers the whole, so that the relative humidity is 100%. Moist air is pumped through the boxes every second day to expel the stale air. These boxes are kept at a temperature of 28°± 2°C.

The soil burial is a standard method of rotting used by Siu (1951), where the cloth is buried in a loop below three inches of soil. The moisture content of the soil in this case is usually about 35%.

Proofed cloth is removed for strength tests after various lengths of time. Eight samples of each treatment is considered the minimum to give a result of any value. The cloth strips fringed down to 2", are conditioned for twenty four hours, and then broken on the Goodbrand cloth testing machine and the percentage strength retained can then be worked out since the initial strength is known.

Soil mixtures used.

Two soil mixtures have been used in the above cloth rotting tests - soil/horse manure and soil/leaf mould.

Soil/horse manure. This is a half and half mixture. To this is added 0.77 gm. urea per 100 gm. mixture (Ashcroft and Bell, 1958-59), as the added nitrogen is known to increase microbiological activity.

Carbon and nitrogen (A4 & 5) determinations have been done on this mixture and the results are as follows:-

Carbon content = 4.6%

Nitrogen content = 0.296% (before urea added)

% C/N ratio = 15.6

pH = 6.5.

Soil/leaf mould. This mixture was done in similar quantities to that suggested by Galloway (1956), i.e.

Soil 70: Leaf mould 20: Sand 10: Calcium carbonate 1.

Carbon content = 3.7%

Nitrogen content = 0.213%

% C/N ratio = 17.6

pH = 7.2

Isolation of Aspergillus terreus and Trichoderma viride from the soil.

Aspergillus terreus was isolated from the soil/horse manure mixture by the dilution plate method (Burges 1958). Trichoderma viride, on the other hand, was isolated from the soil/leaf mould.

All cultures were incubated at 28°C. in a Gallenkamp incubator. The petri dishes were previously sterilised in a Griffin and Tatlock thermostatically controlled oven at 100°C.

After 2 - 3 days, colonies of Aspergillus terreus were visible, but to be sure of a pure culture further subculturing was done and once the parent strain was successfully isolated the experiment could begin. From this stage Czapek Dox agar (A1) pH 6.8 was used since this was known to be a suitable medium from the work of Lockwood et al (1945). On adding 0.1% L.P.C.P. to the Czapek Dox medium the pH was found to have altered only to 6.5.

Trichoderma viride grew better on malt extract agar (A2) pH 5.4. In both cases the agar was made by crushing the prescribed number of agar pellets (made by Oxo Ltd.) and adding distilled water. 20 cc. portions of media were then run into test-tubes for autoclaving at 10 lbs. pressure for 20 minutes. Sterilised petri dishes were then filled with the 20 cc. portions of agar and allowed to solidify. Just before agar pouring, and inoculating began the glass topped bench was swabbed down with industrial spirit and the air was well sprayed with a 2% thymol spray (A6).

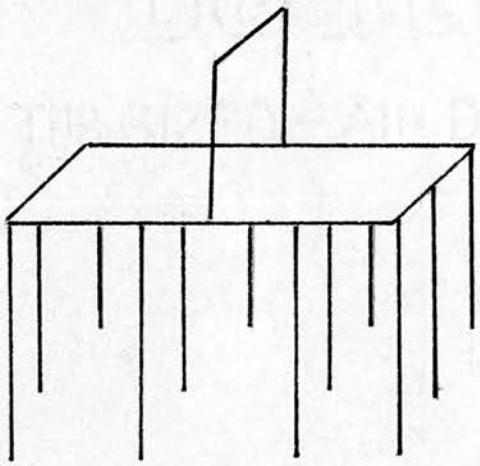
Initially there was some difficulty in separating the conidia from each other, in both fungal species. This was overcome by gently rubbing over the surface of the colony with a sterilised glass rod. The separated spores were then transferred by a flamed platinum wire to a vial which

contained sterile distilled water plus one or two drops of sterilised Teepol as a wetting agent.

A sample of this spore suspension was then pipetted out and examined with a haemocytometer to give an idea of how much dilution would be necessary to allow one ml. of the spore suspension to be taken and inoculated on to normal agar, so that monospore colonies developed. In both fungal species a 10^3/ml. dilution was quite satisfactory for this purpose.

When colonies were transferred from one plate to another, a triangle was cut round the colony, and this was then removed, taking the depth of the agar, to another plate.

"Replica" plating was done using a holder containing twelve needles.



This type of inoculation was of value once growth on L.P.C.P. treated agar had developed. Spores growing on one

concentration of L.P.C.P. could be transferred by this means to a higher concentration of L.P.C.P. in agar.

Spore germination tests.

For examining spore germination transparent cellophane discs were used on top of the agar, and these could then be stripped off the surface quite easily for examination.

Another method for spore growth observation was to cover slides with a thin layer of nutritive medium mentioned in McLean and Cook, 1958 (A7). The spores were then sprinkled on this medium and kept in a petri dish with damp filter papers in its base at 28°C.

Cytology of germinated spores. Spores and hyphae were examined cytologically using Somers, Wagner & Hsu (1960) staining method, i.e. acetic/alcohol 1:3 (A8) followed by 1 N HCl and then acetic orcein.

RESULTS.

Cloth rotting tests.

Tests on cloth proofed with L.P.C.P. were done using the flat plate and soil burial methods of rotting that were described in the introduction.

Soil/horse manure and soil/leaf mould were used and the degree of rotting in each case was recorded (Wilson, 1962).

Flat plate - Soil/Horse manure.

	% strength retained.						
No. of weeks on test	4	8	12	16	20	24	28
L.P.C.P. treatment	82.2	84	83.7	81.1	75.3	73.6	66.5
L.P.C.P. content	0.94	0.8					
Untreated	**0**						

Flat plate - Soil/Leaf mould.

	% strength retained					
No. of weeks on test	4	8	12	16	20	24
L.P.C.P. treatment	78.4	58.3	33.5	24.6	4.5	0
L.P.C.P. content	0.78	0.49				
Untreated	**0**					

Soil burial - Soil/Horse manure.

	% strength retained			
No. of weeks on test	4	8	12	16
L.P.C.P. treatment	88.9	76.1	78.9	76.4
L.P.C.P. content	0.95	0.8		
Untreated	**0**			

Soil burial - Soil/Leaf mould.

	% strength retained					
No. of weeks on test	4	8	12	16	20	24
L.P.C.P. treatment	55	18.4	3.4	0	0	0
L.P.C.P. content	0.65	0.6				
Untreated	0					

L.P.C.P. determinations were done following the method described (A9).

The L.P.C.P. content on the cloth was 1.0%. After one month on the soil/horse manure there was no alteration in concentration. There was a reduction of about 0.2% on the cloth in contact with the soil/leaf mould. It was not thought that this was due to leaching out of L.P.C.P. from the cloth and Mr. Lloyd of Catomance (L.P.C.P. producers) said in a letter that he -"suspects that the leaf mould might contain tannins and consequently might easily have a micro-organic flora tolerant to phenolic substances, or possibly even utilise them". The L.P.C.P. losses in the first four weeks would certainly bear this out.

The soil/leaf mould mixture caused rapid degradation of L.P.C.P. treated cloth.

A further confirmatory test was done again using L.P.C.P. treated cloth in contact with soil/horse manure and soil/ leaf mould. These tests were left for three months and then

the samples were removed and tested. Those in the soil/ horse manure mixture still retained at least 50% of their strength, whilst those in the soil/leaf mould were completely degraded.

The soil/leaf mould mixture, which was used in the rotting test with the L.P.C.P. treated cloth which had been completely degraded within 3 months, was examined.

A soil suspension was formed with sterile distilled water. Only half the dilution recommended by Burges (1958) was used because this soil suspension was to be inoculated on treated agar to see if anything would grow.

1 ml. of the soil suspension was put on each of the plates which contained 0.05% L.P.C.P. to see if any micro-organisms grew.

6th day. No fungal growth, but one or two patches of bacterial growth.

14th day. Petri dish I. All over the surface of the agar there were small bacterial colonies. There was also an area with Trichoderma viride. This fungus did not change colour or form because of the presence of L.P.C.P. in the agar.

Petri dish II. There were similar bacterial patches. One or two areas with mycelial growth although no spore heads.

Petri dish III. Bacteria again and five fungal colonies, two of which were quite large.

Petri dish IV. Two or three patches of well developed bacteria and Trichoderma viride in quite large amounts.

20th day. Fungal colonies which were still not fully developed and were, therefore, unidentifiable were transferred to dishes containing normal agar.

One plate had Scopulariopsis brevicaulis present which is a weak cellulolytic fungus.

3 days later. Examination of the colonies on normal agar showed that growth had occurred in each case.

Aspergillus terreus - cellulose destroying.

Scopulariopsis brevicaulis - weak cellulolytic fungus.

Trichoderma viride - cellulose destroying.

Stysanus sp. - moderate cellulose destroyer.

Penicillium digitatum

Penicillium spp.

Bacteria.

Other petri dishes with normal agar were inoculated with pieces of the degraded cloth. The cloth was black and brittle to the touch.

Similar isolates as mentioned above were found growing on normal agar inoculated with a spore suspension of soil/leaf mould.

4th day. The major organism present was Trichoderma viride. Also present were Aspergillus candidus, A. terreus, A. ustus, and several species of bacteria.

Soil/horse manure mixture was examined.

A soil/horse manure suspension was made up and 1 ml. of this was inoculated on normal agar.

The following micro-organisms developed:

Aspergillus fumigatus - cellulose destroying.

Aspergillus terreus - cellulose destroying.

Aspergillus spp.

Rhizopus nigricans - non-cellulolytic.

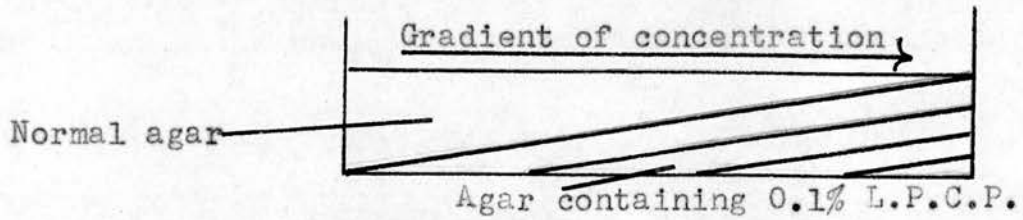
A number of species of bacteria.

The reaction of fungi to rotproofing agent.

Most of the experimental work was carried out on Aspergillus terreus and this will be reported in the first instance. Aspergillus terreus was identified using Thom and Raper's Manual of the Aspergilli (1951).

Gradient Plate Test.

These gradient plates were made up as shown below.



A 10^5/ml. spore suspension was made up and 1 ml. of this was inoculated over the surface of the dishes. Two dishes had the 1 ml. dispersed over the whole surface and two dishes had the 1 ml. restricted to the less concentrated side.

2 days. Growth, like normal Aspergillus terreus, had begun on the less concentrated side in each case.

8 days. In the dishes where the 1 ml. had been restricted to the less concentrated side of the dish the growth had gone half over the plate. Where the 1 ml. had been spread over the whole surface of the plate the growth was $\frac{3}{4}$ across the plate.

Just short of the half way mark in each case yellow mycelium was present. Interspersed with this were white conidial heads.

From half way across the plate to the more concentrated side, growth appeared crinkled and the lower surface of the agar was puckered.

This type of test was not repeated since the actual quantity of L.P.C.P. diffusing through the agar was not known.

Solid Medium.

Throughout the tests which are described below the morphological differences of Aspergillus terreus are noted, when growth in presence of L.P.C.P. occurs, and these are compared with the normal parent strain growth, on normal Czapek Dox agar. The colony size is another feature of importance, as is the time lag for spore germination. These are all adaptations which the fungus has made to allow growth to take place in the toxic environment.

Normal Czapek Dox agar and normal parent strain of Aspergillus terreus

1 ml. portions of a 10^4/ml. spore suspension were inoculated on to the normal agar.

2nd day. A white mat of mycelium had grown over the surface of the agar and a large number of white spore heads were present.

4th day. The spore heads were cinnamon coloured (Plate 1).

Another test was done using normal Czapek Dox agar, with 1 ml. of a 10^3/ml. spore suspension inoculated. Monospore colonies were produced, and their growth rate was no different from the above so this is considered to be the normal rate of growth for Aspergillus terreus on Czapek Dox. The average diameter of a normal colony is about 3 cm.

Czapek Dox agar with 0.1% L.P.C.P. and normal parent strain of Aspergillus terreus.

1 ml. of a spore suspension of 10^6/ml. was inoculated on these plates.

4th day The petri dish was covered by small colonies all growing into one another.

5th day The mycelium was white and crinkled on surface. The undersurface of the agar was also crinkled and a slight yellow colouration had developed. There was no chance of isolating the individual colonies and it was thought that a 10^6 spore suspension was too high.

6th day A very few spore heads were present. They were white in colour and found to be abnormal on examination. (Plate 2).

7th day. Some of the spore heads were cinnamon coloured and they lay in a band of mycelium across the plate. Also a band of yellow, sterile mycelium had grown and on this, small, clear yellow globules of liquid exudate were noticeable. The cells of the sterile mycelium were thicker and contorted and the cell content was very apparent. The mycelium branched irregularly and many parts were swollen. (Plate 3).

Using a 10^3/ml. spore suspension on this concentration of L.P.C.P. gave no growth at all. 10^3/ml. was considered a very suitable concentration on normal agar.

A 10^3/ml. or 10^4/ml. spore suspension was used for the following tests. The spore concentration was too high for monospore colonies in the above test, but there are signs of features which occur repeatedly in the later experiments.

(1) Two day lag in germination of spores.

(2) Yellow mycelium.

(3) Liquid exudate.

(4) Crinkling of agar.

(5) Irregular branching and contortion of mycelium.

The characters of colony appearance are similar to those described by Raper, Coghill and Hollaender (1945) in their work on ultra violet irradiated spores of Aspergillus terreus. The time for these colonies to reach the production of cinnamon spore heads was double that of normal Aspergillus terreus. Further tests showed that, when the colonies had been derived from a single spore, and not as in this case from a group of spores, that the growth rate was even slower.

0.1% L.P.C.P. - N.P.S. Aspergillus terreus

2nd day - germination of spores

4th day - small white colonies

5th day - yellow mycelium

6th day - white conidial heads

7th day - cinnamon heads.

Normal parent strain of Aspergillus terreus on 0.02% L.P.C.P. Czapek Dox agar.

Czapek Dox agar was used throughout these tests and the type of agar will not be mentioned again unless another type has been used.

Inoculated with a 10^3/ ml. spore suspension of normal parent strain.

4th day. The colony growth was on average 7/10 cm. in diameter. The mycelium was white with tinges of yellow. No spore heads were present. Agar was wrinkled on undersurface.

5th day. There was a mixture of white and cinnamon spore heads. There was one furrow in each colony. Spore heads appeared quite normal when examined microscopically.

6th day. Yellow mycelium was still in evidence but cinnamon, cream, and white spore heads present in quite considerable profusion. There were one or two small globules of liquid, also some abnormal heads and one which looked like a species of Penicillium. The average colony diameter = 1½ cm.

It was decided to begin with a really low concentration of L.P.C.P. in the agar and increase the concentration in a stepwise manner.

0.02% L.P.C.P. - N.P.S. Aspergillus terreus.

2nd day - germination of spores.

4th day - white and yellow mycelium

5th day - white and cinnamon spore heads

6th day - more cinnamon heads

Average colony diameter

4th day - 7/10 cm.

6th day - 1½ cm.

The diameter of the colony at the mature cinnamon spore head stage was only half that of the normal parent strain on the normal agar medium.

Normal parent strain of Aspergillus terreus on 0.03% L.P.C.P. agar.

Inoculated with a 10^3/ml. spore suspension.

4th day. Colony growth was restricted, the average diameter being 1/5 cm. The number of colonies was countable - about 260.

White mycelial growth only. Twelve colonies were transferred from 0.03% L.P.C.P. to a plate containing 0.03% L.P.C.P.

5th day. The white mycelium had all turned yellow.

6th day. The hyphae had grown from the transfer on to the plate agar and there were a few young conidial heads.

8th day. Spore heads were quite common and mostly cinnamon. There were one or two heads resembling a species of Penicillium. One head showed a transition stage between the Aspergillus type of head and the Penicillium type (Plate 4). Although a number of the heads were normal, there were a few variations in growth form.

9th day. The growth on the plate agar showed slight indentations and conidial heads. The average diameter of the colonies was 1½ cm.

0.03% L.P.C.P. agar - Normal parent strain Aspergillus terreus.

2nd day - germination of spores.

4th day - white mycelium.

5th day - yellow mycelium

6th day - young conidial heads

8th day - cinnamon heads.

Average colony diameter

4th day - 1/5 cm.

8th day - 1½ cm.

The presence of yellow pigmentation in the mycelium was probably the result of changes in metabolic processes due to the presence of the L.P.C.P. as indeed were all the abnormalities of growth described.

Only 26% of the spore inoculum was viable even at this low concentration of L.P.C.P.

The appearance of the head resembling a species of Penicillium makes it interesting to examine the phylogeny of Aspergillus and Penicillium. They seem to be quite closely associated, both belonging to the Aspergillales.

Spore suspension from 0.03% L.P.C.P. colony inoculated on to 0.03% L.P.C.P. agar. A 10

1 ml. of a 10^3/ml. suspension of spores which had developed on 0.03% L.P.C.P. agar was inoculated on 0.03% L.P.C.P. agar.

3rd day. There were colonies forming. The hyphae were contorted, and flat on the agar. There was no fluffiness.

4th day. Twelve colonies were subcultured on to 0.03% L.P.C.P. agar.

7th day. Mycelium was pale yellow.

8th day. Spore heads had formed.

9th day. Spore heads were cinnamon.

0.03% L.P.C.P. agar - 0.03% L.P.C.P. spores

1st day - germination of spores

4th day - white mycelium

7th day - yellow mycelium

8th day - young conidial heads

9th day - cinnamon heads.

It is of interest to note here that the germination was more rapid, as if the fungus which had already grown in presence of the L.P.C.P. was more able to tolerate the presence of the toxic substance. The reduction of the initial lag period did not, however, increase the growth rate of the colonies. On the third day the hyphae formed a flat mat of mycelium on the agar surface, and this phenomenon had not been observed previously.

Spore suspension from 0.03% L.P.C.P. colony on to 0.05% L.P.C.P. agar. A 10

1 ml. of a 10^3/ml. suspension of spores which had developed on 0.03% L.P.C.P. agar was inoculated on 0.05% L.P.C.P. agar.

3rd day. There were two plates involved here. One produced 170 colonies whilst the other only developed 17.

4th day. Twelve colonies from each plate were transferred to plates containing 0.05% L.P.C.P. agar.

Colonies transferred from 170 plate.

7th day. The colonies showed yellow mycelium. Hyphal growth had just begun on the plate agar. No spore heads.

10th day. Average size of colonies diameter was 1½ cm.

White spore heads were growing on the yellow mycelium. One or two cinnamon heads.

14th day. Average diameter of colonies was 2 cm. Growth irregularities were apparent. Indentations were visible in the agar. Not many globules present.

Colonies transferred from 17 plate.

7th day. The colonies were small and the mycelium white and fluffy.

10th day. The mycelium had grown from the transfer on to the plate agar and was bright yellow, and on top of each colony white spore heads had formed.

14th day. Average diameter of colonies was 1¼ cm. Furrows and some liquid globules present, also cinnamon spore heads.

19th day. All colonies had grown giving an average diameter of 1½ cm.

0.05% L.P.C.P. agar - 0.03% L.P.C.P. spores (17% viability)

1st day - germination of spores

3rd day - white mycelium

7th day - yellow mycelium

10th day - white conidial heads

11th day - cinnamon heads.

Average colony diameter

10th day - 1½ cm.

14th day - 2 cm.

This plate showed 17% viable spores. The development was slightly slower in this higher concentration, although again the spore germination was early.

The plate with only a 1.7% spore viability gave the following rate of growth:-

0.05% L.P.C.P. agar - 0.03% L.P.C.P. spores (1.7% viability)

7th day - white mycelium

10th day - yellow mycelium and white conidial heads

14th day - cinnamon heads.

Average colony diameter.

14th day - 1¼ cm.

19th day - 1½ cm.

In some way this plate had a restriction of growth, and as a result development was retarded. To the best of our knowledge the plates were identical in L.P.C.P. content and both plates were subjected to similar incubation conditions so the difference in results is difficult to interpret.

In both cases furrowing of the colonies was noted. There were rings of active and restricted growth alternately producing zonate colonies.

During the period of active growth the fungus seemed to have built up a resistance to the inhibitor in L.P.C.P. but this was not maintained and a period of slow growth with limited spore head production and restricted growth followed before another active period developed.

It is a response of the fungus to the environment and quite often strains of species of Aspergilli produce conspicuously zonate colonies. (Thom and Raper, 1951).

Colonies growing on 0.03% L.P.C.P. agar transferred to 0.06% L.P.C.P. agar. A 10

After four days growth the colonies were transferred to 0.06% L.P.C.P. agar.

6th day. The mycelium was fluffy and yellow and appeared to be growing quite well although it had not grown from the transfer on to the plate agar.

7th day. No spore heads.

8th day. Conidial heads were just beginning to appear. Minute liquid globules of yellow exudate were present everywhere.

11th day. The colonies had grown from the transfer on to the plate agar and were growing quite well on it. The mycelium had turned yellow, although it was still white at the margins. The colonies had formed

indentations in the agar. All spore heads were white. The average colony diameter was 1¼ cm. The agar was wrinkled on the undersurface.

12th day. Spore heads were cinnamon.

0.06% L.P.C.P. agar - 0.03% L.P.C.P. spores

4th day - white mycelium

6th day - yellow mycelium

8th day - white conidial heads

12th day - cinnamon heads.

Average colony diameter by the 12th day was 1¼ cm.

Although the diameter of the colonies was slightly restricted compared with the previous tests the all over rate of growth was as good and in some cases better than that of Aspergillus terreus on lower concentrations of L.P.C.P. in the agar medium.

Spore suspension from 0.03% L.P.C.P. colony inoculated on to normal agar. A 10

2nd day. White mycelium was evident.

3rd day. Colonies had developed and young spore heads had formed. Colonies were slightly restricted. Microscopical examination showed the spore heads to be normal.

4th day. Spore heads were cinnamon.

Normal agar - 0.03% L.P.C.P. spores.

1st day - germination of spores.

2nd day - white mycelium.

3rd day - white conidial heads.

4th day - cinnamon heads.

There was no sign of yellow pigmentation, and the fungus has practically completely reverted to the growth pattern associated with the normal parent strain. There was no persistance of resistance. It would seem, therefore, that no genetic mutation had taken place, and that any morphological and physiological changes observed were purely of a metabolic nature.

Replica plating technique using needle holder.

Spores from 0.03% L.P.C.P. colony replica plated on to 0.03% L.P.C.P. agar.

3rd day. Central areas were white and fluffy. No spore heads. One area of indentation.

4th day. All colonies had bright yellow centres with white mycelium round about. White spore heads were beginning to form. Average diameter = 1⅛ cm.

5th day. Yellow coloration not so evident and there was a large number of cinnamon spores. The average colony diameter was 1½ cms. Undersurface of agar was wrinkled and indentation evident.

Replica plating.

0.03% L.P.C.P. - 0.03% L.P.C.P. spores.

1st day - germination of spores.

3rd day - white mycelium.

4th day - yellow mycelium and white conidial heads.

5th day - cinnamon heads.

Average colony diameter

4th day - 1⅕ cms.

5th day - 1½ cms.

By using the replica plating technique the growth rate was increased, because a number of spores were deposited at each needle hole. The spore germination was again normal, and the time of growth was more like the 0.02% L.P.C.P. agar colonies described previously. The colony growth was restricted to 1½ cms. but when it is considered that twelve colonies were growing on the plates, it is realised that there is not much more room for expansion.

Spores from 0.03% L.P.C.P. colony replica plated on to normal agar.

3rd day. Colony average diameter was 1½ cm. Spore heads were cinnamon with a trace of yellow mycelium beneath. There was one indentation before the young mycelium.

Replica plating of normal spores on 0.05% L.P.C.P. agar.

2nd day. Germination of spores.

3rd day. A small amount of growth at the needle holes. Hyphae were swollen and contorted.

8th day. Mycelium was white.

10th day. Colonies were minute and white and growth was most irregular.

12th day. Faint yellow tinges in the mycelium.

16th day. White spore heads were visible.

18th day. Some colonies were quite advanced with spore heads cinnamon.

Spores from 0.05% L.P.C.P. colony replica plated on to 0.05% L.P.C.P. agar. A 10

2nd day. Spore germination.

3rd day. Small amount of growth at needle holes.

13th day. Four colonies were developing having a small

amount of mycelium with just the faintest shade of yellow. Microscopical examination showed a few malformed heads but the majority of growth was sterile hyphae.

18th day. Mycelium was bright yellow and there were a number of white and cinnamon spore heads.

Spores from 0.05% L.P.C.P. colony replica plated on 0.05% L.P.C.P. agar. A 10

6th day. Mycelium was white.

8th day. Mycelium was white tinged with yellow, and it was fluffy.

11th day. Colonies were about 1½ cms. in diameter. Spore heads in centre were cinnamon.

Camera lucida drawings were made of abnormalities on the 0.05% L.P.C.P. agar (Plate 5).

Replica plating.

0.05% L.P.C.P. - Normal parent strain

Aspergillus terreus

2nd day - germination of spores.

8th day - white mycelium.

12th day - yellow mycelium.

16th day - white conidial heads.

18th day - cinnamon heads.

0.05% L.P.C.P. - 0.05% L.P.C.P. spores.

2nd day - germination of spores.

13th day - white and yellow mycelium.

18th day - white and cinnamon conidial heads.

0.05% L.P.C.P. - 0.05% L.P.C.P. spores.

2nd day - germination of spores.

6th day - white mycelium.

8th day - white tinged with yellow.

11th day - white and cinnamon conidial heads.

This series of replica tests shows the adaptation of the fungus actually taking place. In the first test the normal parent strain of Aspergillus terreus took some time to develop, as it did in the second test, the spores of the previous test having been used for the inoculation. The third test was using spores from the second test and it is clear that some increased adaptation to the environment had developed since in the third test the fungal growth was more rapid. An increased resistance to the rotproofer has been set up by the fungus, and continued subculturing would have probably reduced the growth rate even further. The development time has increased considerably with increased concentration.

Normal parent strain of Aspergillus terreus on 0.04% L.P.C.P. agar. A 10

A 10^3/ml. spore suspension was inoculated on 0.04% L.P.C.P. agar.

4th day. The colonies were at initial stages of development. They appeared as small flat round dots on the agar surface.

5th day. More growth and the colony numbers were as follows - 113, 189, and 133.

6th day. The mycelium was fluffy and white. On the 6th day individual colonies were inoculated on 0.04% L.P.C.P., 0.05% L.P.C.P., and 0.06% L.P.C.P.

11th day. In all cases the transferred colonies had grown from the transfer on to the level of the plate agar and this mycelium was white. The transferred pieces had yellow mycelium with droplets of liquid exudate. No spore heads had formed.

15th day. In all cases white and cinnamon spore heads were present.

18th day. All spore heads were cinnamon.

0.04% L.P.C.P. - Normal parent strain Aspergillus terreus.

2nd day - germination of spores
5th day - white mycelium
11th day - yellow mycelium
15th day - white and cinnamon conidial heads
18th day - cinnamon heads.

This growth pattern is similar to the replica plate timing of normal parent strain on 0.05% L.P.C.P. agar. It shows that the normal strain takes longer to adapt itself to the toxic medium than strains which have already grown on L.P.C.P. because spores which have grown on 0.03% concentration of L.P.C.P. and have been inoculated on to 0.05% L.P.C.P. agar grow more quickly than the normal parent strain inoculated on to 0.04% L.P.C.P. agar.

Spore germination showed that an average of 14.4% of the spores were viable. This does not vary very much from the 0.03% L.P.C.P. spore viability on 0.05% L.P.C.P. agar (i.e. 17%).

Normal parent strain of Aspergillus terreus on 0.05% L.PCP agar.

A 10 A 10^4/ml. spore suspension was made up of normal Aspergillus terreus and 1 ml. of this was then inoculated on 0.05% L.P.C.P. agar.

2nd day. There was slight hyphal growth and it was possible to pick off individual colonies and transfer these to other dishes.

3rd day. Twelve colonies were transferred from the spore suspension growth on 0.05% L.P.C.P. to another dish containing 0.05%. These transferred colonies were white.

6th day. All the mycelium had turned yellow. A small

amount of white mycelial growth had formed on the new plate agar. There were no conidial heads.

8th day. Growth continued to occur slowly and the mycelium was still yellow and sterile apart from three small areas where white conidial heads were visible. All the yellow mycelium was covered with small yellow liquid globules.

10th day. Yellow mycelium was still prevalent, but intermingled with it were conidial heads which had begun to turn cinnamon.

Fresh mycelial growth had occurred round each colony and this, in each case, was white but without conidial heads. The average diameter size was $1\frac{1}{5}$ cm.

Furrowing of the colonies in the agar was evident (Plate 6).

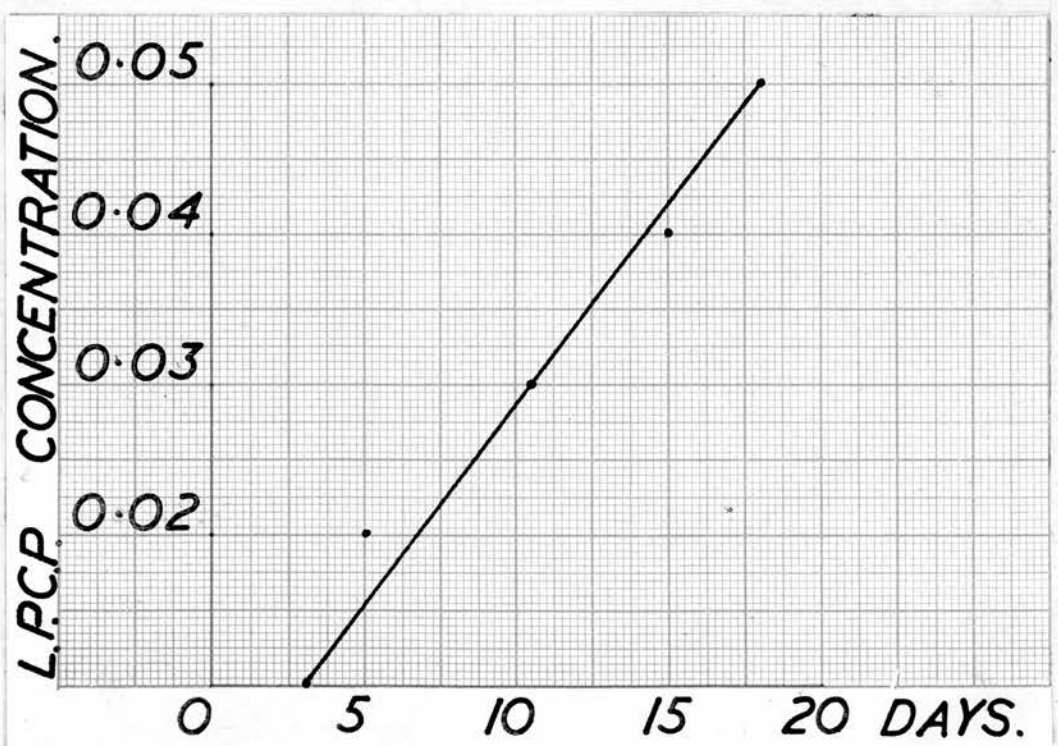
13th day. The colonies had all grown to about $1\frac{3}{4}$ cm. in diameter. Growth of the colonies was in the form of concentric rings, and at each ring portion, there was an indentation of growth further into the agar. The yellow mycelium still persisted, and was particularly prevalent in the furrows; on it grew a few white and cinnamon heads. Conidial heads had also developed on the youngest concentric ring. There were liquid globules present.

The yellow mycelium showed much twisted and irregular branching, without spore head formation.

From an area containing spore heads, irregularities of conidiophore development were apparent. The majority of heads appeared normal, but some had abnormalities (Plate 7 and 8). There were conidiophores with two, three, four and five heads.

15th day. Heads which had developed by this time on the youngest hyphae were found to be quite normal. These heads were white on the 14th day, and were cinnamon coloured by the 15th day. There was no trace of yellow mycelium by now.

This graph shows L.P.C.P. concentration plotted against time in days to reach mature cinnamon heads.



0.05% L.P.C.P. - Normal parent strain of Aspergillus terreus.

2nd day - germination of spores.

3rd day - white mycelium.

6th day - yellow mycelium.

10th day - white conidial heads.

13th day - cinnamon heads.

Average colony diameter.

10th day - 1⅕ cm.

13th day - 1¾ cm.

The young heads which grew on the outer rim of the colonies did not appear abnormal in any way, and it seemed as if all the L.P.C.P. had diffused into the already growing colonies and that the normal growth was due to the fungus growing on virtually normal agar.

The liquid globules were apparent here again, but they have been noted by Raper et al. (1945) in connection with Aspergillus terreus and by Cushing, Schwartz, and Bennet (1949) with indole-resistant mutants of Neurospora crassa.

A test was done using normal agar and normal Aspergillus terreus but with the agar slashed, to see if liquid globules formed along the slashes.

After six days the culture was examined for liquid globules. There were some over the whole surface of the

colony which was quite normal, but there was no greater quantity along the slashes; in fact, if anything, there were fewer in this area.

Yellow mycelium inoculated on normal agar.

Some of the sterile yellow mycelium in the above experiment was inoculated on to normal agar. It was a tough mat of mycelium which was difficult to break through.

2nd day. The yellow coloration had begun to disappear, and young, white conidial heads were developing.

5th day. All the yellow colour had gone, and the central area of each colony was covered with cinnamon coloured heads, and beyond this there was a zone of white mycelial growth with young heads. Microscopical examination of this culture showed that Aspergillus terreus had returned to its normal growth. The average diameter of the colonies was 1½ cm.

Normal agar - sterile yellow mycelium.

2nd day - white conidial heads.

5th day - cinnamon heads.

Average colony diameter.

5th day - 1½ cm.

A transfer of yellow mycelium, which had grown on 0.05% L.P.C.P. agar, was made to 0.05% L.P.C.P. agar. The same sterile yellow mycelium developed.

The sterile mycelium quickly reverted to a normal type of growth on normal agar. This again shows that the sterility was associated with metabolic function, and that spore production was latent till suitable environmental conditions were available. The sterile regions formed concentric rings in the colony growth, and represent the restricted region of development which has already been discussed when zonate colony development was described.

Spore suspension from 0.05% L.P.C.P. colony inoculated on 0.05% L.P.C.P. agar.

1 ml. of a spore suspension made up from the spores which had developed on 0.05% L.P.C.P. agar was inoculated on another dish containing 0.05% L.P.C.P. agar.

4th day. Individual colonies had formed. No spore heads were present, and the mycelial growth was white. Twelve colonies were subcultured at this time to another dish containing the same percentage L.P.C.P.

5th day. No further growth.

6th day. Growth had developed on the piece of transferred agar, but it had not extended to the new plate agar. White spore heads were just developing and pale yellow mycelium was evident; microscopical examination showed growth irregularities.

7th day. Still no growth at plate level. Conidial heads still white. There were small liquid globules present.

11th day. The cultures had all grown on to the plate agar, and in each case at the base of the transfer yellow mycelium occurred, and round this, new white mycelial growth was embedding itself into the agar. The undersurface of the cultures was wrinkled as in previous tests. Colonies were about 4/5 cm. across. The majority of abnormalities were double heads.

13th day. The central part of the colonies had cinnamon heads. The yellow mycelium area was still obvious and beyond this appeared a ring of young white heads. There was white mycelium surrounding this and the average diameter was 1 cm.

15th day. Concentric rings of indentations were again apparent. The yellow mycelium had begun to develop one or two small spore heads. In addition to this there was a brown liquid exudate, which was more prevalent in the areas of the yellow mycelium, which in turn were in the indented areas. The final average diameter was $1\frac{9}{10}$ cm.

Spore suspension from 0.05% L.P.C.P. colony inoculated on to 0.05% L.P.C.P. agar.

1 ml. of a 10^4/ml. spore suspension from the above 0.05% L.P.C.P. agar medium was inoculated on to a petri dish containing 0.05% L.P.C.P. agar.

3rd day. The beginning of growth areas was just visible.

5th day. The colonies had grown to 1½ mm. in diameter. Microscopical examination showed peculiar restricted hyphae (Plate 9). Agar indentation again. No spore heads formed.

6th day. Plate I had 60 colonies.
Plate II had 70 colonies.

8th day. Average diameter of colonies was ½ cm. The colonies were raised in the middle. At the base of each colony the mycelium was yellow and small droplets of exudate could be seen.

9th day. White conidial heads were present.

13th day. Average diameter was 1½ cm. Cinnamon coloured spore heads were abundant. There were no indentations in the last concentric ring and no yellow coloration. There was a large number of liquid globules over the parts in which the yellow mycelium was most common. Microscopical examination showed the usual abnormalities, and a further type of irregular growth was when an

already formed spore head, then went on to produce another conidiophore (Plate 10).

0.05% L.P.C.P. - 0.05% L.P.C.P. spores.

2nd day - germination of spores.
4th day - white mycelium.
6th day - yellow mycelium.
7th day - white conidial heads.
13th day - cinnamon heads.

Average colony diameter

11th day - 4/5 cm.
13th day - 1 cm.
15th day - $1\frac{9}{10}$ cm.

0.05% L.P.C.P. - 0.05% L.P.C.P. spores

2nd day - germination of spores.
5th day - white mycelium.
8th day - yellow mycelium.
9th day - white conidial heads.
13th day - cinnamon heads.

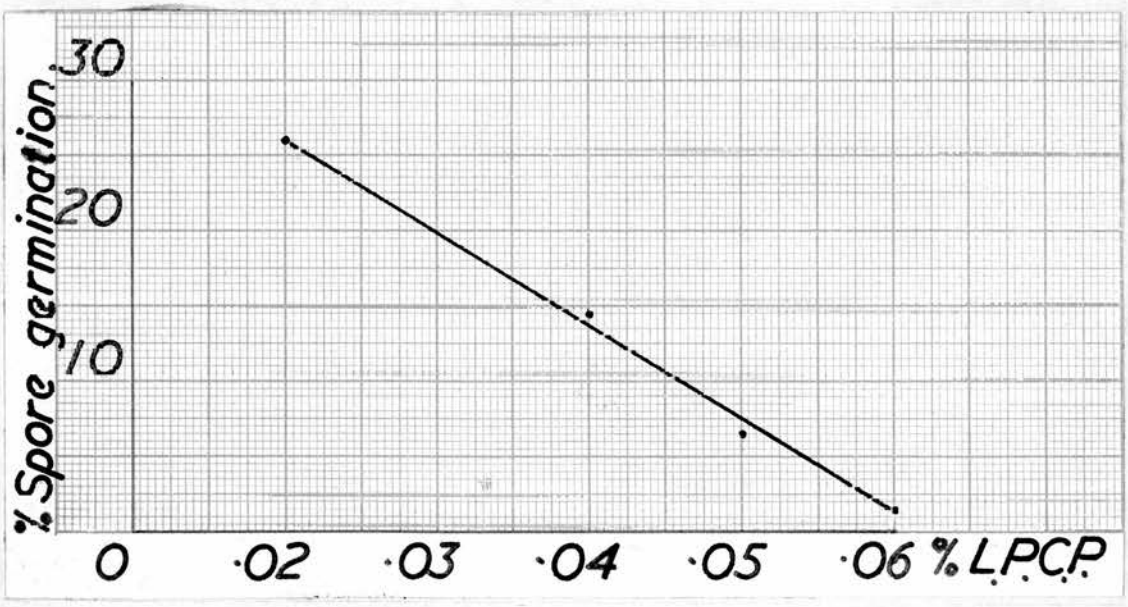
Average colony diameter

8th day - $\frac{1}{2}$ cm.
13th day - $1\frac{1}{2}$ cm.

This was a further series of tests using spore suspensions as opposed to replica plating and in this particular test the

initial growth has taken less time than in the replica plated cultures, but the third subculture here has taken two more days to reach maturity than the third subculture in the replica plates, and there has been no acceleration of growth rate in the third culture. Further subculturing may have increased the fungal adaptation to environment in time.

There was only a 6.5% spore germination, so it appears that increased concentration of L.P.C.P. gives decreased viability. It produces a straight line graph, as shown below:-



Spore suspension from 0.05% L.P.C.P. colony inoculated on to 0.06% L.P.C.P. agar. A 10

1 ml. of a spore suspension made up from the above culture was inoculated on to 0.06% L.P.C.P. agar.

3rd day. Spores germinated.

5th day. A small amount of growth just visible.

6th day. 17 colonies on one plate and 16 on another.

9th day. Small white colonies with agar indentations apparent. The average colony diameter was 0.2 cm.

12th day. Subcultures were made to other plates containing 0.06% L.P.C.P.

14th day. Mycelium was yellow in colour.

15th day. Growth was on an average = ½ cm. The mycelium was bright yellow and growth was fluffy. There was some liquid exudate, but no spore heads.

18th day. Yellow mycelium still prevalent. New growth had occurred and there were young spore heads present.

20th day. Cinnamon heads were produced here and there, but it was not an all over pattern. There were also liquid globules. The agar undersurface was wrinkled, except in the area of the last concentric ring.

Normal parent strain on 0.06% L.P.C.P. agar. A 10

3rd day. The spore suspension had produced initial growth.

8th day. The mycelium was white, and no spore heads had formed. The colonies were raised. The average diameter was 0.2 cm.

12th day. Mycelium was yellow.

16th day. White mycelium as well as yellow and the usual embedding. Spore heads were beginning to develop. Colonies had a diameter of 0.6 cm.

17th day. Colony average diameter was 1 cm. White heads were noted, some of these being abnormal in growth. A hypha which branched in two showed dissimilar heads. One head still retained the typical Aspergillus form whilst the other looked much more like a species of Penicillium (Plate 11).

18th day. Further growth giving a colony diameter of 1.2 cm. There were some cinnamon coloured heads, but the majority were white.

19th day. There were a large number of white and cinnamon heads. Similar rings and embedding as before. Colony diameter was 1.6 cm.

There was a definite gradient of abnormality when slides of outer, middle, and central areas of the colonies were examined.

The outer area produced normal heads, whilst the middle area had a few abnormal heads. In the centre irregular spore heads were most common.

0.06% L.P.C.P. - 0.05% L.P.C.P. spores.

3rd day - germination of spores
9th day - white mycelium
14th day - yellow mycelium
18th day - white conidial heads
20th day - cinnamon heads.

0.06% L.P.C.P. - normal parent strain Aspergillus terreus

3rd day - germination of spores
8th day - white mycelium
12th day - yellow mycelium
17th day - white conidial heads
18th day - cinnamon heads.

There was not a very great difference between the two rates of development here, but mature spore production seems to be becoming a very slow process.

0.05% L.P.C.P. spores inoculated on 0.1% L.P.C.P. agar.

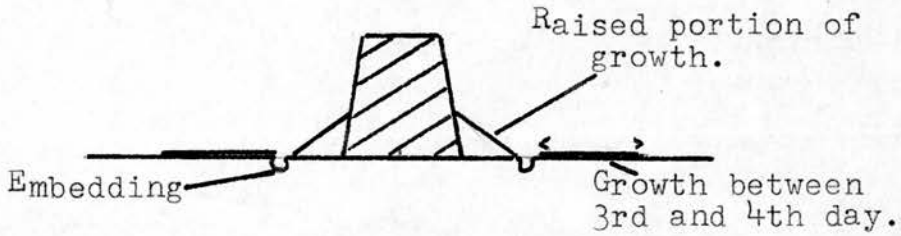
There was no growth after 1 month and since Parry and Wood (1959) consider this a sufficiently long period for testing growth on solid media, the spores were considered non-viable, as examination of the agar medium showed their presence.

Spore suspension from 0.05% L.P.C.P. colony inoculated on to normal agar. A 10

2nd day. Mycelium was slightly yellow.

3rd day. Young conidial heads had developed around the basal region of the pieces transferred on the 2nd day. The mycelium was yellow. The average diameter of the colonies was 1 cm. Microscopical examination of spore heads showed no irregularities.

4th day. New white growth developed round the base of the colonies and the average diameter was 1½ cm. Slight embedding was the feature between the old and the new growth. No globules present. Figure shows type of indentation.



The white conidial heads of the 3rd day had turned cinnamon.

8th day. The colonies were all cinnamon and had grown to 2 cm. in diameter. No yellow coloured mycelium had remained and the undersurface of the colonies appeared to be quite normal.

Normal parent strain on 0.07% L.P.C.P. agar. A 10

A 10^3/ ml. spore suspension of normal Aspergillus terreus was made up and 1 ml. portions were inoculated on 0.07% L.P.C.P. agar. After 22 days there was no growth visible, and the plates, even though they were sellotaped, were beginning to dry out (Parry and Wood, 1959).

1 ml. portions were also inoculated on to normal agar and after one day's growth colonies were subcultured on to agar containing 0.07% L.P.C.P.

After 22 days in this concentration no apparent growth had occurred and when examined microscopically the hyphae seemed to have become submerged into the agar and produced a sort of curling effect (Plate 12).

This curling effect was similar to that recorded by Brian (1960) in his description of the reaction of Botrytis allii to griseofulvin.

Solid medium.

Growth of Trichoderma viride on L.P.C.P. treated agar.

Further experimental work on solid medium was done using the fungus Trichoderma viride which had been isolated from the soil/leaf mould mixture. It was identified using Gilman (1957) and Smith (1954).

This fungus was found growing frequently on treated jute hessian, being particularly prevalent on the cloth exposed to the soil/leaf mould mixture.

A limited number of tests on Trichoderma viride have been done to give an outline of the reaction of this fungus to L.P.C.P. but they have not been carried out in such great detail as the tests on Aspergillus terreus.

Difficulty in separating the spores was encountered to begin with. The use of a slightly older culture, and shaking the spores of this culture with a few drops of Teepol helped. In addition to this some pieces of culture were gently crushed on the side of a vial with a sterile glass rod.

A spore suspension was made up and 1 ml. portions of this were inoculated on to plates containing normal Czapek Dox agar.

By the 5th day growth had occurred but not very luxuriantly. It was found that if malt extract agar was

used which has a pH of 5.4 instead of Czapek Dox, pH = 6.8, much more luxuriant growth took place.

Malt extract agar was used in the rest of the tests.

Normal malt extract agar and normal parent strain of Trichoderma viride.

Malt extract agar was used for all the following tests with Trichoderma viride, so the type of agar will not be mentioned again.

1 ml. of a 10^3/ml. spore suspension was inoculated on petri dishes containing normal malt extract agar.

2nd day. The spores had germinated and produced one or two hyphae.

3rd day. White mycelium was evident.

4th day. Green spore heads had formed.

Normal agar - normal parent strain of Trichoderma viride

2nd day - white mycelium

4th day - green conidial heads.

This is considered to be the normal rate of growth for Trichoderma viride on normal malt extract agar.

Normal parent strain of Trichoderma viride on 0.05% L.P.C.P. agar. A 10

2nd day. Placed twelve individual colonies from normal agar on to agar containing 0.05% L.P.C.P.

7th day. Area of growth was small = 7 mm. The mycelium was sterile, but there was no stunting of hyphal growth, although some of the cells present were enlarged and the hyphae tended to be ropey in appearance.

9th day. At the 12 mm. level a ring of raised hyphae occurred, and on this were green spore heads.

14th day. Prolific growth of Trichoderma viride over the surface of the agar, and another ring of spore heads were produced round the colonies (Plate 13). There was no restricted growth and no abnormal morphology apparent on microscopical examination.

0.05% L.P.C.P. - Normal parent strain of Trichoderma viride

2nd day - white mycelium

9th day - green spore heads.

Average colony diameter

7th day - 7/10 cm.

9th day - $1\frac{1}{10}$ cm.

There was no colour change in the mycelial growth, and the development of Trichoderma viride in the presence of

0.05% L.P.C.P. was more rapid than that of Aspergillus terreus, although the normal growth on normal agar of each fungus was just the same in time factor.

Normal parent strain of Trichoderma viride inoculated on 0.07% L.P.C.P. agar.

A spore suspension of normal parent strain Trichoderma viride was made up and 1 ml. portions were inoculated on to agar treated with 0.07% L.P.C.P. agar. After one month no growth had occurred.

Normal parent strain of T. viride transferred from normal to 0.07% L.P.C.P. agar.

After ten days there was only a very limited amount of distorted hyphal growth (Plate 14). There was no further development.

Again 0.07% L.P.C.P. agar appears to be the critical point in adaptation. The normal parent strain is not able to adapt itself to this higher concentration. The hyphae were again twisted and contorted but in a different way from those of Aspergillus terreus. There was no evidence of the curling effect.

Replica plating.

Spores from 0.05% L.P.C.P. colony replica plated on 0.05% L.P.C.P. agar. A 10

3rd day. One plate showed no growth, but the other showed the beginning of growth.

6th day. Both plates showed the twelve colonies developing. These had grown on an average to 2 cm. diameter. The mycelium was white with yellowish-green spore heads beginning to develop.

7th day. Spore heads were green and concentric rings of growth had developed.

Spores from 0.05% L.P.C.P. colony replica plated on 0.07% L.P.C.P. agar.

Spores from the second growth of Trichoderma viride were needle inoculated on to plates containing 0.07% L.P.C.P. agar.

3rd day. There was white mycelial growth from a few of the needle holes.

6th day. One dish showed no growth at all, whilst the other one had five colonies growing, with an average diameter of 1 cm. The mycelium was greyish-white.

8th day. Colony diameter was 2½ cm. Similar growth to colonies which were photographed previously. Spore heads developed.

10th day. Colonies had 3-4 rings of growth and the average diameter was 3 cm. One other small colony had developed making a total of six formed and six unformed colonies.

In this test it would seem that some of the colonies had been able to adapt to the proofing treatment better than others and therefore those which had adapted, must have had more resistance to L.P.C.P. than the others.

Spores from 0.07% L.P.C.P. colonies of T. viride replica plated on 0.07% L.P.C.P. agar.

6th day. Three colonies on one plate, two on another, and four on a third. Colony diameter was from 1 - 1½ cm. The colonies were stone coloured and there were no spore heads visible. One or two areas of fluffy white mycelium.

7th day. Central areas had green spore heads, also new hyphal growth. The average colony diameter was 2 cm. The rings developed in previous tests were again present.

The rate of growth in this test was slightly greater than the previous test showing the beginnings of further adaptation to L.P.C.P. This resembles the results in some of the Aspergillus terreus work also.

The development of ring growth is of interest. The growth in Trichoderma viride differs from the Aspergillus terreus ring growth. In Aspergillus terreus the rings were formed due to indentations in the agar, but in T. viride there was a band of green spore heads, and then a thin white mycelial band followed by another band of green spore heads (Plate 13 & 14). A 10

In previous discussion on this, it was suggested that the fungus overcame the rotproofer for some time and then had a period of restricted growth when it was again building up a resistance to L.P.C.P. This was not failure of inoculation, since the spores were seen in the agar medium.

During these tests attempts were made to grow Aspergillus terreus for comparative results, but it did not grow on malt agar + L.P.C.P. A test was then done reducing the pH to 3.5 with 10% lactic acid + malt agar.

3rd day. White hyphal growth developed.

6th day. Colonies had an average diameter of 2 cm. The spore heads were a little darker in colour than usual.

A final test was carried out making an extract from Trichoderma viride. This was done by grinding the spores in a mortar and pestle and adding water to form a solution. Even with repeated filtration all the spores of Trichoderma viride could not be removed, as the filter available was not sufficiently fine so the mixture was autoclaved.

This was then added to petri dishes in 1 ml. portions. Some of the dishes contained normal agar whilst others contained 0.05% L.P.C.P. A 10^3/ml. spore suspension of normal parent strain Aspergillus terreus was added to the dishes in 1 ml. portions and these plates were then incubated. The spores on the normal agar plates germinated quite successfully, but the presence of the Trichoderma viride extract did not apparently accelerate growth. Neither did the presence of the extract allow any growth of Aspergillus terreus on 0.05% L.P.C.P. after 3 weeks incubation. The reason for this is difficult to understand, especially since the malt extract agar is a more nutritive medium than Czapek Dox. Raper (1943) has found the same feature occasionally in his tests on Aspergillus terreus and his only explanation is that, because of an alteration in metabolism there might arise a production of an excess of staling products, thus making it impossible for the spores to germinate successfully.

When Trichoderma viride which had developed on 0.05% L.P.C.P. agar was subcultured on to normal agar it grew normally and there was little difference between it and normal parent strain of Trichoderma viride. It had not, in fact, retained its resistance but had reverted to its original type of growth.

Spore germination tests.

Two methods of test were used here.

(1) Sterile cellophane discs (Yuill, 1950) were laid over the surface of the agar and spores were then streaked on to these. The discs allowed the nutritive material to pass through and the fungus grew on these discs which could then be pealed off when necessary.

Some petri dishes with a layer of cellophane paper were inoculated with Aspergillus terreus. By the second day white mycelial tufts had developed. These germinated spores were examined cytologically using Somers, Wagner and Hsu's (1960) staining method, i.e. acetic/alcohol 1:3, followed by 1 N. HCl and then acetic orcein.

The use of the cellophane discs was not altogether satisfactory, as it was difficult to take the growing material off the cellophane.

(2) A more suitable way of studying spore germination was using slide cultures.

Slides were covered with a thin layer of medium mentioned in McLean and Cook (1958). The spores were sprinkled on to the surface of the medium and placed in petri dishes which had wet filter papers in their base.

Cytological examination.

Some Aspergillus terreus spores were inoculated at 4.30 p.m and examined at 9.30 a.m. on the next day. Many of the spores had enlarged slightly and the nuclei had also enlarged.

Initial spore size when examined microscopically with oil immersion 2.5 μ . All the initial spores had one nucleus and this agrees with Yuill's (1950) findings. (Plate 15).

Oil immersion (after 5 hours) average size was 5μ.

By 22 hours the spores had produced hyphae which were too advanced for measuring.

Examination of the overnight cultures (4.30 p.m. - 9.30 a.m.)

In many of the enlarged spores could be seen thin strands of a darker colour which resembled a prophase in higher organisms. One of the spores appeared to have four(?) chromosomes in its centre (Plate 15). There were a few spores which had not developed in any way from the original. There were also some spores which had not produced hyphae but which appeared to have prophase nuclei.

Slides with 0.05% L.P.C.P. agar and Aspergillus terreus.

The McLean and Cook mixture was made up as before and to this was added 0.05% L.P.C.P. This mixture on the slides was then inoculated with normal Aspergillus terreus spores. Spores were inoculated at 5 p.m. By 3 p.m. on the next day

slides were examined. No germ tubes had developed, but a number of the spores had enlarged. Oil immersion after 22 hours gave average spore size as 4μ.

A large number of spores had not altered in size at all, although one spore was 7μ.

Forty-eight hours after inoculation showed that germination had occurred but that growth was stunted and abnormal. (Plates 16 and 17).

One spore had two nuclei and another had three, but all others had one nucleus. These extra nuclei appeared after germination.

5th day. The hyphae had grown by this time but they were very stunted and gnarled looking. The ends were branched in a peculiar way (Plates 18 and 19).

Trichoderma viride spore germination.

Slides with 0.05% L.P.C.P. and nutritive medium.

The initial size of Trichoderma viride spores was 3μ using oil immersion.

After 22 hours the following sizes were noted under oil immersion: 4μ , 5μ , 5μ , 5μ , 3μ , 3μ , 4μ , 5μ , 5μ .

A few of the spores had formed germ tubes, but there was no stunting in growth as in Aspergillus terreus. A parallel experiment using Aspergillus terreus was done at

this time and the same results as before were noted.

The percentage spore germination for Trichoderma viride on 0.05% L.P.C.P. was 18% which was slightly higher than Aspergillus terreus at the same level of concentration.

Again, the time lag in spore germination was noticed, but a stunted gnarled growth occurred with Aspergillus terreus and not with Trichoderma viride. Could this be another feature suggesting a better adaptation to L.P.C.P. by Trichoderma viride?

A note on the cytological features of Aspergillus terreus will be given in the discussion. Trichoderma viride was not examined cytologically.

Cloth squares test.

Since the "training" was a slow process and difficulties of growth occurred at relatively low concentrations of L.P.C.P. it was thought that if spores of Aspergillus terreus were streak inoculated on to normal agar and then some treated cloth was placed over this, the results might be interesting.

Some cloth was treated with 0.05% L.P.C.P. and was cut up into squares and autoclaved. Inoculation was made by using a platinum loop containing a number of spores and streaking along the surface of the agar.

2nd day. White mycelium had developed but not on the cloth.

3rd day. White mycelium patches growing thinly on the cloth. No spore heads.

7th day. A small quantity of growth had occurred on cloth. Spore heads were produced, some abnormal (Plate 20), but the majority seemed normal. The fungal growth under the cloth was yellow and sterile.

The jute fibres which had been attacked by the fungal growth were examined and showed degradation when treated with 15% NaOH (Doree, 1947).

Cloth treated with 0.07% L.P.C.P. - Aspergillus terreus.

Czapek Dox agar medium was inoculated in the usual way and a square of cloth treated with 0.07% L.P.C.P. was placed on top.

3rd day. There was quite a lot of mycelial growth and some white spore heads.

7th day. Examined under the dissecting microscope small white heads had developed over the surface of the cloth square. There were a few heads beginning to turn cinnamon, but they were smaller in size than the normal heads growing outside the area of the square. On lifting up the square of cloth it was seen that the

undersurface of the square was infected to an even greater extent and some spore heads were intermingled with the sterile mycelium. The undersurface of the petri dish showed that the agar was wrinkled in the area where the cloth was placed.

Cloth treated with 0.7% L.P.C.P. - Aspergillus terreus.

Inoculations were done in usual way.

4th day. The growth outwith the area of the jute square was quite normal with cinnamon coloured spore heads; nearer the square of cloth the hyphae were yellow. There was no hyphal nor spore growth on the upper surface of the cloth.

5th day. Still no growth on the upper surface of the cloth pieces. Radiating from the square were bright yellow crescents of hyphae which had quite a number of liquid globules. On the outer surface of the crescents were bands of small white spore heads (Plate 21).

The undersurface of one of the pieces of jute was examined and it was noticed that a few of the fibres on this surface were attacked by the fungus. It was interesting to notice that the fungal growth had been restricted to the inoculating lines (Plate 22). There were many indentations into the agar and here liquid globules were present. The bright yellow hyphae were examined microscopically and found to be sterile, but appeared to be attempting to make vesicles here and there. The intensity of the yellow pigment was

much greater than in the lower concentrations of L.P.C.P. on the cloth.

8th day. Tiny patches of white spore heads were growing on the upper surface of the cloth.

15th day. Cinnamon spore heads had grown over the whole upper surface of the cloth. The fungal growth on the plate agar had developed further, so that most of the sterile mycelium had developed spore heads.

Cloth treated with 1% L.P.C.P. - Aspergillus terreus.

The cultures were left in the incubator for 1 month. In each case the fungus had grown over the surface of the cloth and the conidial heads varied in appearance considerably - long thin, short thin, medium and fat heads were all visible.

These tests have shown that the fungus can overcome the toxicity of the L.P.C.P. and can still grow on the cloth and degrade it. As the number of spores in the petri dishes may well be similar to the number found in the soil, it is not surprising that cloth treated with a normal amount of L.P.C.P. will eventually be degraded.

Cloth treated with 1% L.P.C.P. - Trichoderma viride.

Trichoderma viride spores were streak inoculated on to malt agar and a square of 1% L.P.C.P. treated cloth was put on top.

3rd day. Trichoderma viride showed good growth and did not appear to be in any way inhibited, but there was no fungal growth on the upper surface of the cloth. There was no difference in culture colour as was experienced with Aspergillus terreus.

7th day. Fungal growth had begun on the cloth.

12th day. The upper surface of the cloth had fungal growth which, however, did not cover the whole cloth as with Aspergillus terreus, but small balls of white mycelium had formed. This mycelium had begun to develop conidial heads and some of the growth had turned green.

Trichoderma viride has reacted in the same way as Aspergillus terreus, by growing, after a short lag period, on the treated cloth and so degrading it.

Cloth squares test.

These tests showed how well the fungi adapted themselves to the high concentration of L.P.C.P. on the hessian. Aspergillus terreus produced all the features characteristic in the adaptation to L.P.C.P. but spore heads were developed and it was not long before the culture was growing over the upper, as well as the lower, surface of the hessian, which had been treated with 1% L.P.C.P., and degraded it.

The same was found to be the case with Trichoderma viride and in neither was a limit reached by the presence of L.P.C.P. concentration, as was the result with individual spore colonies on agar with percentage L.P.C.P. present.

Untreated cloth samples were exposed to both fungi as a control. The samples were autoclaved, and then placed over the inoculum in the same manner as the treated tests. The cloth was rapidly degraded by Aspergillus terreus on Czapek Dox and Trichoderma viride on malt extract agar.

DISCUSSION.

It has been of great interest from an industrial point of view to make these fungal studies. The jute firms are all anxious to improve the lasting qualities of their cloth, since there is so much competition in the textile market these days with the introduction of man-made fibres. Pentachlorophenol laurate has been used by the jute firms a lot in recent years, but it still cannot be considered as good a proofing treatment as copper naphthenate. These tests which have been carried out show that the two cellulose destroying fungi examined reacted to the presence of L.P.C.P. by taking longer to grow, and producing abnormalities, even at low concentrations. There was evidence of adaptation to the environment, and if time had allowed, continued subculturing till growth on a high concentration of L.P.C.P. occurred, would have been of value. These colonies, after all, were produced from a single spore. In the cases where spores were streaked on to the agar and were then in contact with cloth treated with 1% L.P.C.P.; the results show that the fungus in time adapts itself to the presence of the L.P.C.P. and begins to grow in quite large amounts on the cloth, causing rapid degradation.

In order to compare the efficiency of various rotproofing

agents, treated samples of jute hessian are exposed to soil, under the conditions already stated, and this has been considered the best laboratory method of testing against micro-organism attack. There are, however, very many variables involved and no two sets of results can be compared with any degree of certainty, - thus, when various proofing treatments are tested, copper naphthenate is always included as the standard.

The results of the L.P.C.P. treated cloth on soil/horse manure and soil/leaf mould respectively show how difficult it is to correlate results at times. There did appear to be something present in the soil/leaf mould which caused very rapid and severe damage to the cloth. From the isolates made from the soil it looked as though Trichoderma viride and some bacteria were phenol tolerant, and the phenomenon of L.P.C.P. disappearance in the presence of the soil/leaf mould bears this out.

Smith (1954) says that phenol is probably more efficient for preventing bacterial, rather than mould growth, although it does show a fairly high toxicity to most fungi. He goes on to say - "the halogen substituted phenols are very much more efficient antiseptics than phenol itself, the best, and most readily available being pentachlorophenol."

Gradient plate.

In the gradient plate tests the concentration of spore suspension was too high to allow monospore colony growth, but the behaviour pattern gave an idea of the features which were to occur again and again throughout the tests that followed. The gradient tests were not repeated since the actual amount of L.P.C.P. diffusing through the agar was not known.

Spore colonies on treated agar - Aspergillus terreus.

The work done by Raper, Coghill, and Hollaender (1945) on ultraviolet induced mutations of Aspergillus terreus was of great interest in relation to the work done with Aspergillus terreus in the presence of L.P.C.P. There are many characteristics about the various mutants produced by them which are very similar to the morphological features observed during the present work.

The cultures which were grown and examined here showed a number of features, each one of which was found in a different mutant in the Raper et al (1945) irradiation tests.

The following are the characteristics of Aspergillus terreus mutants due to irradiation which are similar to Aspergillus terreus in L.P.C.P. agar.

(1) "Granulated mutant - Irregular development of conidial heads. Heads are small, and usually irregular in form. Sterigmata are coarse and commonly do not show a normal separation into primary and secondary series."

In our tests there were many irregular formations of conidial heads and lack of sterigmata in the secondary series (Plate 10 and 11). Fewer spores appeared to be produced on each head, but there were four or five heads developed on one conidiophore in many cases in the presence of the L.P.C.P.

(2) "White to yellow floccose. - Colonies floccose, often but not consistantly restricted, white to yellow in colour, light sporing. Heads are not only limited in number but are commonly atypical in pattern; conidiophores may fail to develop vesicles, or vesicles, when formed, may fail to produce sterigmata and conidia."

The fluffy yellow hyphae with few spore heads were also a feature in our colonies. Plate 7 shows an unusual sort of pitting on the vesicle, which might be aborted sterigmata. Although the rate of growth was decreased the fungus compensated for this by producing a number of heads from one original head. (Plate 7).

(3) Leathery - Colonies upon Czapek's agar are restricted, very close-textured, are strongly buckled and wrinkled and produce few and atypical conidial structures."

In our colonies, growth was restricted and close textured with the result that the mycelium mat was tough to break through. The furrowing of the cultures was mentioned in the results and wrinkling of the upper and lower surfaces of the culture was clearly defined.

(4) "Thiamin deficient - Colonies are thin, spreading, consisting almost exclusively of coarse, heavy walled, submerged hyphae."

The growth of Aspergillus terreus which occurred on 0.07% L.P.C.P. agar was of a similar nature to the thiamin deficient mutant (Plate 12).

(5) "Maroon exudate. - Colonies often produce an excessive amount of deep brown to maroon exudate."

This production of excessive amounts of liquid globules was noted in our tests. Our globules, however, were yellow in colour. Cushing, Schwartz, and Bennet (1949) found droplets of a pinkish liquid appearing at irregular intervals on indole-resistant mutants of Neurospora crassa.

Raper (1945) said that about 80% of these mutants remained stable, whilst the rest just reverted to the normal

on subculturing. They are of the opinion that "abnormal growth and development of certain mutants which grow normally on normal agar indicate that these mutations probably result from some type of nutritional deficiency." In our case this is most probably true, since the presence of the L.P.C.P. may have inhibited some of the usual metabolic processes.

Raper found that the white and yellow floccose mutant was the one which was most unstable. In our case these features were strong, and this may account for the lack of persistent resistance.

The yellow pigment produced by Aspergillus terreus is called citrinin (Anderson, 1946), and this particular pigmentation was developed throughout the tests. Pigmentation is thought to be associated with respiration (Wolf and Wolf, 1949). Fungal metabolism, as a result, is also involved (Hawker, 1950). Spore production may be inhibited and mycelial growth restricted if some of the essential trace elements are missing or are marked.

There was no evidence of any sudden metabolic change, such as might be expected in a gene mutation and this was similar to the results of Parry and Wood (1959) work on the reaction of Botrytis cinerea to chlorinated nitrobenzene.

They found in these tests that the susceptibility of the fungus to the fungicide was affected by the depth of the medium. Throughout our experiments we endeavoured to keep the same amount of medium (20 mls.) present in each petri-dish, and when the colonies were transferred from one plate to another the whole piece of agar that the colony was growing on was removed each time.

Bartlett (1959) who has worked with Penicillium roqueforti said that although resistance to different drugs, such as proflavine or sodium azide, was acquired gradually there was no evidence of mutation and the stability of the resistance was low. The fungus quickly returned to its original state of growth, when inoculated on drug-free medium. This happened every time in our tests, and both the resistant strain and the parent strain grew at the same rate on drug-free medium. This is similar to results by Parry and Wood (1959).

Czapek Dox agar was used in most of Raper's work on Aspergillus terreus, although malt extract was used also.

In our tests Czapek Dox was used for Aspergillus terreus but malt extract agar was used for Trichoderma viride as this fungus grew better on this medium.

It was hoped to carry out parallel tests with

Aspergillus terreus for comparative purposes, but Aspergillus terreus would not grow on malt extract agar + 0.05% L.P.C.P. Tests were done by reducing the pH of normal malt extract agar from 5.4 to about 3.5 to see if pH had anything to do with the lack of growth. The pH was lowered by adding 10% lactic acid. The fungus grew quite normally after about one week. A further attempt to grow A. terreus on malt extract agar + 0.05% L.P.C.P. + 1 ml. of Trichoderma viride extract also failed to produce growth. Raper (1945) noted that in a limited number of cases better growth of A. terreus was obtained on Czapek Dox than on malt agar. They find the explanation of this difficult and say "the possibility of an altered metabolism, resulting in the production of an excess of staling products should not be overlooked."

The "curling factor".

As the concentration of L.P.C.P. in the agar medium increased, growth of Aspergillus terreus altered, and by the time the 0.07% L.P.C.P. level was reached the hyphae were very restricted in growth, and were partly submerged in the agar. At 0.07% the limits of resistance of the fungus to the L.P.C.P. seemed to be reached. There were no apparent signs of growth on the plates after one month, but on microscopical examination it was observed that hyphae

had grown producing a helical curling effect (Plate 12), similar to these described by Brian (1960) in connection with the action of griseofulvin on Botrytis allii. Brian states that he knows "of no substance other than griseofulvin and a few related compounds which produce helical curling of fungal hyphae", but we find a similar result using pentachlorophenol.laurate, which is not related to griseofulvin chemically. The only difference, as regards the curling, was that in our experiments the highest concentration of chemical (0.07% L.P.C.P.) upon which the fungus was able to grow gave this curling effect whilst in Brian's case (1960) it was the lowest concentration of griseofulvin (0.1 - 0.2 μg./ml.) that gave the similar result. Because of this peculiar hyphal growth that is produced in the presence of griseofulvin, the latter, which is isolated from the fungus Penicillium Janczewskii can be termed the "curling factor" (10 & 11.)

Phylogeny of Aspergillus and Penicillium.

Both fungi belong to the same family of Aspergillaceae and are therefore, closely associated. There does not seem to be information about a possible origin of one genus from another in Smith (1955) or Bessey (1950). They describe the origin of the Ascomycetae only, but there is nothing about

the phylogeny within the Ascomycetae. The monographs by Thom and Raper on Aspergilli and Penicillia give no information on the phylogeny.

The fact, however, that the two genera are so closely linked, might make it possible to explain the likeness to Penicillium of some of the Aspergillus terreus heads. (Plates 4 & 11). One plate shows a head which appears to be in a transition stage between an Aspergillus and a Penicillium head. The other plate shows a Penicillium-like head on a branched hypha, the other part of the branch having an Aspergillus head. If time had allowed it would have been interesting to isolate the Penicillium-like heads and subculture.

Lag period in germination.

This lag was of one or two days. The two days lag was in the higher concentration of L.P.C.P., i.e. 0.06% L.P.C.P. Horsfall (1945) is of the opinion that a lag period is induced by most poisons. Often a log. of percentage retardation against log. of concentration will give a reasonably straight line graph.

Wild and Hinshelwood (1956) have found a lag period in culturing Saccharomyces cerevisiae with sodium azide and they also note that the lag increases in higher

concentrations of toxic medium.

Priest and Wood (1961) have noted the same effect with Botrytis allii in association with tetrachloronitrobenzene. The spore germination was retarded and the mycelium which was eventually produced was swollen and distorted and often vacuolated.

Brian et al (1960) on the other hand found that the presence of the "curling factor" did not produce a lag and that 100% germination was reached at the same time as the controls, i.e. in about 7 hours. Even though the stunting was severe the Botrytis allii still produced the germ tube without any retarding effect.

In our tests there was a timelag, and in addition to this there was a low percentage germination. The germ tubes of Aspergillus terreus were very swollen, stunted and distorted (Plates 16, 17 and 18). Trichoderma viride also had a low percentage germination but the germ tubes produced were normal, and showed no stunting.

Secondary Colonies.

Wild and Hinshelwood (1956) noted an interesting phenomenon in their cultures of Saccharomyces cerevisiae known as "secondary colonies". These arose in the presence of sodium azide and developed later and were smaller and

slower in growth than the rest of the culture. There was no sign of this type of growth in the tests with Aspergillus terreus or Trichoderma viride.

Trichoderma viride is a fast growing soil fungus, and in the soil it is known to parasitize other fungi.- Smith (1954). It has been known to grow on jute treated with L.P.C.P. (Bhattacharyya and Bose, 1954). It is known to be very active in decomposing cellulose (Henrici, 1948) and is listed in Siu (1957) as a strong cellulose destroyer.

It is, therefore, not surprising to find that Trichoderma viride grows on treated jute and appears to adapt itself quite easily to the L.P.C.P. in the agar.

Hawker (1950) states that many fungi have been tested for antibacterial activity, and a few, including species of Trichoderma have been shown to be active. This is of interest from the fact that bacteria were also thought to be causing degradation, but whether the activity of Trichoderma viride in this respect is sufficiently great to be significant, it is difficult to assess. Probably bacterial degradation is involved to a certain extent in the jute rotting tests.

Although normal cultures of Aspergillus terreus and Trichoderma viride on normal agar grow at the same rate, T. viride adapts itself more quickly to the presence of L.P.C.P. and this is probably the reason for so frequently finding it on rotting test samples.

There was no appreciable colour change in the colony growth, although the green colour did have a slightly more blue-green appearance than the parent strain. On microscopical examination there was no apparent morphological change in the lower concentrations of L.P.C.P. The hyphae, were however, twisted and stunted at the 0.07% L.P.C.P. level as shown in Plate 14.

Cytology of Aspergillus terreus.

Noticeable changes in growth and morphology of Aspergillus terreus in the presence of L.P.C.P. show fungal adaptation to environment and, therefore, to include a cytological investigation was of interest to see if any chromosomal abberations were formed.

Normal parent strain spores of Aspergillus terreus were examined at an early stage of development, as hyphae of older cultures were vacuolated and there was nuclear degeneration. As Somers et al. (1960) say "Since many of these fragments are within the size limits of chromosomes,

it is desirable to avoid examining cultures of this age for evidence of mitosis."

The enlarging spores showed prophase nuclei, and in Plate 15 are shown what appear to be four chromosomes. This was the only time, anything resembling chromosomes was seen, and there was no sign at any time of anaphase or telophase.

Plate 15 shows four nuclei situated in the vegetative mycelium.

Bakerspigel believes that fungal cytology differs from that of higher plants and is more like fission division. This he has noted with Neurospora crassa, Saprolegnia and others.

Yuill (1950) states that Aspergillus terreus is a uni-nucleate fungus, as are most of the Aspergilli with spores which average less than 4μ. Those species with spores having an average diameter of more than 5μ are substantially multi-nucleate. Yuill goes on to say "Even uni-nucleate species may not be invariably uni-nucleate - an exception is occasionally found: Aspergillus chevalieri typically uni-nucleate shows two nuclei in a very small proportion of its conidia." This was found in some of the tests where the spores were in the presence of L.P.C.P. when 2 and 3 nuclei were seen.(Plate 15).

Dowding (1960) says that in Neurospora she has seen stages of meiosis and mitosis in the ascus which appear to resemble those processes in higher plants, but she has not observed this in the vegetative mycelium.

Somers et al. (1960) are of the opinion that normal mitosis occurs in the vegetative mycelium. This is a difficult phenomenon to prove or disprove and the interpretation of results by different people may give very varying descriptions. In addition to this the nature of the material being examined is difficult.

Trichoderma viride was not examined cytologically.

SUMMARY.

The reason for this project was to see how two cellulose destroying fungi reacted in the presence of a widely used textile rotproofing agent called pentachlorophenol laurate.

Cloth rotting tests.

These tests where the cloth was in contact with micro-organisms from various soil mixtures showed that treated cloth could become rapidly degraded under certain circumstances. L.P.C.P. treated cloth deteriorated quickly in the presence of soil/leaf mould mixture which contained a number of cellulose destroying bacteria and Trichoderma viride in addition to other micro-organisms. The T. viride was seen growing on the cloth samples.

Fungal cultures.

The two cellulose destroying fungi isolated for particular examination were Aspergillus terreus and Trichoderma viride.

Morphological adaptations of Aspergillus terreus to L.P.C.P. in agar medium are as follows:

(1) Irregular and atypical development of conidial heads. Examination of conidial heads showed that a number from 2 - 6 extra heads would develop from one original head, so this was one way of increasing the spore output.

(2) Vesicles did not always form sterigmata.

(3) Yellow pigmentation of hyphae, which were close textured.

(4) Furrowing and wrinkling of colonies.

(5) A liquid exudate developed.

(6) A Penicillium-type of head formed sometimes.

In addition to these features above, there was a lag period in development, the colonies were half the size of the normal parent strain colonies, and spore viability decreased with increased concentration of L.P.C.P.

Growth of Aspergillus terreus

		No. of days.				
Types of spore	Conc. of L.P.C.P.	Germ-ination	White mycelium	Yellow mycelium	White heads	Cinnamon heads
Normal	0.00%	1	2	-	2	3
Normal	0.02%	2	4	4	5	5 & 6
Normal	0.03%	2	4	5	6	8
Normal	0.04%	2	5	11	15	15 & 18
Normal	0.05%	2	8	12	16	18
Normal	0.06%	3	8	12	17	18
Normal	0.05%	2	8	12	16	18
0.05%	0.05%	2	13	13	18	18
0.05%	0.05%	2	6	8	11	11

The above tables show the trend in development of Aspergillus terreus. With increase in concentration there was a corresponding decrease in growth rate. A straight line graph has been drawn (p.38) to show that the time taken to reach mature cinnamon heads is proportional to the concentration of L.P.C.P. present. The table showing the growth at 0.05% L.P.C.P. indicates that continued subculturing on the same concentration reduces the time taken to reach maturity.

0.07% L.P.C.P. was the limit of growth for single spore colonies of normal parent strain Aspergillus terreus on toxic medium. A "curling" of the hyphae occurred at 0.07% L.P.C.P. agar.

Replica plating technique was used and found successful, especially in growth and adaptation of Trichoderma viride to L.P.C.P. This fungus grew well on agar containing 0.07% L.P.C.P.

Strains of Aspergillus terreus and Trichoderma viride were made resistant to L.P.C.P. but this resistance was unstable and reverted to the parent strain type of growth on drug-free medium.

Spore germination in Aspergillus terreus showed gnarled, twisted hyphae in the presence of L.P.C.P., whereas Trichoderma viride germ tubes were quite straight.

Examination of spores after 22 hours incubation in the presence of 0.05% L.P.C.P. showed the percentage of spores which were viable.

6.5% viable spores of Aspergillus terreus at 0.05% L.P.C.P.

18% " " " Trichoderma viride " " "

Treated cloth tests.

These tests showed that cloth treated with L.P.C.P. at 1% concentration which is that used in commerce was still degraded by growth of Aspergillus terreus and Trichoderma viride, as both these fungi had adapted themselves to the presence of L.P.C.P. and had overcome the toxic nature of the proofing treatment.

APPENDICES.

Appendix 1.

Czapek Dox Agar (modified)

Oxoid Tablets

Formula -	Gms. per litre
Sodium nitrate	2
Potassium chloride	0.5
Magnesium glycerophosphate	0.5
Ferrous sulphate	0.01
Potassium sulphate	0.35
Sucrose	30
Oxoid agar No.3	12

pH - 6.8

One tablet added to 5 ml. of distilled water and sterilised by autoclaving for 20 minutes at 10 lbs. per square inch, gives the correct concentration of medium.

Appendix 2.

Malt Extract Agar.

Oxoid Tablets

Formula -	Gms. per litre
Malt Extract (Oxoid L39)	30
Mycological peptone (Oxoid L40)	5
Agar	15

pH - 5.4

Again, one tablet added to 5 mls. of distilled water.

Appendix 3.

Lacto-phenol cotton blue

Lactic acid	100 mls.
Phenol	100 gm.
Glycerine	100 mls.
Water	100 mls.

Dissolve the phenol in water without heating, to prevent oxidation. Then add the glycerine and lactic acid. Dissolve 1% cotton blue (soluble blue) in lacto phenol.

Appendix 4.

Carbon Analysis of soil.

0.2 gm. of soil are weighed out and transferred to a test tube. 20 mls. of chromic acid solution are added; this solution is prepared by dissolving 8.1 gms. of potassium chromate in 300 mls. of concentrated sulphuric acid, and heating the mixture at 165°C. in a glycerine bath for half an hour. The soil is mixed well with the solution by vigorous shaking. The oxidation of the organic matter is brought about by inserting the test-tube in the glycerine bath maintained at 165°C. for ten minutes. A blank with 20 mls. of chromic acid solution is also inserted. The test tubes are then taken out and allowed to cool. Meanwhile 100 mls. of recently boiled and cooled water are taken in a 600 ml. flask and 10 mls. of 85% phosphoric acid solution are added.

The contents of the test tube after cooling are transferred to the flask with 150 mls. of boiled and cooled water. Ten drops of diphenylamine solution (prepared by dissolving 0.5 gm. in 100 mls. of concentrated sulphuric acid and 20 mls. of water) are added. The indicator turns the solution deep blue. The solution is titrated with ferrous ammonium sulphate prepared by dissolving 79.5 gm. in 1000 mls. water containing 40 mls. of concentrated sulphuric acid. The titration is continued to the total disappearance of blue from the green solution, i.e. until the solution turns muddy green. Fifteen to sixteen samples may be oxidised in the same glycerine bath.

(1 ml. ferrous ammonium sulphate solution equals 0.0005955 gm. of carbon.)

Example:

Blank = 36.0 mls. ferrous ammonium sulphate

Blank titration = 22.0 mls. " " "

Carbon equivalent = 14.0 mls. " " "

= 0.00834 gm. carbon

% Carbon = 4.17

Appendix 5.

Nitrogen analysis of soil.

Kjeldahl - Gunning-Jodlbauer Method.

Take 4 - 5 gm. soil, and place in a round bottomed Kjeldahl flask of 300 ml. capacity. Add a freshly prepared mixture of 2 gm. salicylic acid and 30 mls. of concentrated sulphuric acid through a long stemmed funnel so as to cover all the soil at once. Shake contents of flask until thoroughly mixed and allow them to stand in the cold with occasional shaking for 30 minutes. Should the mixture become warm at any time during the mixing process, cool it immediately by placing the flask in ice water. If the substance has not dissolved at the end of 30 minutes standing, heat the flask on a boiling water bath until solution is complete.

Cool the solution and add 2 gm. of zinc dust, little by little, through the long-stemmed funnel, shaking and cooling the contents of the flask meanwhile by immersion in cold water. When all the zinc has been added, shake the flask at intervals of 15 minutes for about 1½ hours, and then allow it to stand overnight in the cold, the mouth being closed with a loosely fitting, pear-shaped, hollow glass stopper.

When reduction is complete, support the flask in an inclined position on a piece of asbestos millboard, having a circular hole 1" in diameter, and heat it with a low, naked flame for 1½ - 2 hours. Turn up the flame and boil the contents of the flask for 1½-2 hours rotating the vessel at intervals. Remove the flask to an asbestos mat, add 1 gm. of copper sulphate, return the flask to the flame, and boil the contents for 1½ hours or until the acid becomes colourless. Remove the flask to an asbestos mat, add 7.5 gm. of dry potassium sulphate and 10 mls. of concentrated sulphuric acid; boil the mixture for a further 1½ - 2 hours. If mixture is colourless, the digestion is complete, but if there are any brown specks, add 1 gm. of dry, coarsely powdered potassium sulphate and continue the boiling for about 1 hour. Cool the flask and dilute the contents with about 200 mls. water.

Transfer to ammonia distillation apparatus. Add caustic soda (30% w/v) to make solution strongly alkaline (120 mls. required). Distil over into boric acid solution (20 mls. of 2% boric acid in receiver). Titrate with N/10 HCl, using methyl red bromo cresol green (200 mls. sufficient) as indicator.

Indicator

5.0 mls. of 0.1% solution in alcohol of bromo cresol green and 2.0 mls. of 0.1% solution in alcohol of methyl red and dilute the mixture to 30 mls. of 95% alcohol.

Appendix 6.

Thymol Spray.

Thymol	2 gms.
Methyl alcohol	100 mls.

Appendix 7.

Nutritive medium for spore germination.

Agar	0.5 gm.
Sucrose	1 gm.
Powdered gelatin	0.5 gm.
Water	25 mls.

Boil agar with sucrose in 25 mls. water. Stir in the powdered gelatin. Smear on warm slide and dust on spores. Germinate in moist chamber at 28°C.

Appendix 8.

Cytological staining

Fixative - Acetic acid/alcohol

Glacial acetic acid	1 part
Absolute alcohol	3 parts

Stain - 1 gm. orcein in 50% acetic acid.

Method of staining spores. Spores were fixed in acetic acid/alcohol for 25 minutes.

Spores then hydrolyzed in 1N HCl for 8 minutes at 60°C.

Then spores were stained with acetic orcein stain. This was sometimes heated gently over the bunsen.

Appendix 9.

Determination of pentachlorophenyl laurate

2 gms. of jute material, cut up into small pieces, were weighed and put in a 700 ml. round bottomed flask, together with 40 mls. 4N NaOH and some anti-bumping granules. This was boiled vigorously under reflux for 2 hours. By this means the pentachlorophenyl laurate was saponified to liberate pentachlorophenol and lauric acid as their sodium salts. The solution was then acidified with 60 mls. concentrated hydrochloric acid to liberate the free phenol, which was separated from other organic debris by steam distillation and concentrated by extracting the 250 mls. of distillate with chloroform.

After thorough shaking, the distillate was poured into a 500 ml. separating funnel and the bottom chloroform layer was transferred to a 50 ml. separating funnel. 20 mls. chloroform had been used to wash out the condenser and the measuring cylinder. Another 20 mls. were also shaken up.

3 mls. copper sulphate and 2 mls. pyridine were made up to 25 mls. and added to the chloroform in the 50 ml. separating funnel and shaken well and allowed to separate.

The coloured chloroform layer was then filtered into a standard flask and the colour intensity was measured on the Spekker and the percentage of L.P.C.P. calculated.

Appendix 10.

Throughout all the tests with spore suspension and replica plates involving agar plus different concentrations of L.P.C.P., controls were set up to ensure that the environmental conditions were not altered in any way to give misleading results. In every experiment normal parent strain was inoculated on normal agar.

The following controls were omitted in the general text.

Spore suspension from 0.03% L.P.C.P. colony inoculated on 0.03% L.P.C.P. agar. p. 24

Control

Normal parent strain on 0.03% L.P.C.P. agar.

Spore suspension from 0.03% L.P.C.P. colony on to 0.05% L.P.C.P. agar. p. 25

Spore suspension from 0.03% L.P.C.P. colony on to 0.06% L.P.C.P. agar. p. 28

Control

Spore suspension from 0.03% L.P.C.P. colony on to 0.03% L.P.C.P. agar. Also normal parent strain on 0.03% L.P.C.P. agar.

Spore suspension from 0.03% L.P.C.P. colony inoculated on to normal agar. p. 29

Control

Apart from normal parent strain on normal agar there was normal parent strain on 0.03% L.P.C.P. agar.

Spores from 0.05% L.P.C.P. colony replica plated on 0.05% L.P.C.P. agar. pp. 32 & 33

Control

Normal parent strain on 0.05% L.P.C.P. agar.

Spore suspension from 0.05% L.P.C.P. colony inoculated on to 0.06% L.P.C.P. agar. p. 46

Control

Normal parent strain on 0.05% L.P.C.P. agar.

Spore suspension from 0.05% L.P.C.P. colony inoculated on to normal agar. p. 49

Control

Apart from normal parent strain on normal agar there was normal parent strain on 0.05% L.P.C.P.

Spores from 0.05% L.P.C.P. colony replica plated on 0.07% L.P.C.P. agar. p. 55

Control

Spores from 0.05% L.P.C.P. colony replica plated on 0.05% L.P.C.P. agar. Also normal parent strain on 0.05% L.P.C.P. agar.

On pages 53, 56, and 57 it is mentioned that Trichoderma viride formed a ring growth and it was suggested that this might be a reaction to the presence of the L.P.C.P.

In the Review of Applied Mycology Vol. 37, 1958, p. 453, Y. Gutter discusses the effect of light on sporulation of T. viride. An exposure of T. viride colony to 20-60 seconds of light is sufficient to induce sporulation. Alternating conditions of light and dark

induced the formation of concentric rings of sporulating and non-sporulating mycelium.

Since the cultures described on p. 57 were subjected to these conditions because of daily examination, the suggestion made on p. 57 is therefore, not valid.

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Plate 1.

Fig. 1 and Fig 2 show normal heads of *Aspergillus terreus* x 600.

PLATE

Fig. 1.

Fig. 2.

Plate 2.

Figs. 1, 2 and 3 all show abnormal growth of Aspergillus terreus in presence of L.P.C.P. x 1,200.

Fig. 1 shows a vesicle which has not produced sterigmata but has gone on to develop a conidial head later.

Fig. 2 shows a bulge on the conidiophore which is another example of a malformed vesicle.

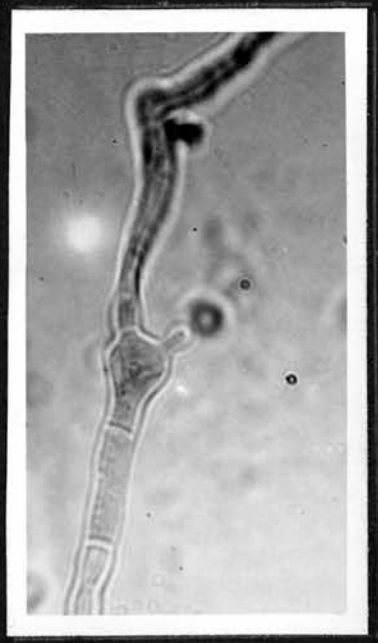


Fig. 1.

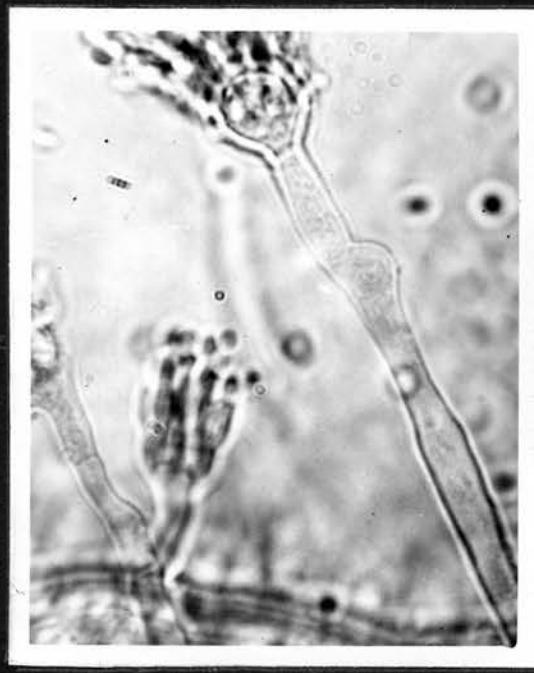


Fig 2.

Fig. 3.

Plate 3.

Fig. 1 shows swollen mycelium of Aspergillus terreus in presence of L.P.C.P. x 1,200.

Fig. 2 shows swollen and contorted mycelium of Aspergillus terreus x 720.

PLATE

Fig. 1.

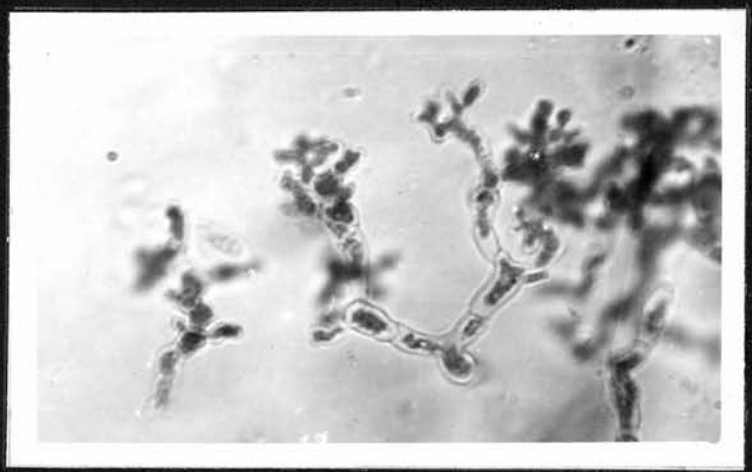


Fig. 2.

Plate 4.

Fig. 1 and Fig 2 show head of Aspergillus terreus in presence of 0.03% L.P.C.P. which is a transition between the Aspergillus and Penicillium type of head. x 400.

Fig. 1.

Fig 2.

Plate 5.

Camera lucida drawings of abnormal heads of *Aspergillus terreus* produced in presence of 0.05% L.P.C.P.

x 600

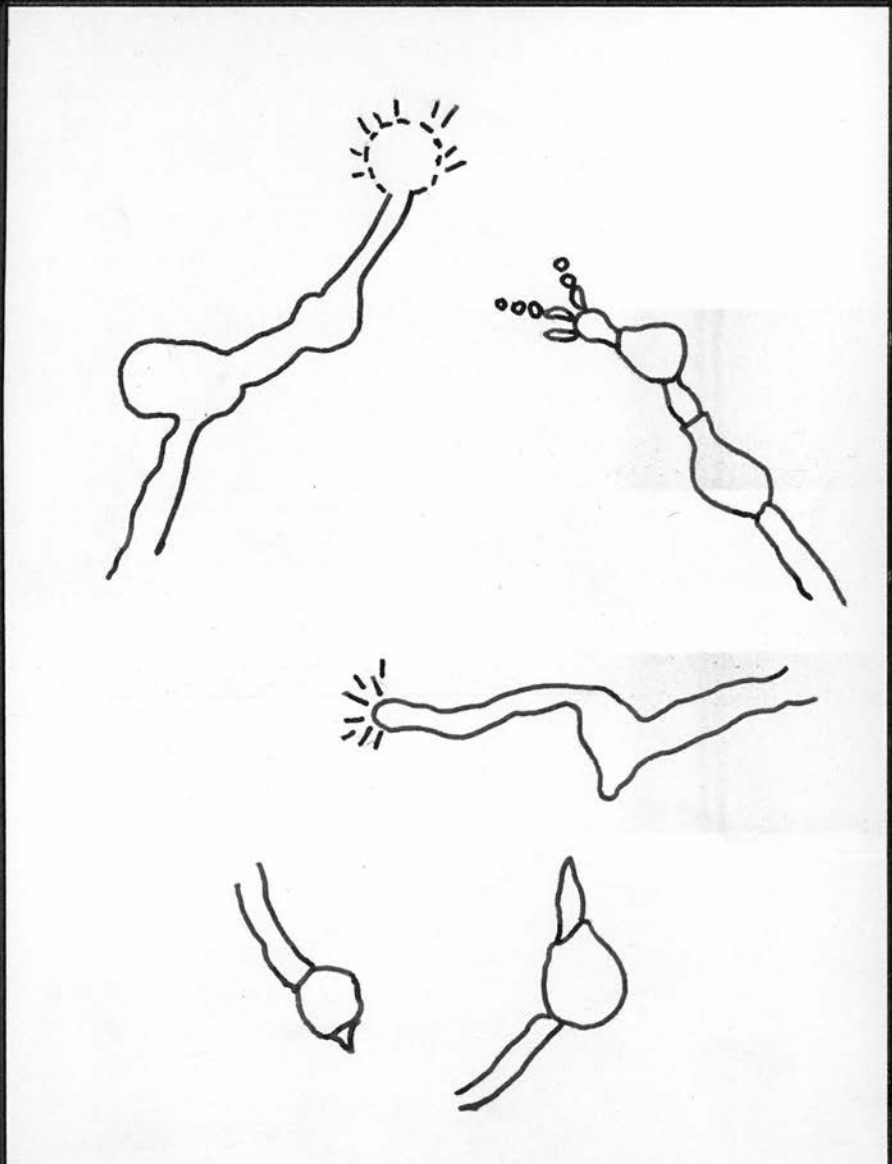


PLATE 3

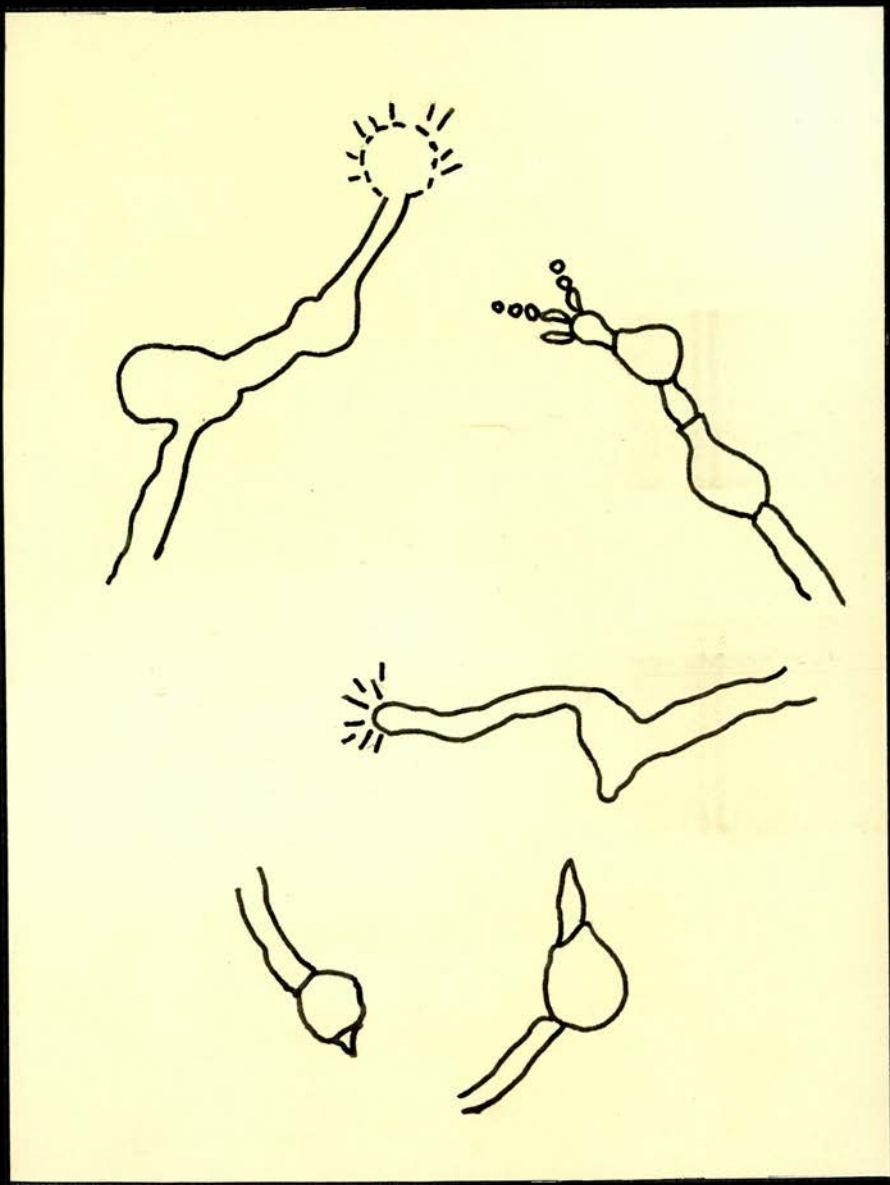


Plate 6.

Colony of Aspergillus terreus on 0.05% L.P.C.P. x 5.

No. 1. Brown spore heads cover the whole top.

No. 2. Furrows with yellow mycelium which only has a few spore heads present.

No. 3. Spore heads present at different stages of development.

No. 4. Young mycelial growth.

PLATE

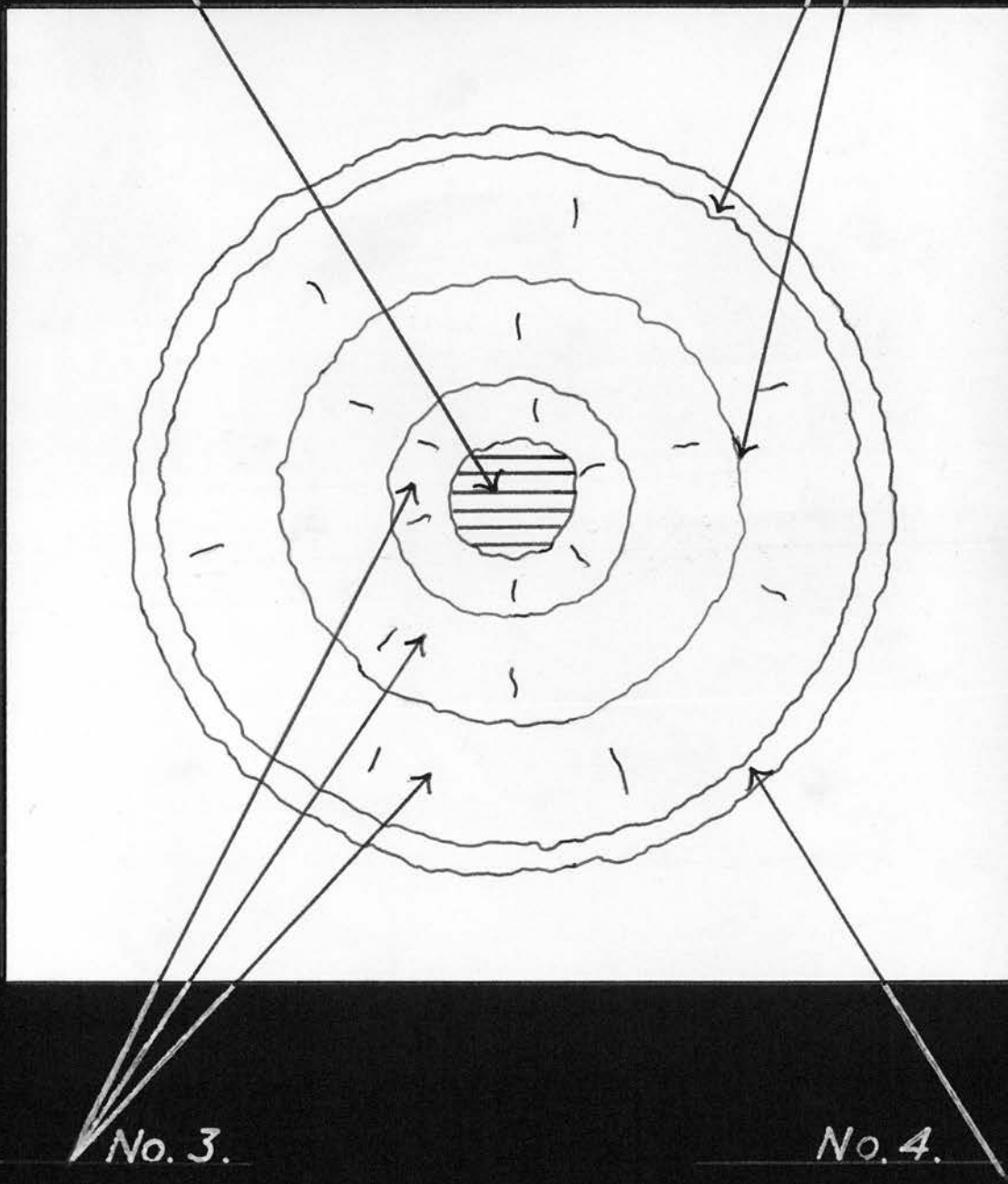


Plate 7.

Fig. 1. Two heads produced from one vesicle in presence of 0.05% L.P.C.P. Pitted region where sterigmata should have developed. x 730.

Fig. 2. Abnormal head with five conidiophores developed. x 730.

PLATE

Fig. 1.

Fig. 2.

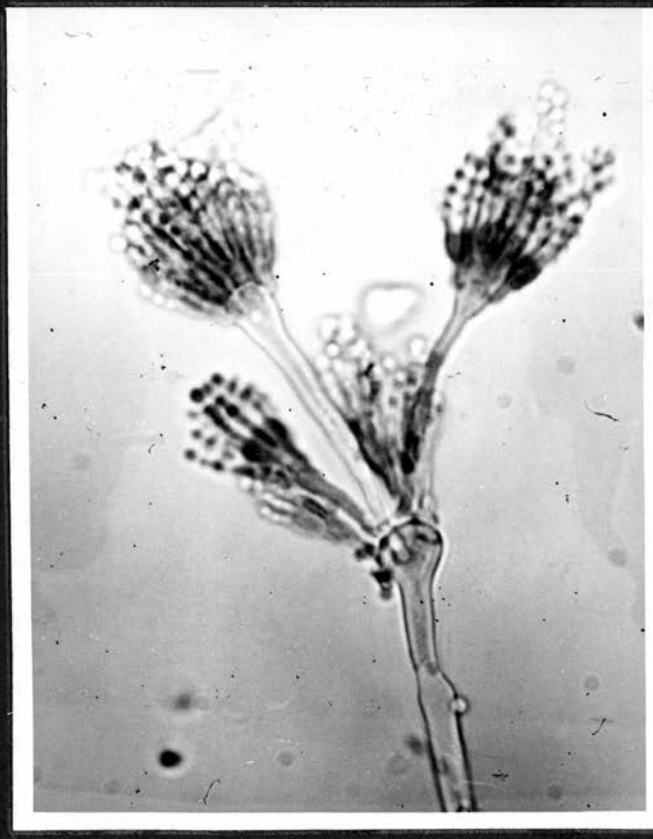


Plate 8.

Abnormal heads of Aspergillus terreus growing on 0.05% L.P.C.P. Camera lucida drawings x 600.

PLATE 8

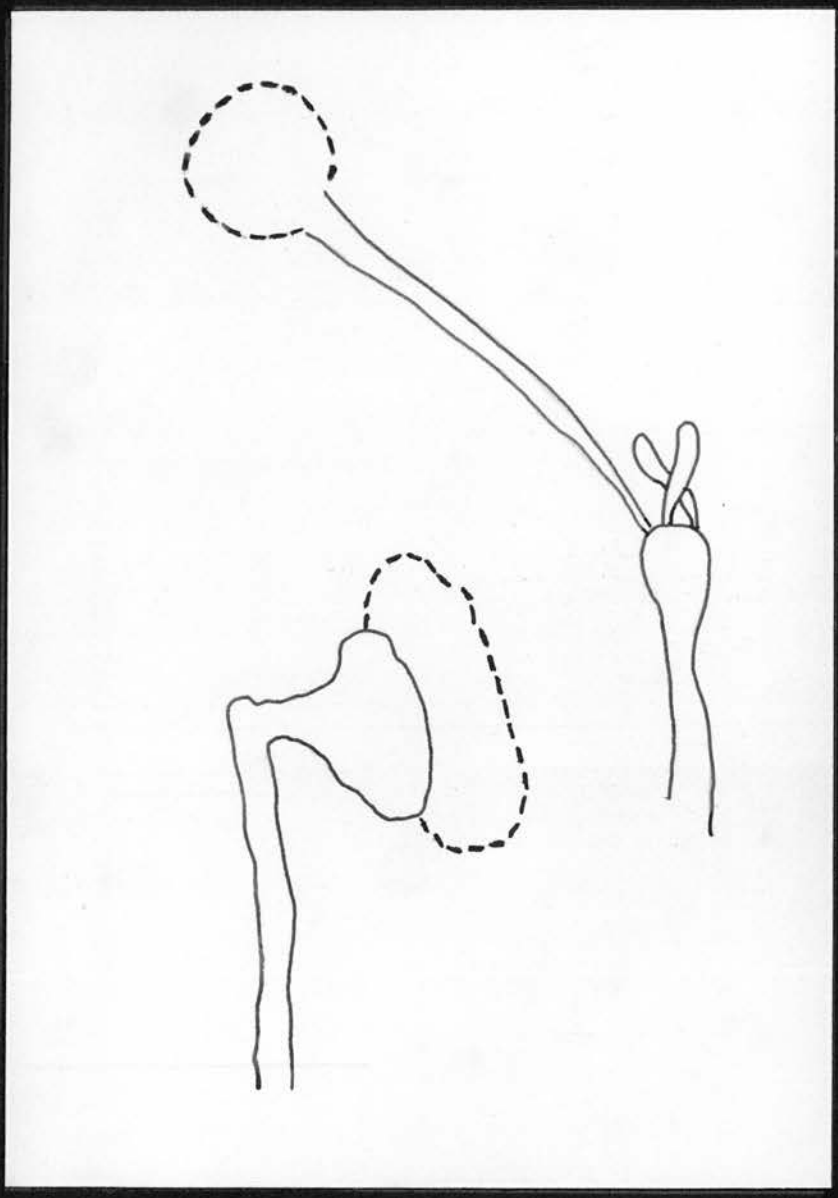


Plate 9.

Restricted hyphae of Aspergillus terreus growing on 0.05% L.P.C.P. Camera lucida drawing x 600.

PLATE

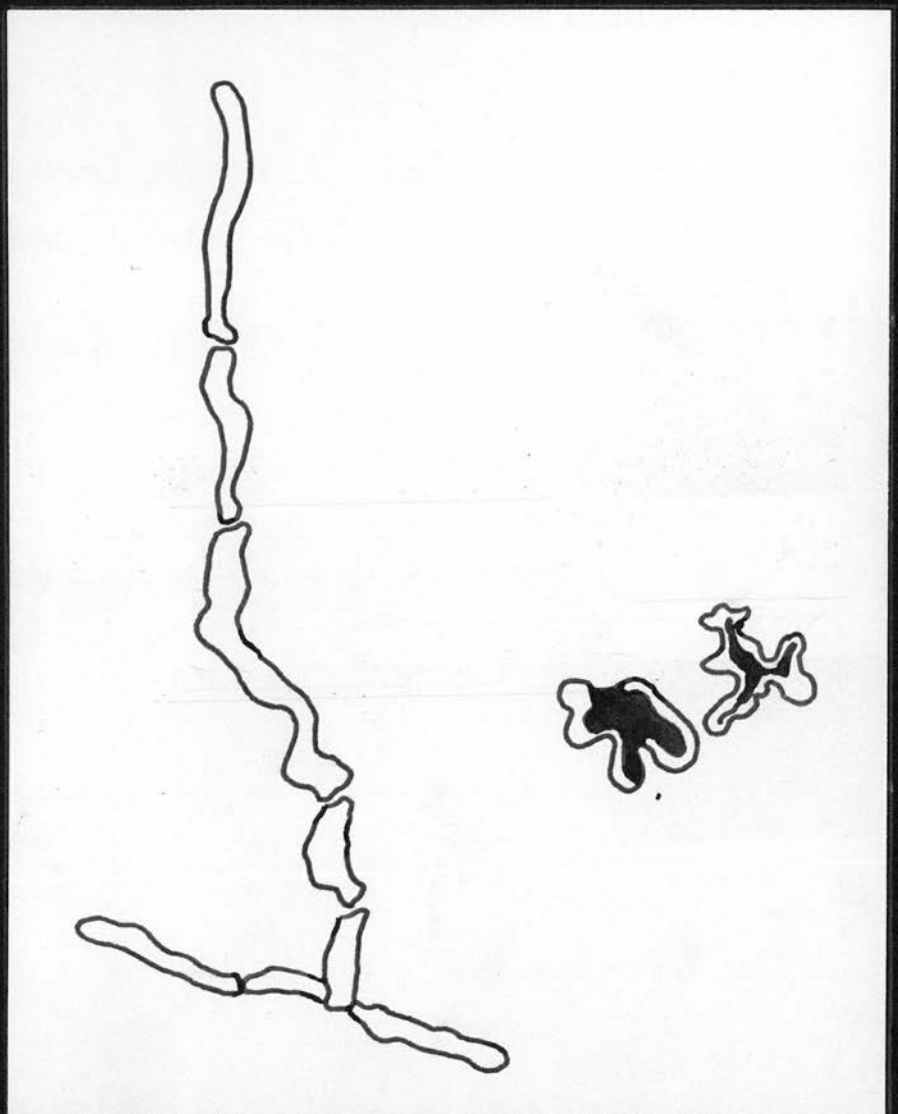


Plate 10.

Conidiophore of Aspergillus terreus developing another conidiophore in presence of 0.05% L.P.C.P. x 600.

PLATE 10.

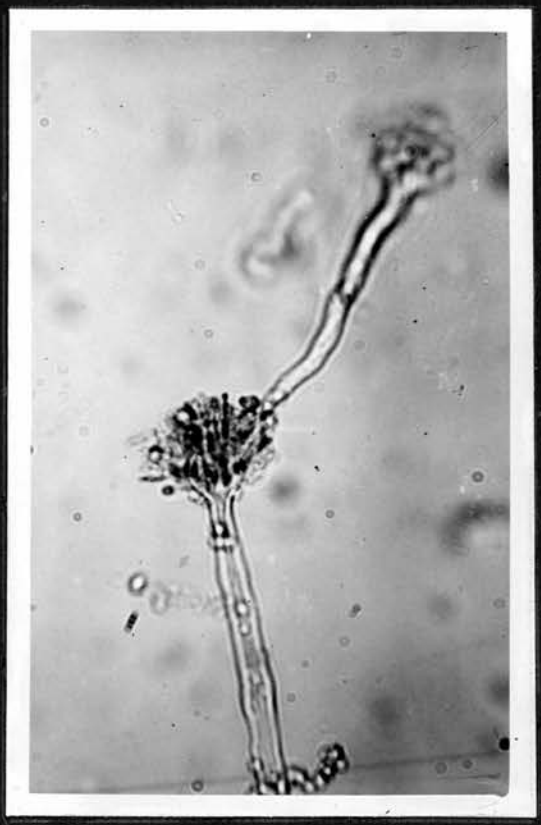


Fig. 1.

Fig. 2.

Plate 11.

Aspergillus terreus head developing on 0.06% L.P.C.P. with a Penicillium type of head branching from it.

x 600.

Fig. 1. Aspergillus head

Fig. 2. Branching of the heads

Fig. 3. Penicillium like head.

PLATE II.

Fig. 1.

Fig. 2.

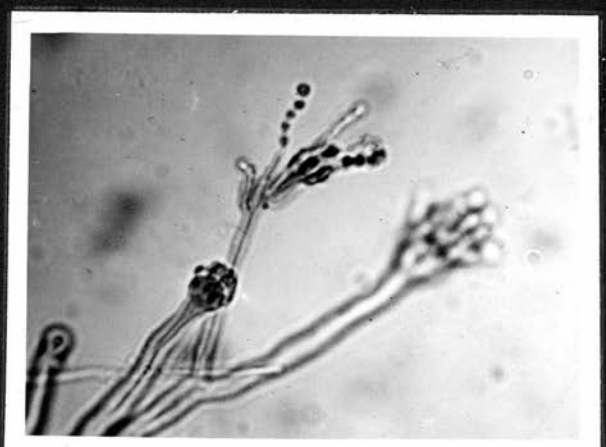


Fig.

Plate 12.

Figs. 1 and 2 show curling factor in *Aspergillus terreus* in the presence of 0.07% L.P.C.P. x 580.

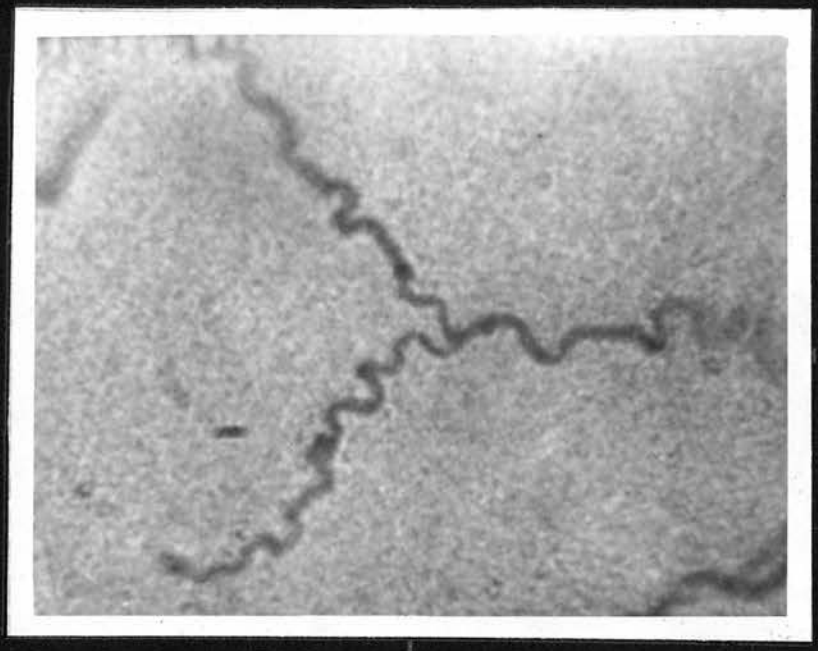


Fig. 1.

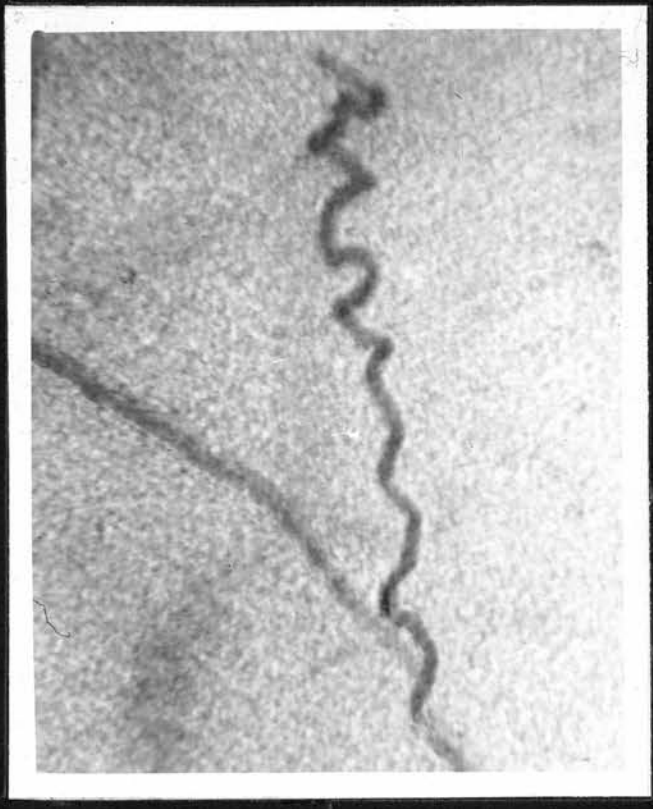


Fig. 2.

Plate 13.

Petri dishes of Trichoderma viride growing on 0.05% L.P.C.P. This shows the concentric rings of growth. x ¾ original size.

PLATE 13.

Plate 14.

Restricted hyphae of Trichoderma viride in 0.07%

L.P.C.P. x 580.

PLATE 14.

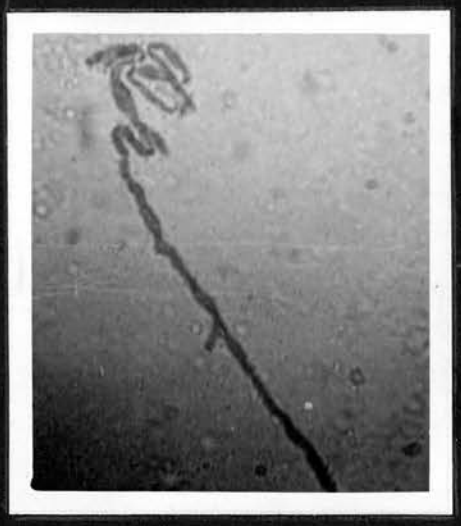


Fig. 1.

Fig. 2.

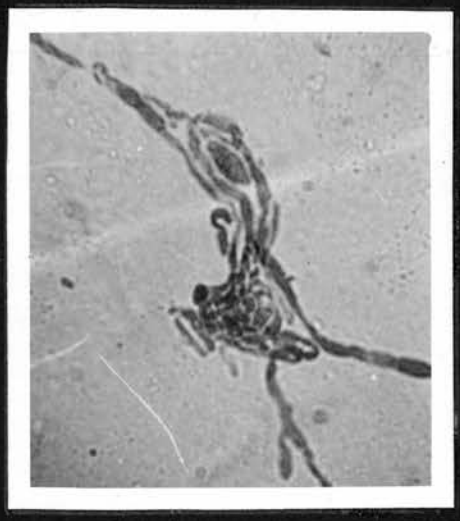


Plate 15.

Fig. 1. Four nuclei visible in hypha of Aspergillus terreus x 1,000.

Fig. 2. Enlarged spore of Aspergillus terreus with 4? chromosomes. x 1,000.

Fig. 3. Aspergillus terreus spores showing one nucleus in each case. x 950.

Fig. 4. Three nuclei in Aspergillus terreus spore instead of the usual one nucleus. x 1,000.

PLATE 13.

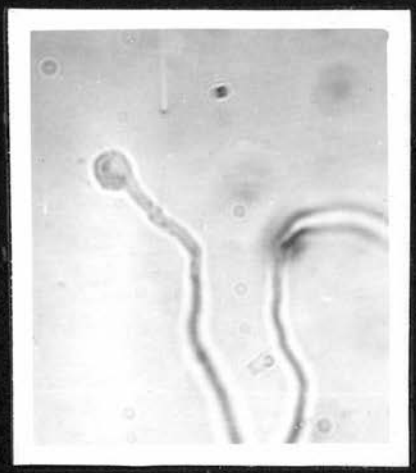


Fig. 1.

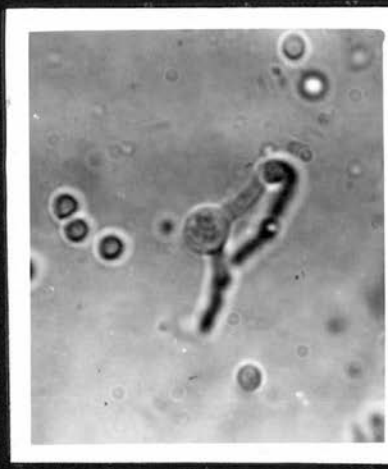


Fig. 2.

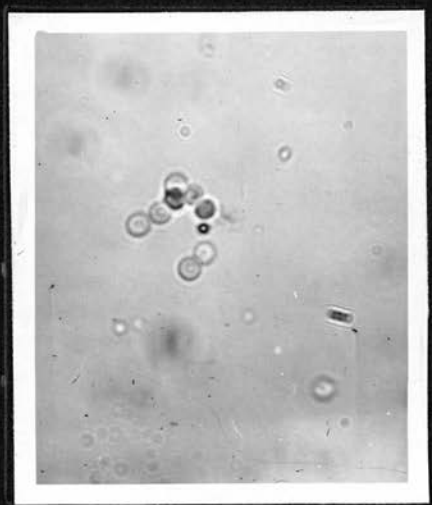


Fig. 3

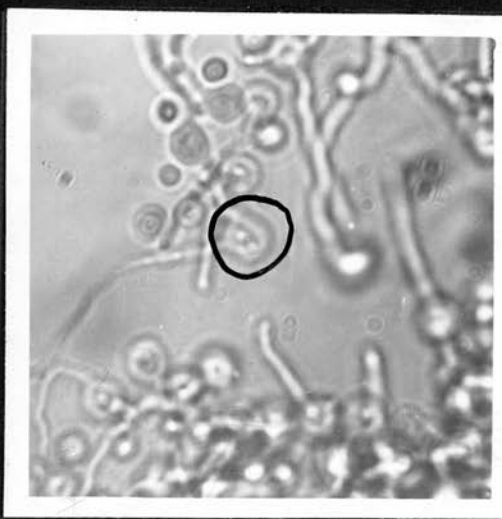


Fig. 4

Plate 16.

Figs. 1 and 2. Aspergillus terreus spores which have germinated in the presence of 0.05% L.P.C.P.

x 1,100.

PLATE I

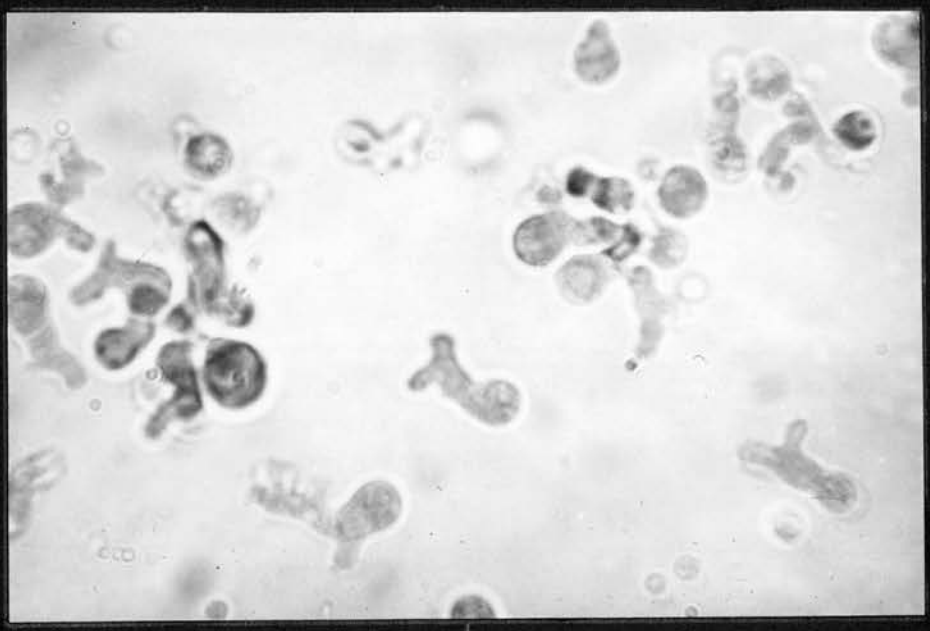


Fig. 1.

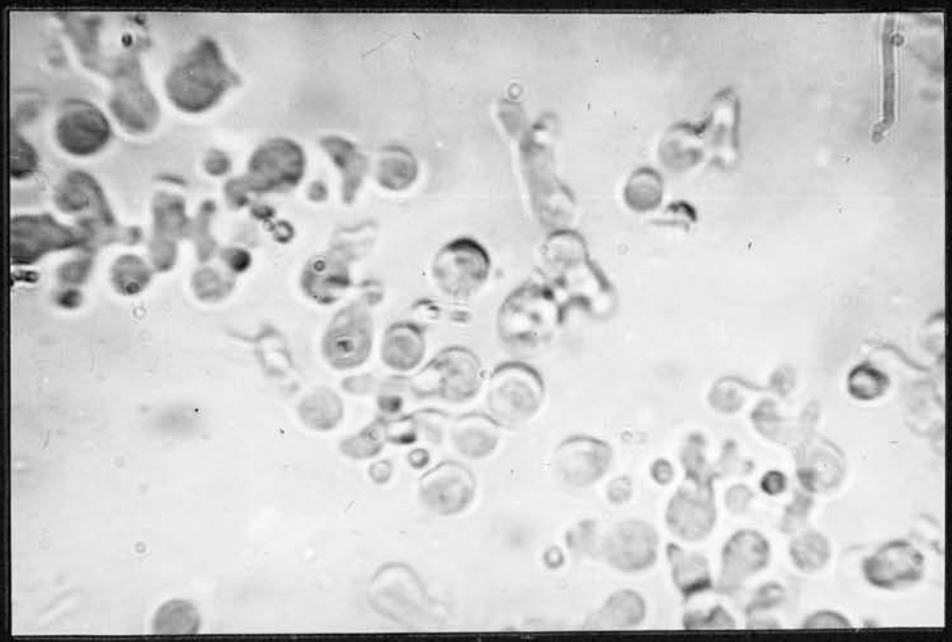


Fig. 2.

Plate 17. Camera lucida drawings of germinated spores of *Aspergillus terreus* in presence of 0.05% L.P.C.P.

x 1,500.

PLATE 1

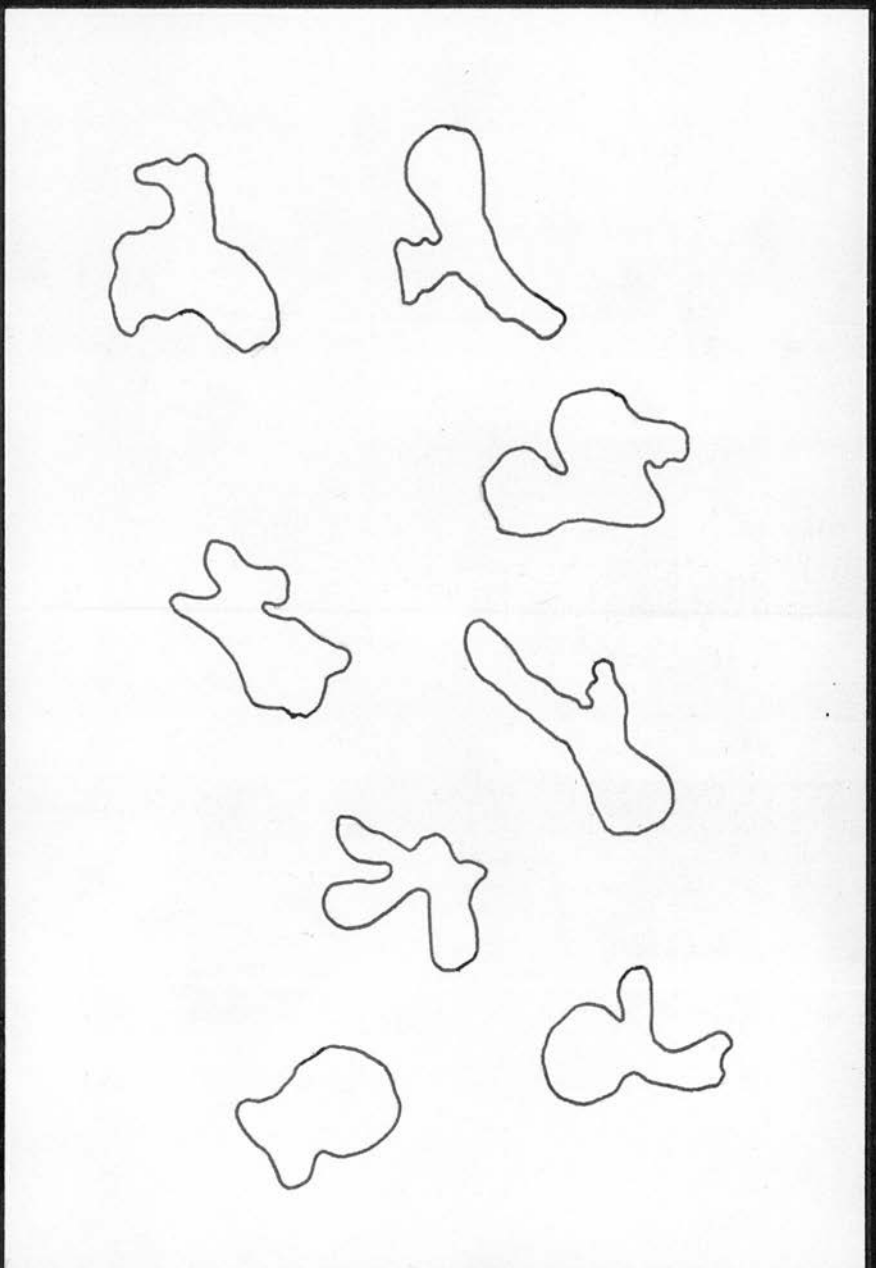


Plate 18.

Stunted hyphae of Aspergillus terreus x 640.

PLATE

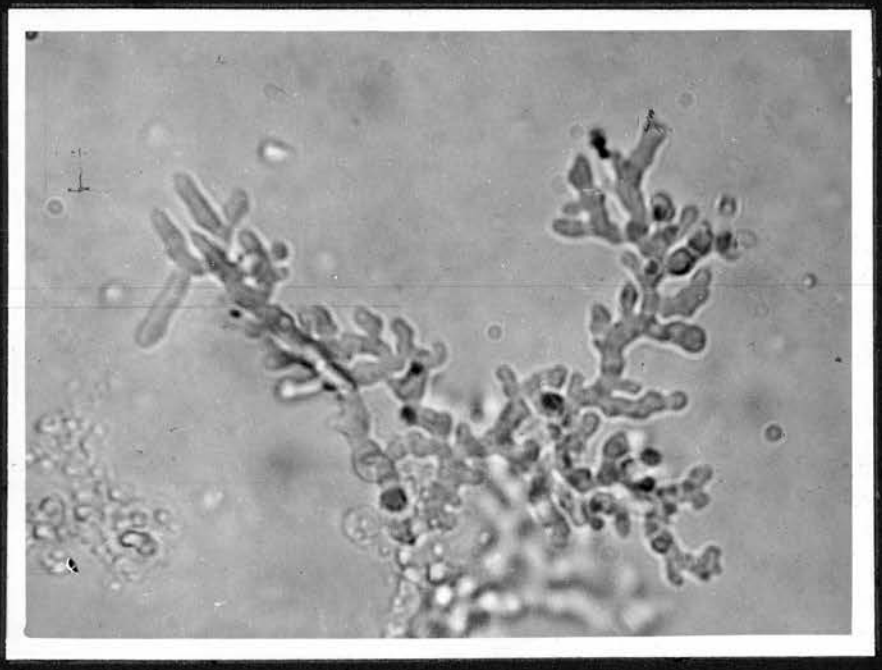


Plate 19.

Figs. 1 and 2 show distorted hyphae of *Aspergillus terreus*. Camera lucida drawing x 800.

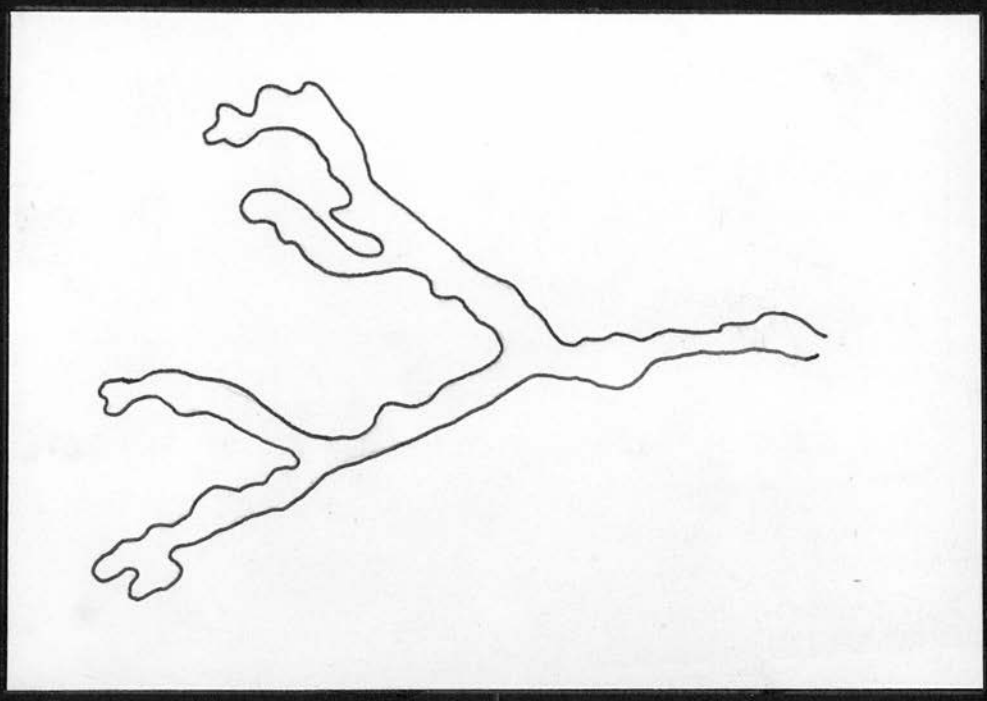


Fig. 1.

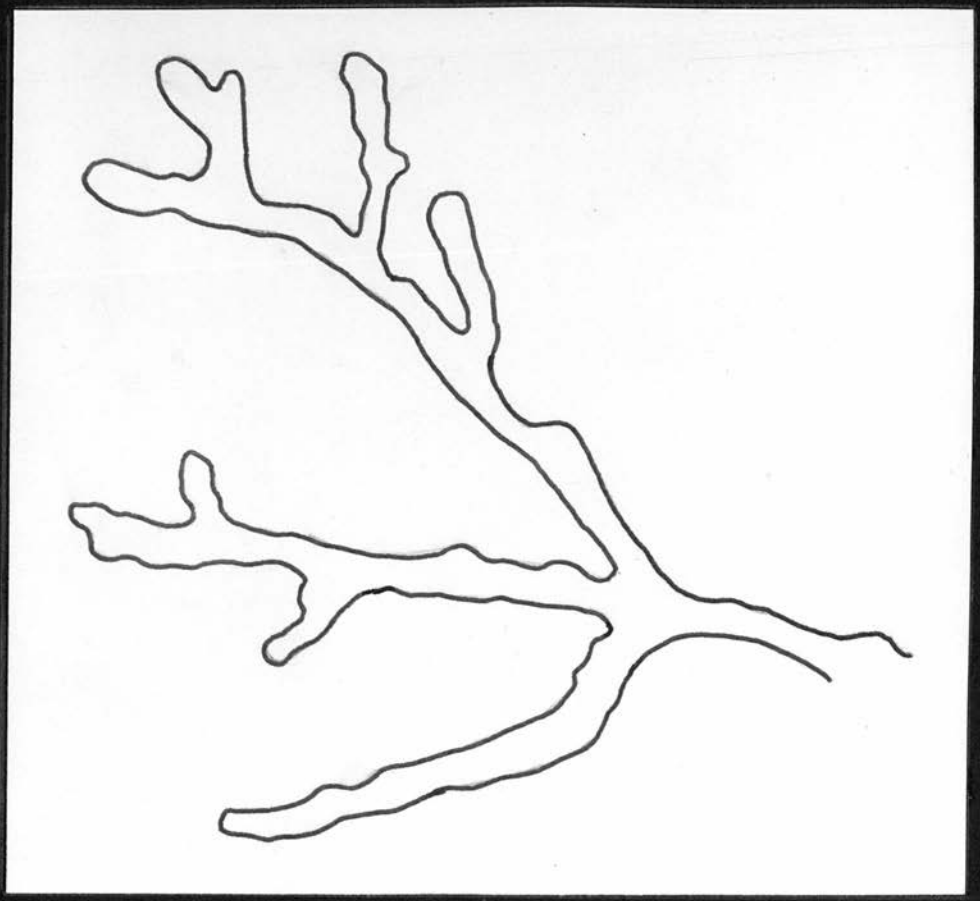


Fig. 2

Plate 20.

Spore heads of Aspergillus terreus growing on jute fibres treated with 0.05% L.P.C.P. x 500.

PLATE 2

Plate 21. Growth of Aspergillus terreus which has been streak inoculated on agar and 0.7% L.P.C.P. treated cloth laid on top.

No. 1. Jute cloth treated with 0.7% L.P.C.P.

No. 2. Area where small white spore heads were present.

No. 3. Yellow mycelium which was sterile. Actual size.

PLATE

No. 1.

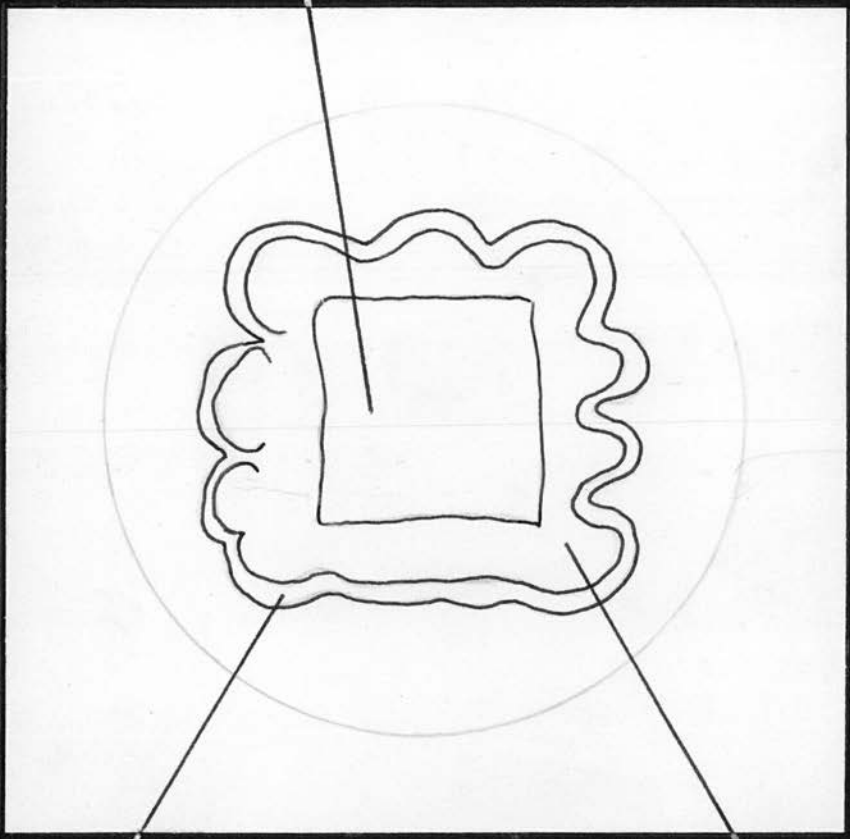


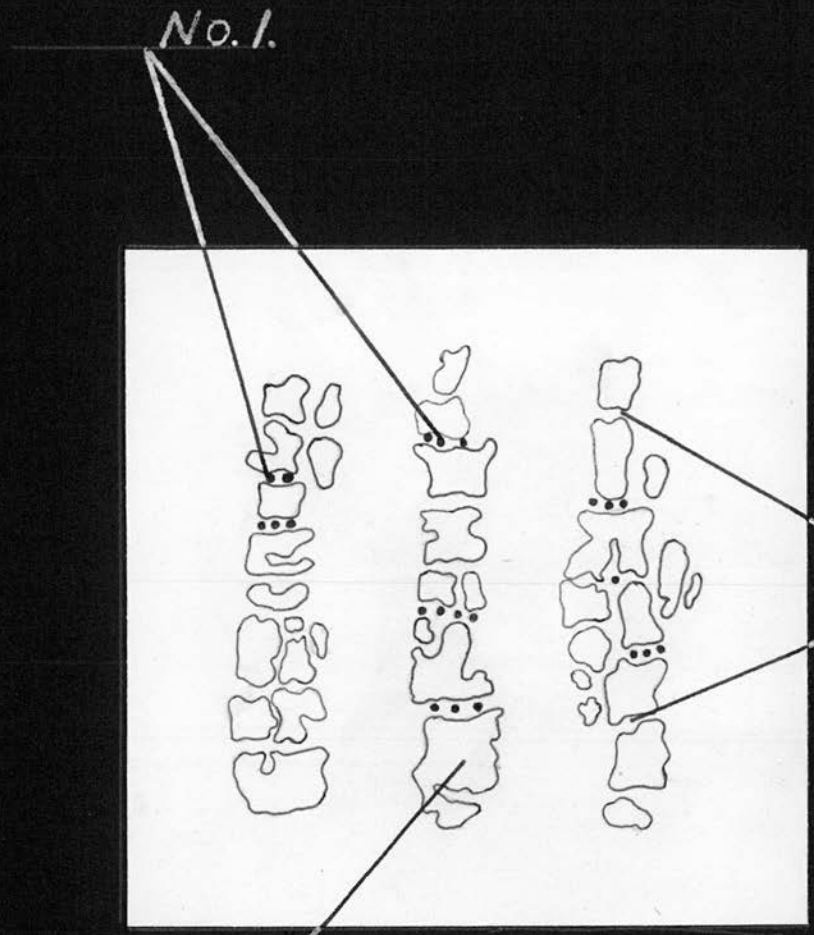
Plate 22.

Drawing of culture underneath cloth treated with 0.7% L.P.C.P.

No. 1. Liquid globules.

No. 2. Indentations in agar.

No. 3. Sterile yellow mycelium.


No. 1.