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STUDIES IN THE NATURE AND EFFECTS OF VOLATILE FUNGAL METABOLITES

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy
in the Faculty of Science.

by

CHRISTINE MARY MARTIN

July 1963

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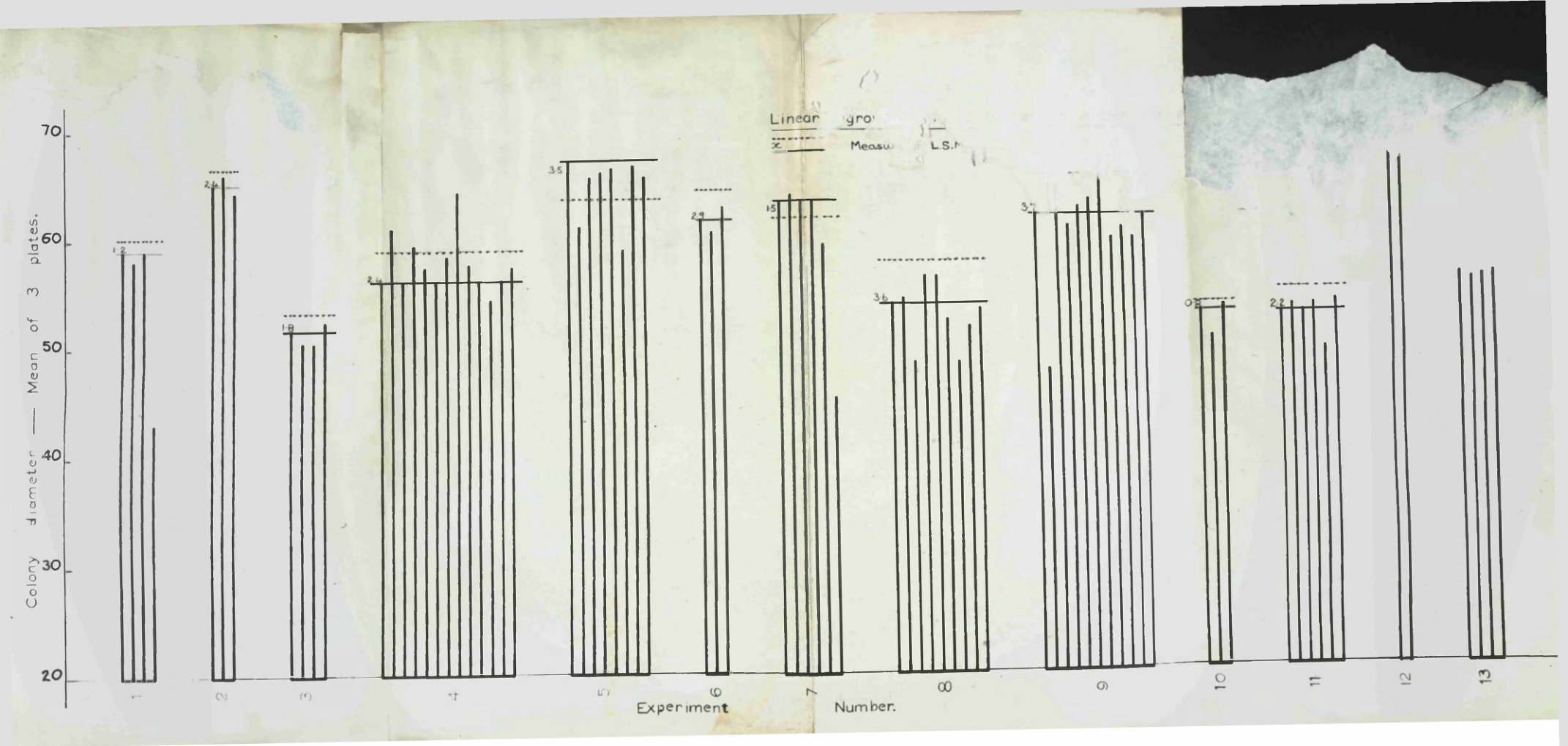
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Colony diameter — Mean of 3 plates.
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2.6
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1.5
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Experiment Number.
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INTRODUCTION.

Previous work carried out in this Department has been concerned with the action of volatile fungal metabolites on the growth of the green plant, and on germination of fungal spores. Several other workers in the past have examined instances of the production of volatiles other than CO_2; but none has made more than an isolated observation on the effect of one metabolite on the growth of another fungus, and/or an examination of its chemical nature.

This thesis reports an extension of such work on the following lines:-

1. A general survey of fungi to determine the prevalence of production of volatile metabolites having effects on the growth and/or morphogenesis of other fungi.

The fungi reported as having biologically active emanations appear to be scattered at random throughout all groups. As a first step in the investigation it was decided to plan a general examination of the occurrence of these interactions. This was designed to discover whether or not there was any pattern of activity which might arise within a certain group and whether any effect observed was specific against a certain assay organism.

2. An investigation of the nature of volatile fungal metabolites.

This problem was tackled on two lines:

a. Analysis of the total gases given off by certain species.

This was thought to be the most satisfactory procedure, as any test of synthetic substances must always leave an uncertainty about undetected action of unknowns.

b. Examination of the effect of synthetic chemicals which, judging by previous reports, were likely to be concerned in some reaction.

This would, of course, be a necessary corollary to (a), but it was originally hoped that it would be simply a confirmatory test.

Section 2 of the thesis reports the work which was done on the development of techniques for condensing the volatile fractions from 'active' fungi, and on the attempt to analyse these by Mass spectrometry, Gas Liquid Chromatography and by Chromatography in a Flame Ionising Detector. Successful methods of concentration were eventually designed, but the problem of analysis has not yet been overcome.

Section 3 reports the work on the reactions of fungi to volatile organic substances. It gives a measure of the type of reaction which might be seen with pure samples of compounds possibly present, but undetected, in the metabolic products.

PART 1.

A SURVEY OF FUNGI TO DETERMINE THE PREVALENCE OF PRODUCTION OF VOLATILE METABOLITES HAVING EFFECTS ON THE GROWTH AND/OR MORPHOGENESIS OF CERTAIN FUNGI.

REVIEW OF LITERATURE:

An examination of the relevant literature has revealed relatively few reports of the biological effects of volatile fungal metabolites.

Bitancourt and Rosetti (1951) stated that a gas produced by Mucor spinosus enhances the growth of Phytophthora citrophthora, whilst Allen (1955,1957) and French, Massay and Weintraus (1958) examined a self inhibitor of the germination of rust uredospores. Bilai (1956) and Dymovych (1960) demonstrated that many strains of Trichoderma are inhibitory to the growth of a large number of fungi and bacteria. McTeague, Hutchinson and Reed (1959) identified a substance produced by Agaricus campestris which stimulated the germination of Agaricus spores. Stanek (1959) also reported this stimulatory effect, whilst Lockard and Kneebone (1962) analysed volatile metabolites of A. campestris but did not agree with McTeague et al. as to the identity of these compounds.

Page (1959) showed that a gas produced by Mucor plumbeus stimulates asexual reproduction in Pilobolus spp., whilst Hepden and Folkes (1960), and Hepden and Hawker (1961) discussed the stimulation of sexual reproduction of Rhizopus sexualis by a volatile substance produced per se. They also reported that several related species of fungi produced similar, if less marked, effects in R. sexualis.

There are several papers dealing with the effects of volatiles from higher plants on fungi, e.g. those of Russian workers on the action

of phytonicides. These are not discussed here since they do not refer specifically to the action of volatile fungal metabolites. Reports on the analysis of fungal volatiles are discussed elsewhere in this work.

In the light of the above work, and also in response to an invitation from P.M. Hepden to co-operate in the examination of volatile metabolites of R. sexualis, it was decided to conduct a survey of fungi representative of the main taxonomic groupings in order to determine the prevalence of production of volatile metabolites having effects on the growth and/or morphogenesis of fungi.

EXPERIMENTAL:

1. Assay species.

For the purposes of this survey five species were selected as assay organisms on which an examination of the effects of various fungi could be based. These were selected to include representatives of the main sub-classes, and species with varying growth and sporing habits.

(a). Rhizopus sexualis (Smith) Callen.

(b). Chaetomium globosum Kunze ex Fr.

(c). Stereum hirsutum (Willde) Fr.

(d). Aspergillus niger van Tiegh.

(e). Botrytis cinerea Pers. ex Fr.

The fungi included in the survey were tested against each of the assay species and scored for effects on selected facets of growth and morphogenesis.

2. Method of testing for effects of volatile metabolites.

(a). Growth medium.

All tests in the general survey were carried out using a standard medium of the following composition:-

'Oxoid' malt extract	20gm.
'Oxoid' agar	20gm.
Distilled water	1000 ml.

Sterilized at 15 lbs. pressure for 20 mins.

25ml aliquots of this were pipetted into 9 cm. diameter Petri dishes.

This medium was chosen as one on which a large number of fungi would grow satisfactorily though not necessarily under optimum conditions.

Phan Chan Ton (1960) reported differential production of ethylene by Penicillium digitatum when grown on media enriched with alanine, glycerol and ethanol. Thus, any effects noted here using malt medium may not be evident with other sources of nutrient, and conversely. However, for the purpose of comparison in this survey, it was thought advisable to keep as many conditions constant as possible.

b. Assay Chambers.

The fungus to be tested for volatile production was inoculated centrally on a Petri dish and incubated at room temperature until a mature colony was formed - i.e. when it covered the dish and/or was sporulating freely. (In the case of the slower growing organisms, e.g. the Aspergilli, three or five inocula were made to give a greater mass of test fungus; the number of inocula being kept constant in all tests.) Hence, due to differential growth rates, cultures of fungi being tested were of different ages; but cultures of similar age were used throughout the series of assays with any one species. This was thought necessary since Bilai (1956) found that strains of Trichoderma showed variation in volatile antibiotic activity against a number of fungi and bacteria according to the age of the Trichoderma culture used.

When a 'mature' colony of the test fungus was obtained the lid of the Petri dish was removed and replaced with a sterile cellophane disc which had previously been washed in acetone to remove any varnish.

Another base inoculated with one of the assay species was then inverted over the membrane, and the two dishes held together by rubber bands.

The cultures were then incubated at 20°C in the dark.

By means of this simple apparatus in which there was no physical contact between the cultures, any volatile substance produced by the fungus on the lower plate passed into the upper part of the chamber where it might have effect on the assay fungus. Observations on the growth and form of this were made and compared with another in which the lower part of the chamber contained only sterile agar; all tests were carried out in triplicate.

c. Test species.

Fungi tested were selected to represent all the main sub-classes, with some bias towards those which grow well on the standard malt medium, but not so prolifically as to penetrate the cellophane membrane and contaminate the test plate.

A full list of species examined is appended at the end of the thesis in Appendix A.

d. Growth responses of assay fungi.

The main features of growth studied in each fungus are given below. Any other deviations from normal growth were monitored by empirical observation over the assay periods.

1. Rhizopus sexualis.

Inoculation: Spot inocula were made from a sporing culture on malt agar.

Main facet of growth studied: Zygospore formation, as defined below, was the main facet studied.

Assay period: Zygospore numbers were counted after 4 days growth in the double chamber, i.e. when the fungus had covered the plate and the spores were mature. The dishes were then separated and 5 counts of zygospores per unit area made over one traverse of the Petri dish using a moveable stage. (Unit area was defined as one field of a Watson Stereoscopic Microscope at a magnification of X100; i.e. an area of $\pi \times 10^6$ u. The counts were then totalled to give a representative number per dish.)

As a test for accuracy in assessment of zygospore numbers a traverse was made over each of ten dishes in random order, and this process repeated 3 times with traverses over different diameters of the dishes. A table of results is given as Table A1 of the appendix. Empirical observation of these shows no significant differences in the total numbers of spores recorded for each of the dishes on the 3 counts. Hence, the method of recording appears to be sufficiently accurate and consistent for the purpose of this investigation.

a. Preliminary study.

The culture of R. sexualis tested was one obtained from Hepden and Hawker, who had shown (1961) that it produced a volatile metabolite active against Rhizopus. As the fungus had been continually sub-cultured since this time, a preliminary study was made to determine if the culture

was still active and if this activity was shown on the proposed 2% malt medium, as opposed to the malt/peptone one used by these authors.

Ten test chambers and controls were set up using malt peptone, and ten with malt, agar media. These were then placed in random order and incubated in the dark at 20°C for four days. Zygospore formation was noted at the end of this period in the manner described above.

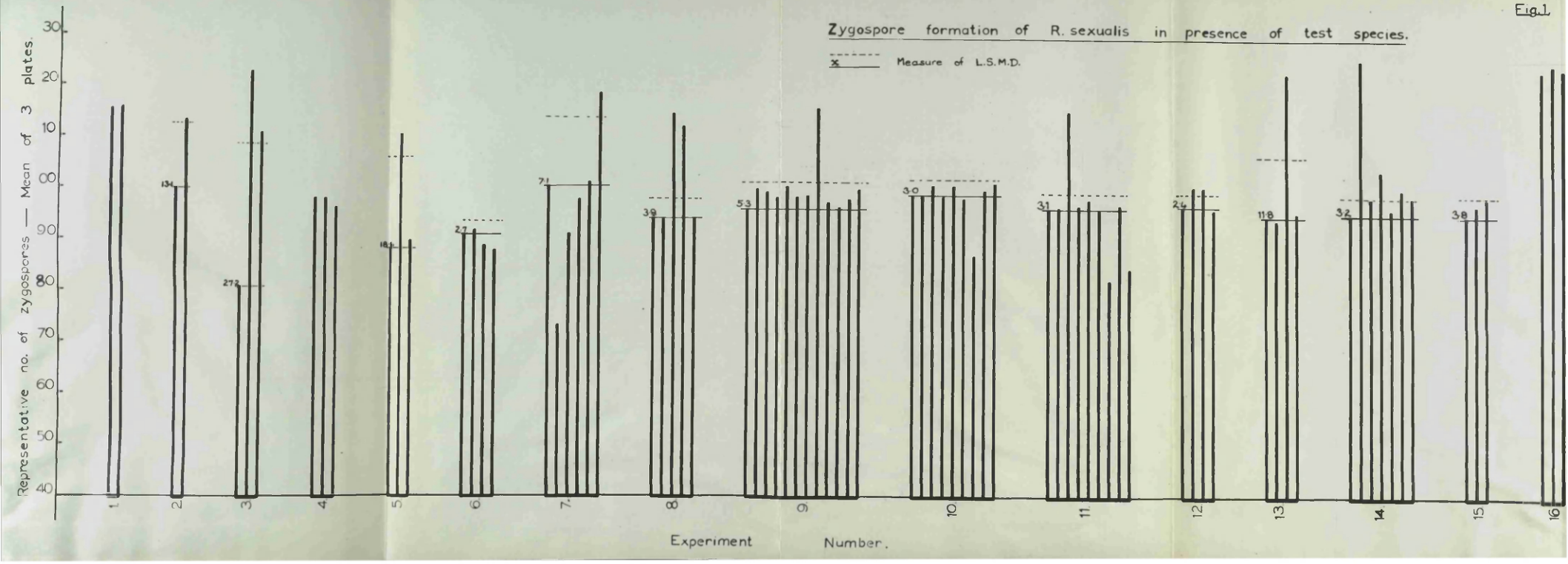
Results of this test are given as tables A2 and A3 of the appendix, and may be summarised as follows:-

	Malt peptone medium		Malt medium	
	Test	Control	Test	Control
Representative No. of zygospores per 10 plates.	2213	1748	1311	981

From these results it was concluded that the R. sexualis culture was still producing the effective metabolite, and that malt agar would be a satisfactory medium for the investigation.

Empirical observation of the amount of mycelium produced by R. sexualis on malt/peptone as opposed to malt medium suggested that the differences in numbers of zygospores seen on the two media might be a reflection of the more prolific general growth with the addition of peptone. (In fact, a reduction in the amount of aerial mycelium produced on the malt medium greatly facilitated the counting of zygospores,

Fig.1


Zygospore formation of R. sexualis in presence of test species.
x Measure of L.S.M.D.
Representative no. of zygospores — Mean of 3 plates.
30
20
10
90
80
70
60
50
40
134
27½
18½
27
71
39
53
30
31
24
11·8
32
38
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Experiment Number.

Control
Chaetomium globosum

Control
Agaricus campestris

Control
Rhizopus sexualis
Botrytis cinerea

Control
Polyporus sulphureus
Caldariomyces fumago

Control
Mucor plumbeus
Saccharomyces cerevisiae

Control
Pyronema domesticum
Trichothecium roseum
Penicillium digitatum

Control
Trichoderma viride
Penicillium claviforme
Aspergillus fumigatus
Sordaria fimicola
Ceratocystis coerulescens

Control
Aspergillus terreus
Thielaviopsis basicola
Gibberella fujikuroi
Cladosporium herbarum

Control
Mucor hiemalis +
M. hiemalis -
Pythium ultimum
Pleospora androgyna
Piptocephalis cylindrospora
Schizosaccharomyces octosporus
Helminthosporium sativum
Fusarium culmorum
Verticillium sp.
Alternaria tenuis
Pericystis apis

Control
Stereum hirsutum
Tilachlidium nigrum
Hyssochlamys fulva
Ophiobolus graminis
Carpenteles sp.
Armillaria mellea
Cephalosporium cretatum
Verticillium psalliotae

Control
Botrytis tulipae
B. spectabilis
B. fabae
Choanephora circinans
Curvularia lunata
Trichoderma koningi
Rhodotorula rubra
Aeremoniella atra

Control
Colletotrichum lindemuthianum
Cunninghamella echinulata
Rectria haematococca

Control
Hansfordia grisella
Cordyceps militaris
Rhizopus stolonifer -

Control
Fomes annosus
Botrytis allii
Absidia spinosa
Cordyceps ophioglossoides
Rhizopus stolonifer +
Pullularia pullulans

Control
Aspergillus niger
Merulius lacrymans

Control
Mortierella ramanniana
Zygorhyncus moelleri

which tend to be produced near to the surface of the substrate.)

b. General survey.

Each of the test species was examined for action of volatile metabolites, using the double chamber method. Growth patterns of the Rhizopus were observed over four days and counts of zygospores plus colony morphology noted at the end of this period.

A complete table of results is appended (Table A4), whilst a summary of these in graph form is given below. (Fig. 1)

2. Chaetomium globosum.

Inoculation: Spot inocula were made from mature, perithecial colonies on malt agar.

Main facets of growth studied:

Linear growth, as measured by the mean of 2 previously marked diameters, and perithecial production were noted.

Assay period:

Colony diameters were measured after 4, 6, 8, and 10 days growth in the double chamber and an estimate of perithecial production made after 10 days.

Individual perithecia were too densely massed to be counted, so that the numbers produced were estimated on the following scale:-

Fig. 2.

Linear growth of Chaetomium globosum in presence of test fungi.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Time, days.

Test species: (a). Choanephora circinans

(b). Verticillium psalliotae

(c). Sordaria fimicola

(d). Fusarium culmorum

Fig. 2.

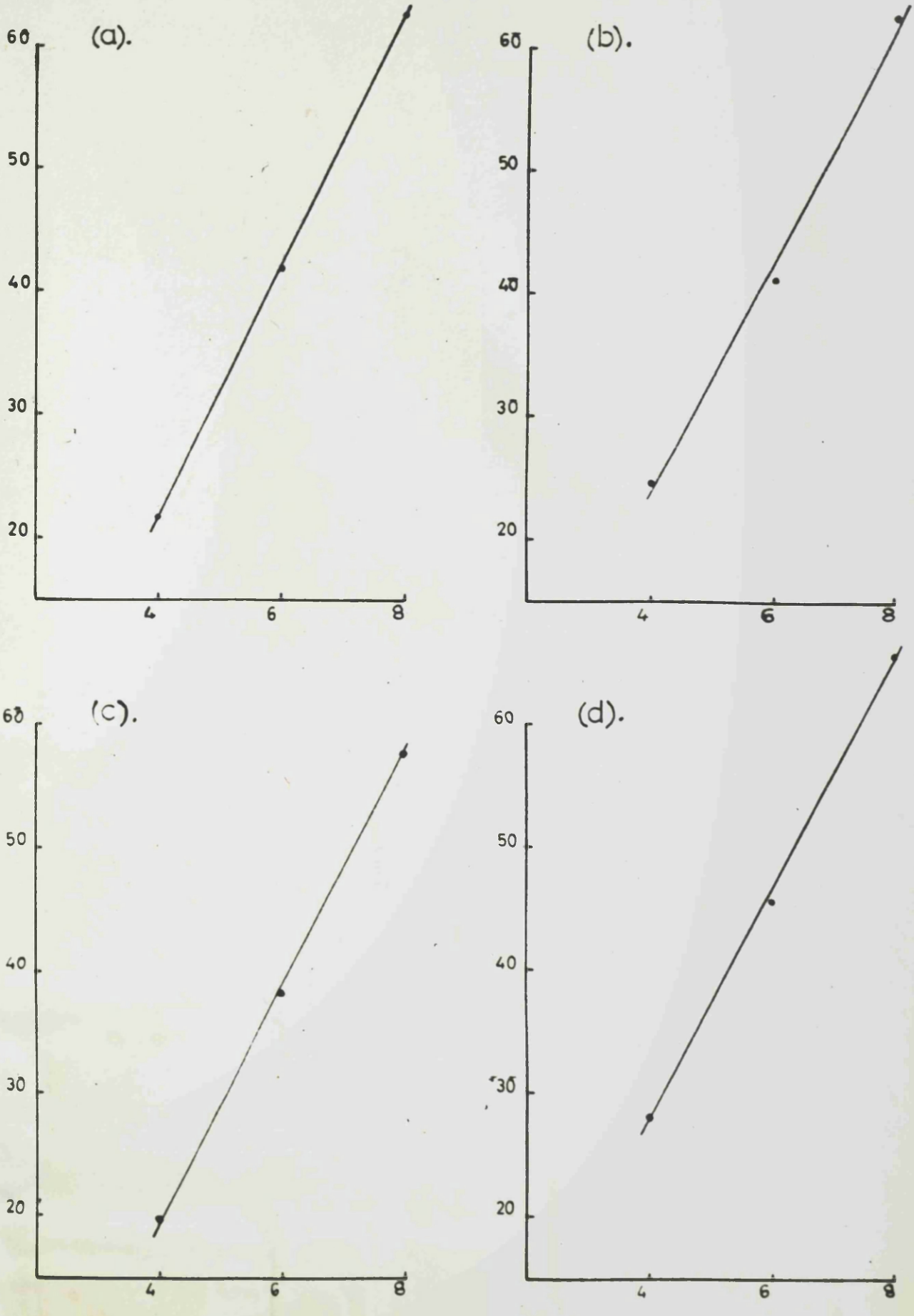
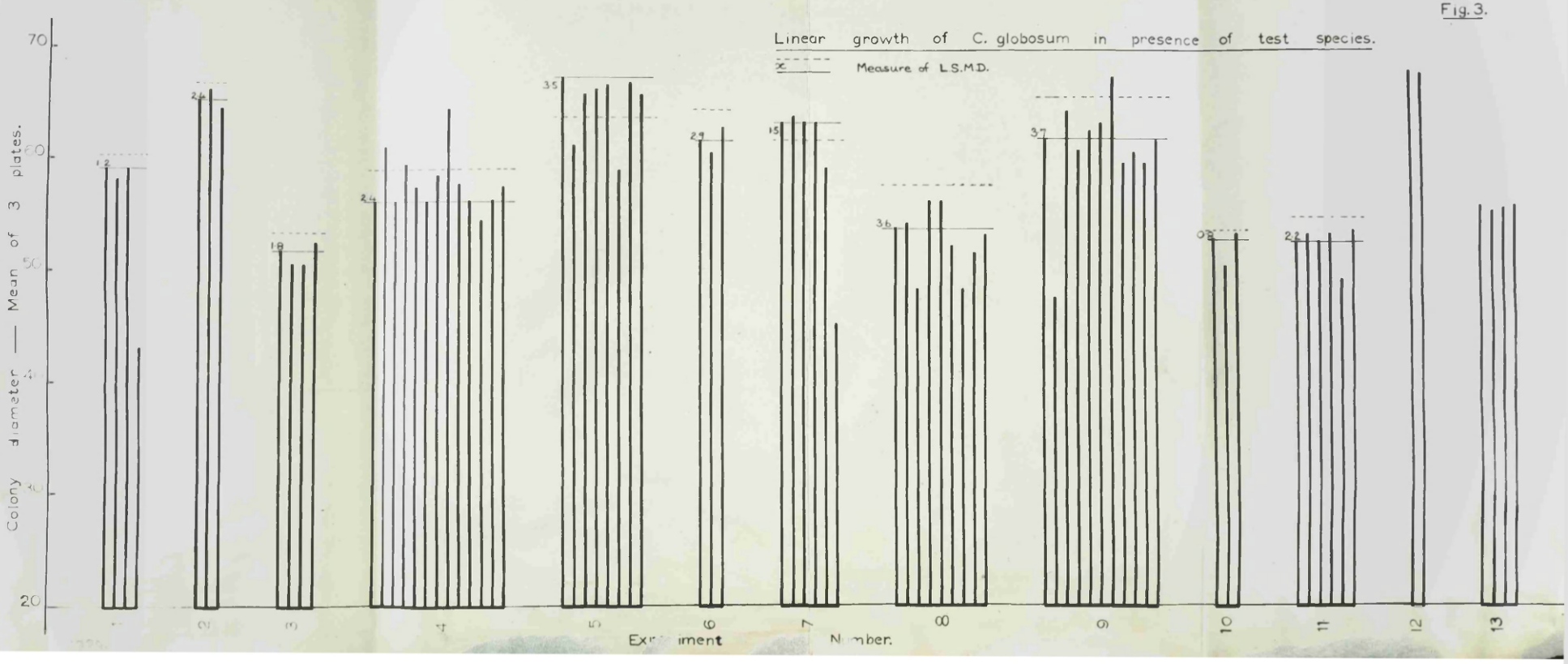

(a).
60
50
40
30
20
4
6
8
(b).
60
50
40
30
20
4
6
8
(c).
60
50
40
30
20
4
6
8
(d).
60
50
40
30
20
4
6
8

Fig. 3.


Linear growth of C. globosum in presence of test species.
x Measure of L.S.M.D.
Colony diameter — Mean of 3 plates.
70
60
50
40
30
20
1.2
2.4
1.8
2.4
3.5
2.9
1.5
3.6
3.7
0.8
2.2
1
2
3
4
5
6
7
8
9
10
11
12
13
Experiment Number.

Control
Chaetomium globosum
Botrytis cinerea
Saccharomyces cerevisiae

Control
Mucor plumbeus
Aspergillus niger

Control
Zygorhynchus moelleri
Tilachlidium nigrum
Agaricus campestris

Control
Cordyceps ophioglossoides
Cladosporium herbarum
Stereum hirsutum
Sordaria fimicola
Aspergillus terreus
Gibberella fujikuroi
Carpentoles sp.
Pericystis apis
Polyporus sulphureus
Ceratocystis moerulescens
Pyronema domesticum
Penicillium claviforme

Control
Colletotrichum lindemuthianum
Helminthosporium sativum
Alternaria tenuis
Fusarium culmorum
Byssochlamys fulva
Ophiobolus graminis
Verticillium psalliotae

Control
Aspergillus fumigatus
Acremoniella atra

Control
Choanephora circinans
Rhodotorula rubra
Trichoderma koningi
Schizosaccharomyces octosporus
Fomes annosus

Control
Pleospora androgyna
Botrytis allii
Rhizopus stolonifer +
Mucor hiemalis +
M. hiemalis -
Cephalosporium cretatum
Pythium ultimum
Cunninghamella echinulata

Control
Trichoderma viride
Botrytis spectabilis
B. tulipae
Armillaria mellea
Verticillium sp.
Penicillium digitatum
Hansfordia grisella
Curvularia lunata
Merulius lacrymans
Nectria haematococca

Control
Caldariomyces fumago
Trichothecium roseum

Control
Rhizopus sexualis
Thielaviopsis basicola
Pullularia pullulans
Absidia spinosa
Piptocephalis cylindrospora

Control
Mortierella ramanniana

Control
Cordyceps militaris
Rhizopus stoloifer -
Botrytis fabae

\+ perithecia occupying 0.25% of the field.

2+ " 25-50% "

3+ " 50-75% "

4+ " 75-100% "

Analysis of consistency of results was not made since the effects observed were usually quite distinct and could be adequately recorded using the above notation.

All test species were examined for effects of volatile metabolites on Chaetomium, and growth patterns noted as above. The results of this survey are given in Table A5 of the appendix as mean colony diameter at 8 days after inoculation, and perithecial production at 10 days. Fig. 2 shows linear growth patterns of C. globosum which are typical of those recorded in the presence of all test fungi.

It can be seen from this that the mean diameter at 8 days may reasonably be taken as representative of the reaction to any volatile metabolites. Perithecia were recorded at a time when examination of the cultures showed that production was at a maximum.

A summary of results of effects of volatile metabolites from the test fungi on the growth of Chaetomium, is presented in graph form as Fig. 3.

3. Stereum hirsutum.

Inoculation: Discs 3mm in diameter cut from the edge of a vigorously growing culture on malt agar were used as inoculum. Subculturing by spot mycelial inocula was not found to be

Fig. 4.

Linear growth of Stereum hirsutum in presence of test fungi.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Time, days.

Test species: (a). Helminthosporium sativum

(b). Saccharomyces cerevisiae

(c). Sordaria fimicola

(d). Mucor hiemalis

Fig. 4.

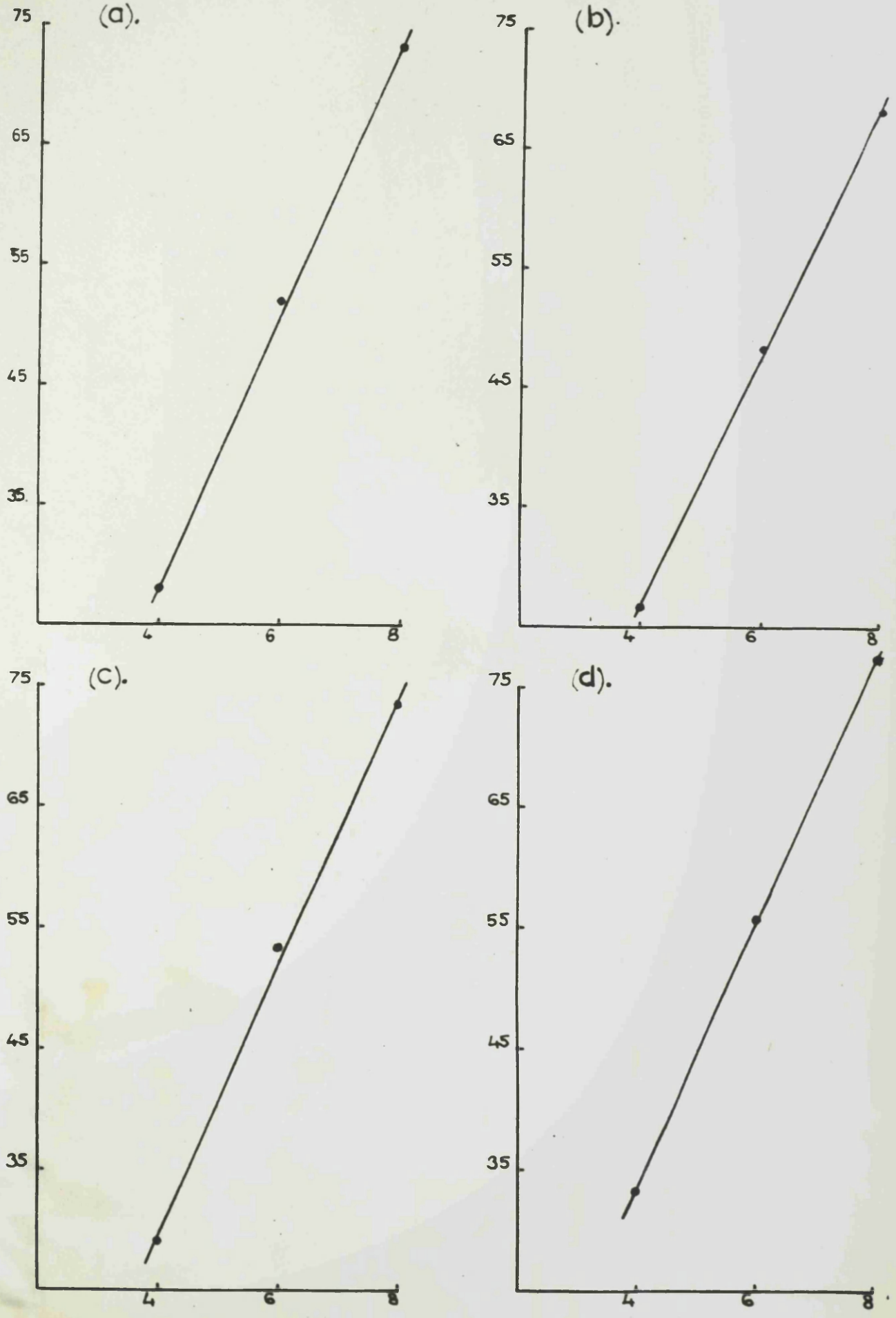
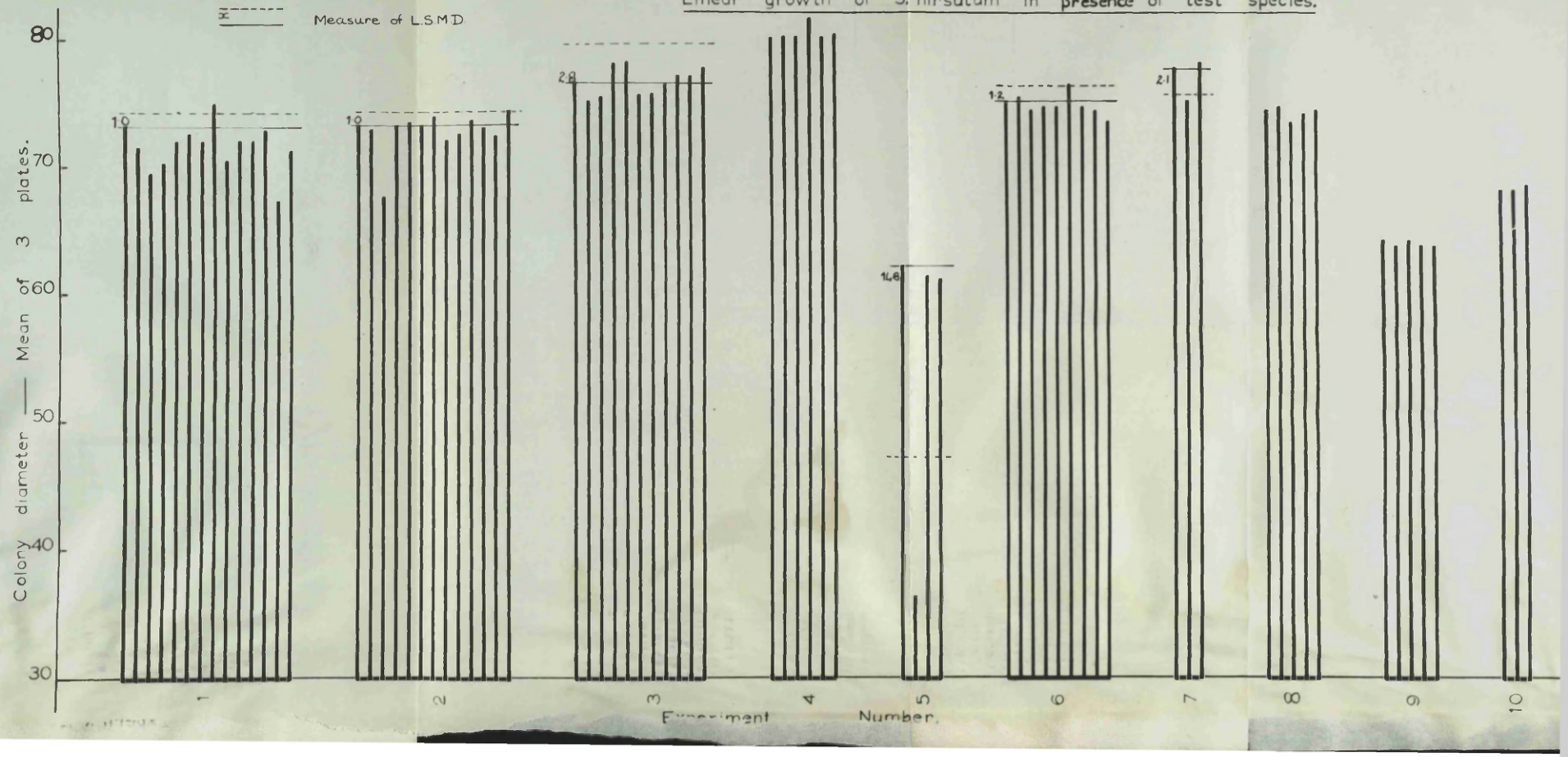

(a).
75
65
55
45
35
4
6
8
(b).
75
65
55
45
35
4
6
8
(C).
75
65
55
45
35
4
6
8
(d).
75
65
55
45
35
4
6
8

Fig. 5.

Linear growth of S. hirsutum in presence of test species.



Control
Carpenteles sp.
Shizosaccharomyces octosporus
Aspergillus terreus
Verticillius sp.
Fusarium culmorum
Zygorhynous moelleri
Pythium ultimum
Cunninghamella echinulata
Gibberella fujikuroi
Cladosperium herbarum
Penicillium digitatum
Mucor plumbeus

Control
Helminthosporium sativum
Saccharomyces cerevisiae
Sordaria fimicola
Stereum hirsutus
Absidia spinosa
Polyporus sulphureus
Aspergillus fumigatus
Alternaria tenuis
Caldariomyces fumago
Cordyceps ophioglossoides
Pyronema domesticum
Ceratocystis coerulescens

Control
Botrytis allii
B. tulipae
B. spectabilis
B. fabae
Hansfordia grisella
Pullularia pullulans
Trichoderma koningi
Acremoniella atra
Mucor hiemalis +
M. hiemalis -

Control
Verticillium psalliotae
Penicillium claviforme
Byssochlamys fulva
Rhodotorula rubra
Choanephora circinans

Control
Fusa[illegible]
[illegible]
Nectria haematococca

Control
Aspergillus niger
Rhizopus nexualis
Pleospora androgyna
Chaetomium globosum
Cordyceps militaris
Cephalosporium acremum
Rhizopus stolonifer +
R. stolonifer -

Control
Trichoderma viride
Ophiobolus graminis

Control
Merulius lacrymans
Curvularia lunata
Colletotrichum lindemuthianum
Trichotheciua roseum

Control
Agaricus campestris
Thielaviopsis basicola
Pistocephalis cylindrospora
Botrytis cinerea

Control
Armillaria mellea
Mortierella rammaniana

convenient with this fungus.

Main facets of growth studied:

Linear growth as measured by the mean of two previously marked diameters was the chief facet of growth studied in this fungus, since under the given culture conditions growth was solely vegetative.

Assay period: Colony diameters were measured after 4, 6, 8, and 10 days growth in the double chamber.

All ~~assay~~ test species were examined for effects of volatile fungal metabolites on Stereum and growth patterns noted as above. Results of this summary are given in Table A6 of the appendix. Fig. 4 shows linear growth patterns which are typical of those recorded in the presence of all test fungi. It can be seen from this that the colony diameter at 8 days may reasonably be taken as representative of the reaction to any volatile metabolites. A summary of results of effects of volatile metabolites from the test fungi on the growth of Stereum is presented in graph form as Fig. 5.

4. Aspergillus niger.

Inoculation: Spot inocula were made from vigorously growing conidial cultures on malt agar.

Main facets of growth studied:

Linear growth as measured by the mean of two previously marked colony diameters, and sporulation, were noted.

Fig. 6.

Linear growth of Aspergillus niger in presence of test fungi.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Time, days.

Test fungi: (a). Gibberella fujikuroi

(b). Verticillium sp.

(c). Caldariomyces fumago

(d). Cladosporium herbarum

Fig. 6

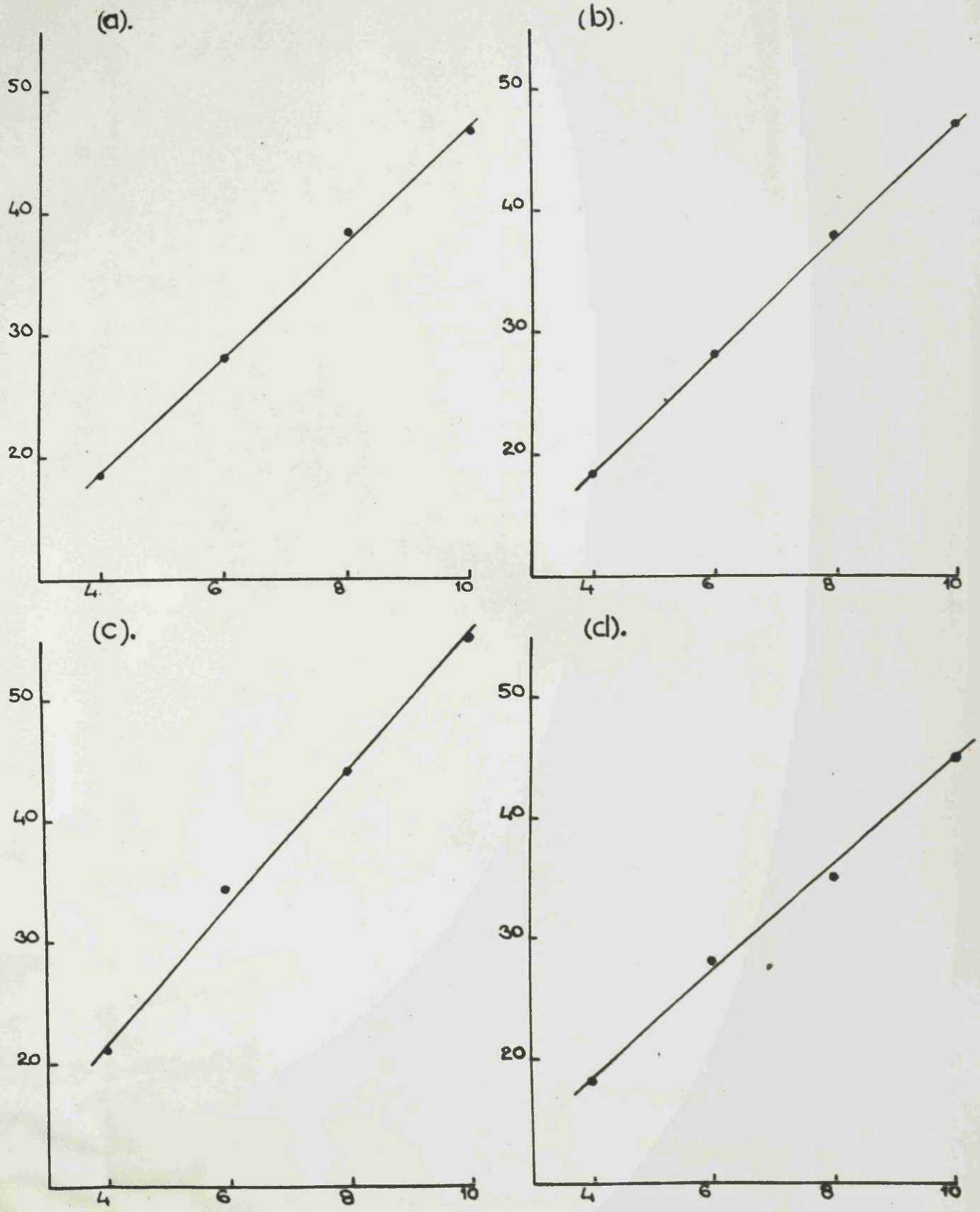
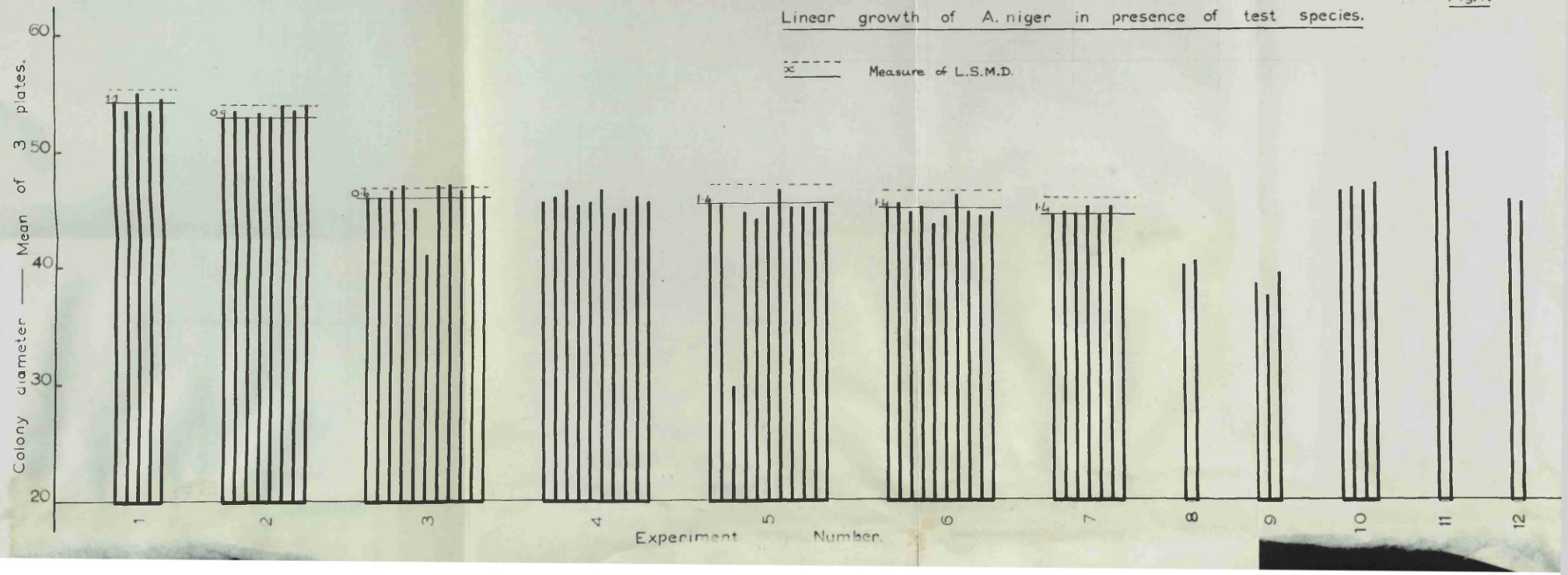

(a).
50
40
30
20
4
6
8
10
(b).
50
40
30
20
4
6
8
10
(c).
50
40
30
20
4
6
8
10
(d).
50
40
30
20
4
6
8
10

Fig. 7.

Linear growth of A. niger in presence of test species.

Measure of L.S.M.D.



Control
Trichoderma viride
Agaricus campestris
Penicillium digitatum
Caldariomyces fumago

Control
Aspergillus niger
Mucor plumbeus
Tilachidium nigrum
Absidia spinosa
Chaetomium globosum
Stereum hirsutum
Polyporus sulphureus

Control
Mucor hiemalis +
M. hiemalis -
Verticillium sp.
Helminthosporium sativum
Shizosaccharomyces octosporus
Rhizopus stolonifer -
R. stolonifer +
Sordaria fimicola
Pericystis apis
Pythium ultimum

Control
Aspergillus fumigatus
Ceratocystis coerulescens
Thielaviopsis basicola
Aspergillus terreus
Gibberella fujikuroi
Cladosporium herbarum
Cunninghamella echinulata
Penicillium claviforme
Pyronema domesticum

Control
Armillaria mellea
Fomes annosus
Cephalosporium crotalum
Verticillium psalliotae
Zygochlamys fulva
Ophiobolus graminis
Carpenteles sp.
Cordyceps militaris
Piptocephalis cylindrospora
Cordyceps ophioglossoides

Control
Botrytis spectabilis
B. tulipae
Pullularia pullulans
Rhodotorula rubra
Botrytis fusca
B. allii
Rhizopus nonsalis
Trichoderma koningi
Acremoniella atra

Control
Colletotrichum lindemuthianum
Choanephora cirrinans
Curvularia lunata
Nectria haematococca
Trichothecium roseum
Saccharomyces cerevisiae

Control
Mycorhynchus koelleri

Control
Hansfordia grisella
Pleospora androgyna

Control
Fusarium culmorum
Sterulina lacrymans
Botrytis cinerea

Control
Alternaria tenuis

Control
Mortierella ramanniana

Assay period: Colony diameters were measured after 4, 6, 8, and 10 days growth in the double chambers, and an estimate of amount of sporulation made after 10 days using the following scale:-

+	conidial heads occupying	0-25% of the field
2+	"	25-50% "
3+	"	50-75% "
4+	"	75-100% "

Analysis of consistency of results was not carried out since the effects observed were usually quite distinct and could be adequately recorded using the above notation.

All test ~~assay~~ species were examined for effects of volatile metabolites on Aspergillus and growth patterns noted as above. Results of this survey are given in table A7 of the appendix as mean colony diameter at 10 days after inoculation.

Fig. 6 shows linear growth patterns of Aspergillus which are typical of those recorded in the presence of all test fungi. It can be seen from this that the mean colony diameter at 10 days may reasonably be taken as representative of the reaction to any volatile metabolites.

A summary of results of effects of volatile metabolites from the test fungi on the growth of Aspergillus is presented in graph form as Fig. 7.

Fig. 8.

Linear growth of Botrytis cinerea in presence of test fungi.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Time, days.

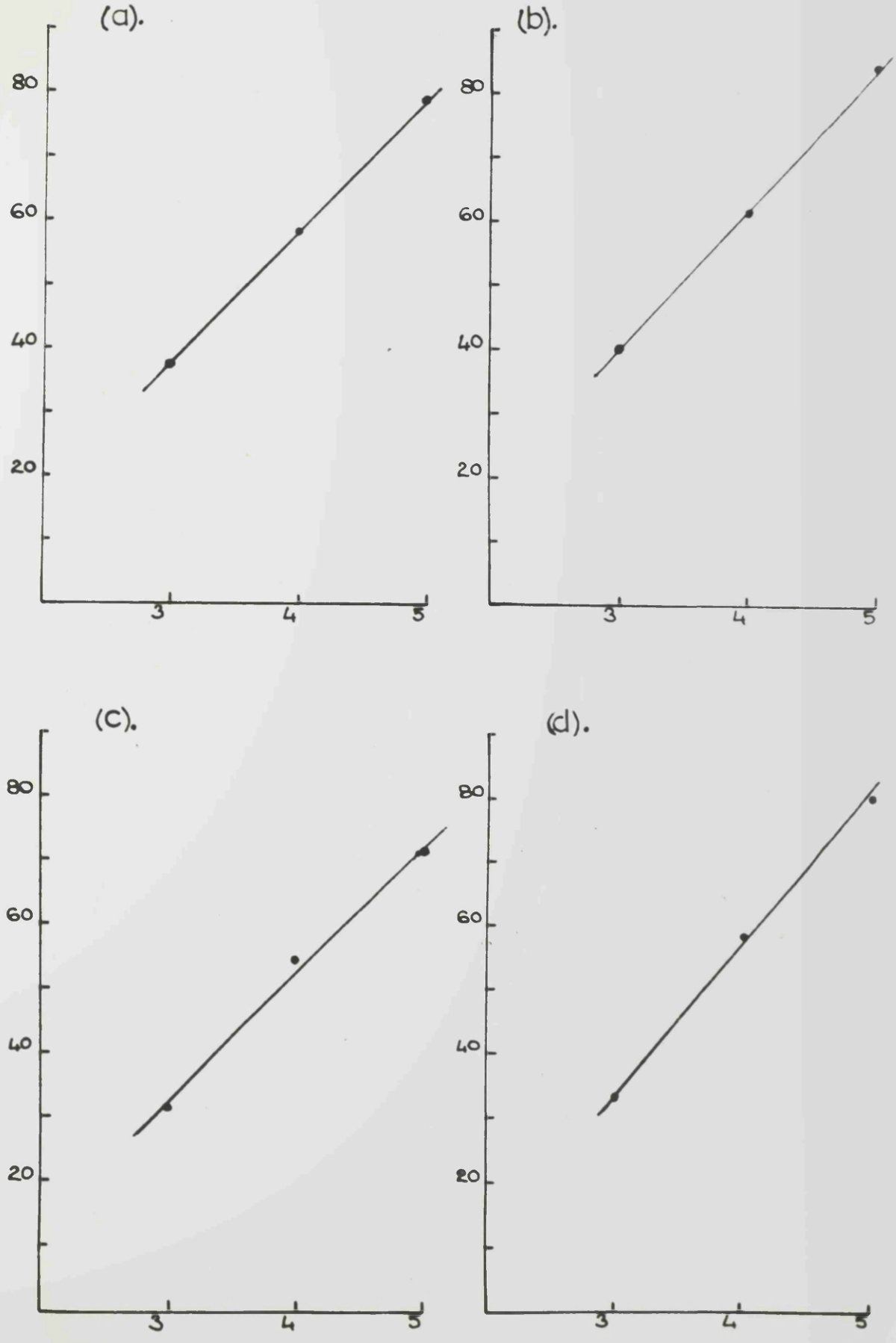
Test fungi: (a). Stereum hirsutum

(b). Byssochlamys fulva

(c). Saccharomyces cerevisiae

(d). Pyronema domesticum

Fig. 8.



5. Botrytis cinerea.

Inoculation: Spot mycelial inocula were made from cultures on malt agar.

Main facets of growth studied:

Linear growth, measured as the mean of two previously marked colony diameters, and sporulation, were noted.

Assay period: Colony diameters were measured after 3, 4 and 5 days growth in the double chamber and an estimate of amount of sporulation made after 7 days, using the symbols as before:-

+	Conidial heads occupying	0-25% of the field.
2+	"	25-50% "
3+	"	50-75% "
4+	"	75-100% "

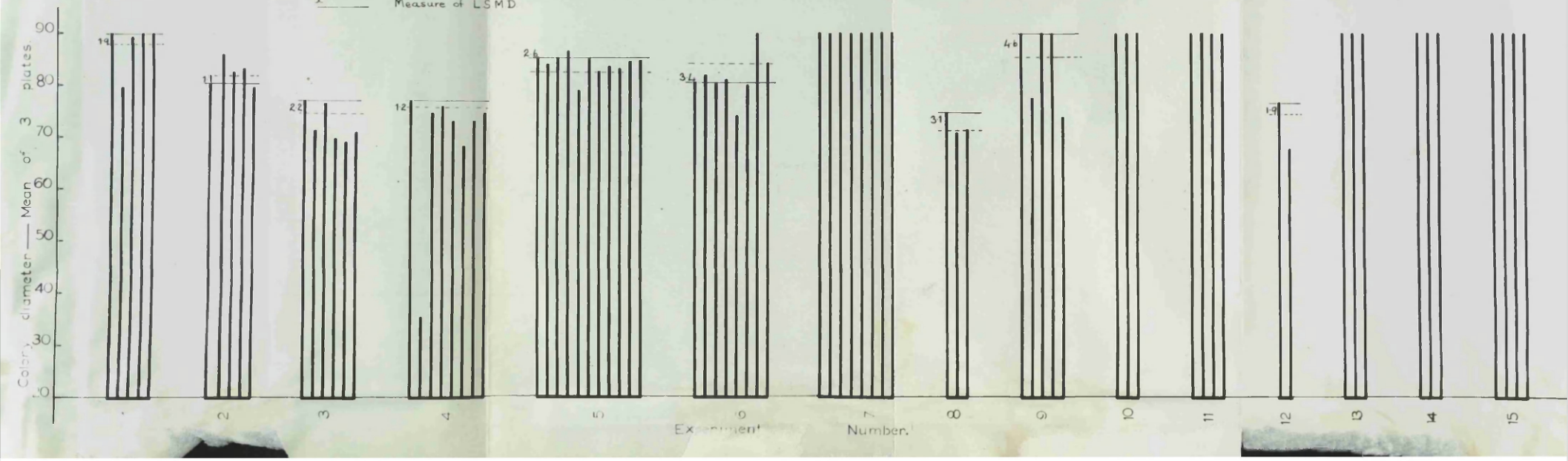
Analysis of consistency of results was not carried out since the effects observed were usually quite distinct and could be adequately recorded using the above notation.

All test species were examined for effects of volatile metabolites on Botrytis and growth patterns noted as above. Results of this survey are given in Table A8 of the appendix as mean colony diameter at 5 days, and sporulation at 7 days after inoculation.

Fig. 8 shows linear growth patterns of Botrytis which are typical of those recorded in the presence of all test fungi. It can

Fig. 9.

Linear growth of B. cinerea in presence of test species.



be seen from this that the mean colony diameter at 5 days may reasonably be taken as representative of the reaction to any volatile metabolites.

A summary of results of effects of volatile metabolites from test fungi on the growth of Botrytis is presented in graph form as Fig. 9.

Control
Saccharomyces cerevisiae
Chaetomium globosum
Botrytis cinerea
Agaricus campestris

Control
Penicillium digitatum
Trichothecium roseum
Mucor plumbeus
Pyrenema domesticum

Control
Ceratocystis coerulescens
Aspergillus terreus
Cunninghamella echinulata
Thielaviopsis basicola
Gibberella fujikuroi

Control
Trichoderma viride
Cordyceps ophioglossoides
Penicillium claviforme
Sordaria fimicola
Polyporus sulphureus
Pericystis apis
Aspergillus fumigatus

Control
Byssochlamys fulva
Pythium ultimum
Absidia spinosa
Stereum hirsutum
Carpenteles sp.
Fusarium culmorum
Helminthosporium sativum
Verticillium sp.
Rhizopus sexualis
Alternaria tenuis

Control
Botrytis fabae
B. tulipae
B. spectabilis
Rosas annosus
Botrytis allii
Aeremonialla atra
Verticillium psalliotae

Control
Rhodotorula rubra
Trichoderma koningi
Pullularia pullulans
Mucor hiemalis +
M. hiemalis -
Rhizopus stolonifer +
R. stolonifer -

Control
Ophiobolus graminis
Nectria haematococca

Control
Armillaria mellea
Colletotrichum lindemuthianum
Hansfordia grisella
Cordyceps militaris

Control
Curvularia lunata
Choanephora cirsinans

Control
Aspergillus niger
Zygorhyncus moelleri
Tilachlidium nigrum

Control
Rhizosaccharomyces octosporus

Control
Merulius lacrymans
Cladosporium herbarum

Control
Piptocephalis cylindrospora
Mortierella ramaniana

Control
Cephalosporium cretatum
Caldariomyces fumago
Pleospora androgyna

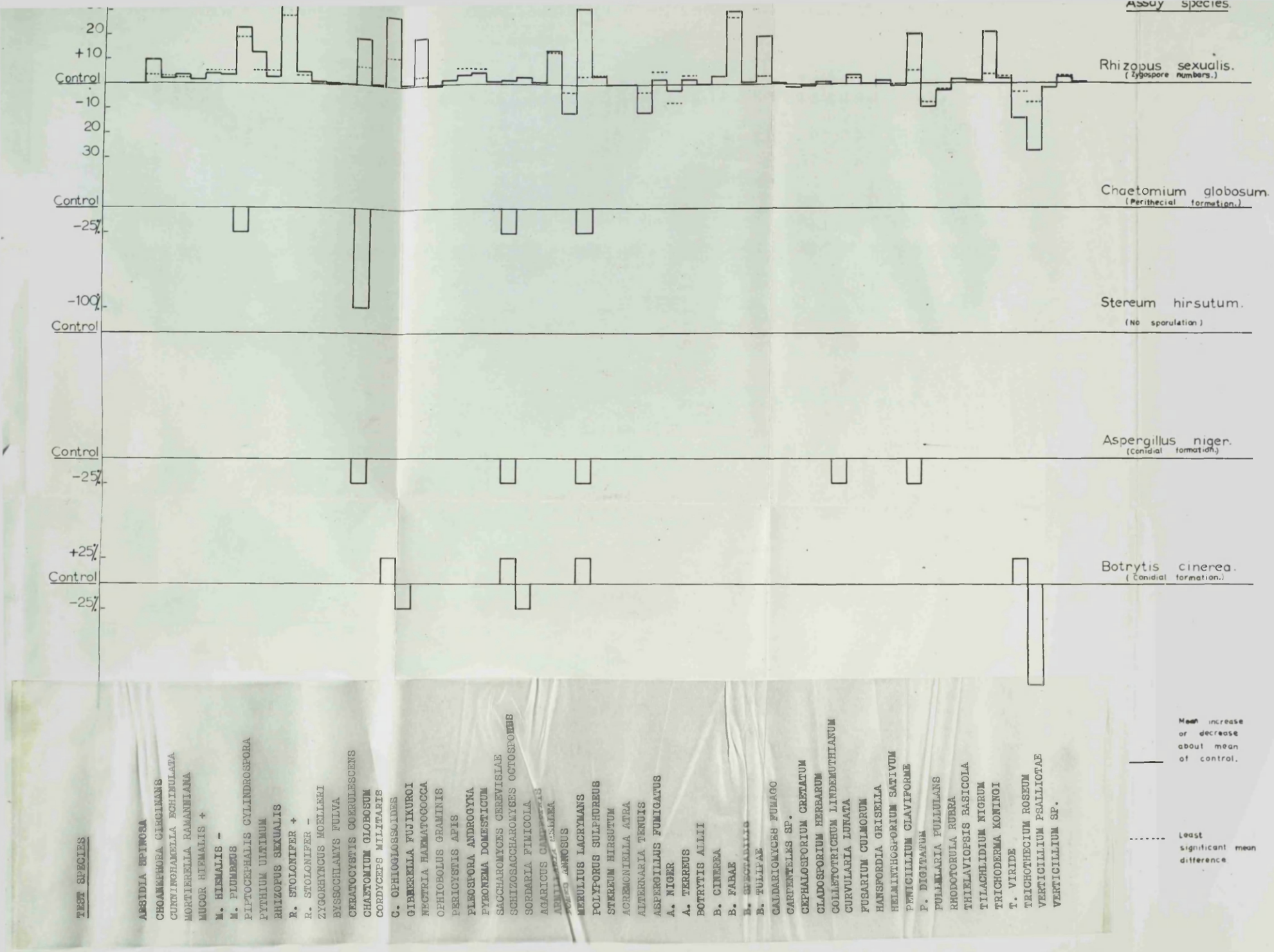


Fig. 11.

Effect of volatile fungal metabolites on sporulation of assay species.

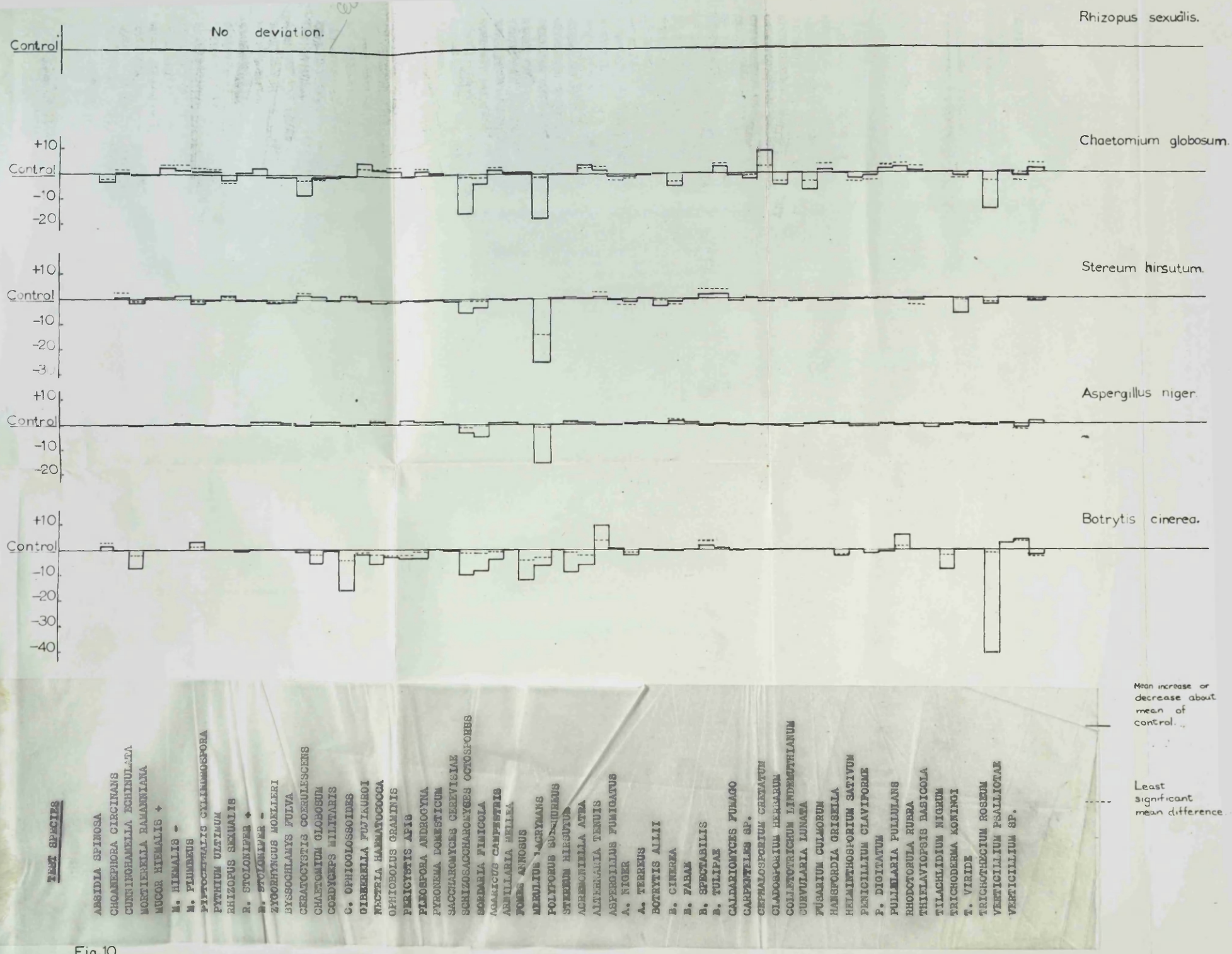


Fig.10.

Effect of volatile fungal metabolites on growth of assay species Linear growth in mm.

SUMMARY AND DISCUSSION OF RESULTS OF GENERAL SURVEY.

Since all 65 species were tested for effects on the five assay organisms, Figs.10 and 11 have been compiled from Tables A9 and A10 to facilitate comparison of the overall action of fungal volatiles detected. Fig.10 indicates increase or decrease in linear growth of the assay species as affected by the test fungi, and is plotted as mean differences in growth of the assay species from that of the controls. Fig. 11 shows increase or decrease in sporulation of the assay fungi as affected by volatile metabolites of the test fungi.

In the following summary the results are considered firstly as prevalence of effects on the individual assay species, and then as action overall.

1. Rhizopus sexualis.

The results show that 13 (20%) of the species tested produced a volatile metabolite which stimulated zygospore production and 5 (8%) inhibited this. Ceratocystis coerulescens stimulated the production of aerial mycelium, but no other effect on vegetative growth was seen.

Those species which gave a mean number of zygospores per plate significantly more than the controls (95% level) were, in decreasing level of activity: Rhizopus sexualis, Fomes annosus, Botrytis cinerea, Cordyceps militaris, Thielaviopsis basicola, Mucor plumbeus, Botrytis spectabilis, Absidia spinosa, Rhizopus stolonifer-, Colletotrichum lindemuthianum, Cunninghamella echinulata, Ceratocystis coerulescens, and Gibberella fujikuroi. The increases in presence of these latter four were only slightly greater than the Least Significant Mean Difference (95% level) as compared with the effect of the other nine which gave increases much greater than the L.S.M.D. Species causing decrease in zygospore formation were: Trichoderma viride, and T. koningi, Armillaria mellea, Acremoniella atra and Penicillium claviforme; the first two giving greatest inhibition.

The results obtained do not indicate any obvious taxonomic relationships between the species affecting zygospore production of Rhizopus; nor does level of activity in enhancing or retarding zygospore production appear to be connected with the amount of fungal material producing the metabolites.

2. Chaetomium globosum.

The results show that 4 (6%) of the species tested produced volatile metabolites increasing linear growth of Chaetomium whilst 10 or (17%) inhibited this facet of growth. No increase in perithecial production was noted, but 6 species (10%) inhibited the formation of fruiting bodies.

Increase in linear growth was brought about by the metabolites of Carpenteles sp., Cordyceps ophioglossoides, Penicillium digitatum, and Stereum hirsutum, in decreasing order of activity.

The inhibitory effects were more marked, especially in the cases of Fomes annosus, Saccharomyces cerevisiae, and Trichoderma viride. Other species inhibiting linear growth, though to a much lesser degree, were: Absidia spinosa, Botrytis allii, Byssochlamys fulva, Caldariomyces fumago, Cephalosporium cretatum, Colletotrichum lindemuthianum and Shizosaccharomyces octosporus.

Of the species inhibitory to linear growth, S. cerevisiae and F. annosus also gave decreased perithecial production; as did Mucor plumbeus and Ceratocystis coerulescens, two fungi having no apparent effect on linear growth.

No relationship is apparent between the test fungi producing these effects above.

The fact that few of the fungi affecting linear growth also affected sporulation suggests that at least two different types of

Of those species found to affect zygospore production only three, namely Rhizopus sexualis, Mucor plumbeus, and Trichoderma viride have been noted previously for the production of volatile metabolites affecting the activity of other fungi.

Hepden and Folkes (1960) indicated that in addition to stimulation of zygospore formation in R. sexualis by mature cultures of that fungus, other 'related' fungi produced volatile substances with similar, but less marked effects. However, in the present work no such relationships have been evident; although the results are in agreement with the greatest stimulation to zygospore production being produced by Rhizopus itself.

Several Russian workers have found unidentified volatiles from T. viride to be inhibitory to the growth of a large number of fungi and bacteria, and results obtained here are thus in agreement with the antagonistic activity of Trichoderma.

Page (1959) showed ammonia to be the substance produced by M. plumbeus which stimulated asexual reproduction of Pilobolus spp. Thus, there may be some correlation here between the stimulatory effect on the growth of one Mucoraceous fungus by an aminating compound, and a similar stimulation in another, Rhizopus, by a substance postulated to be methylamine. Confirmation of this speculation may only be made when positive identification of the nature of the volatile metabolite of R. sexualis is made.

volatiles were active on these different processes. This would seem reasonable, since physiological requirements for vegetative and reproductive growth are usually quite distinct and vary from fungus to fungus.

Penicillium digitatum, which is reported to produce ethylene; Ceratocystis coerulescens producing isobutyl acetate and methylheptenone, and Trichoderma viride producing unknown volatiles, are the only species assayed here which have previously been reported as producing volatile metabolites. Results are in agreement with previous reports that T. viride produces fungistatic substances.

Kamoen (1959) studied factors influencing perithecial production in C. globosum. He did not survey the effects of volatile substances, but noted that when extracts from a number of fungi were incorporated into the growth medium an increase from few to many fruiting bodies occurred. Amongst the fungi studied were T. viride and A. niger which were incorporated in the present survey, but did not give similar effects. Thus, it may be that substances which stimulate perithecial formation in C. globosum are not volatile at low temperatures; and hence the lack of any stimulation by the fungi tested here for volatile production.

3. Stereum hirsutum.

This fungus appears to be fairly resistant to any change in the normal growth pattern produced by volatile metabolites of other fungi. Of the species tested, only Pythium ultimum gave any increase in linear growth of the colony.

Decrease in linear growth was effected by 10 or (17%) of the total number of species tested. As with other assay organisms, Fomes annosus gave greatest decrease in growth; Tilachlidium nigrum and Saccharomyces cerevisiae also being active. Carpenteles sp., Shizosaccharomyces octosporus, Aspergillus terreus, Cunninghamella echinulata, Mucor plumbeus, Rhizopus stolonifer +, and Trichoderma viride all decreased colony diameter to a degree slightly more than that required for significance at the 95% level.

In addition to the retardation of linear growth, the action of F. annosus produced a marked reduction in the amount of aerial mycelium in cultures of Stereum.

No regular pattern of effects produced by the test species was apparent since activity appeared to be spread throughout all fungal groups.

Mucor plumbeus and Trichoderma viride are the only test organisms giving action against the assay fungus which have previously been reported to produce volatiles, and results noted here again confirm the inhibitory action of T. viride against other fungi.

4. Aspergillus niger.

This has proved to be the most stable of the five assay organisms. None of the test species seemed to produce volatile metabolites capable of stimulating either linear growth or sporulation. (4) species, or 6% of the total tested decreased linear growth, F. annosus once again being the most active: the others being S. octosporus, S. cerevisiae and Helminthosporium sativum. With the exception of S. octosporus, these species, plus Cladosporium herbarum and Ceratocystis coerulescens, decreased the amount of sporulation by approximately 25%.

No regular pattern of effects emerges from these results, showing that volatile production is a property of fungi from all groups.

C. coerulescens is the only fungus active here which has been previously shown to produce volatile metabolites.

There appears to be a close relationship in this fungus between colony diameter and amount of sporulation. Thus, it seems possible that only one volatile substance is being produced by the 'active' test species which affects both facets of growth of the Aspergillus.

Total growth, and thus physiological activity of this fungus in general, is much less than that of the other assay species over the assay period, and so any changes in growth pattern brought about by volatile substances are minimised.

5. Botrytis cinerea.

This appears to be the assay species whose total growth is most affected by volatile metabolites of the test fungi. A total of 5 or 8% were active in increasing linear growth, whilst 15 or 22% had the effect of decreasing it.

Increase in linear growth was observed in the presence of Absidia spinosa, Acremoniella atra, Mucor plumbeus, Penicillium digitatum and Trichothecium roseum, although all results were only slightly greater than the L.S.M.D. at the 95% level. A.atra also stimulated sporulation.

Inhibition of linear growth was considerable in the cases of Trichoderma viride, Cordyceps militaris, Armillaria mellea, Saccharomyces cerevisiae, and less marked in the presence of Ceratocystis coerulescens, Cunninghamella echinulata, Fomes annosus, Gibberella frujikuroi, Ophiobolus graminis, Pericystis apis, Polyporus sulphureus, Shizosaccharomyces octosporus, Sordaria fimicola, Stereum hirsutum and Thielaviopsis basicola.

Sporulation was also affected by a number of these: T. viride gave complete inhibition, whilst S. octosporus inhibited by 25%. A. mellea, C. militaris and S. cerevisiae had the effect of increasing sporulation in that production of conidial heads was widespread over the plate and not confined to the peripheral region as in all the controls. C. ophioglossoides and T. koningi respectively

inhibited and increased spore production without affecting linear growth.

Fungi from all groups were active in producing volatile substances affecting the growth of B. cinerea, so that no regular pattern of effects emerges from the results.

Volatile metabolite production has been previously reported from T. viride, M. plumbeus and P. digitatum of the above species affecting the growth of B. cinerea, and results here confirm that M. plumbeus produces a stimulatory action and T. viride a strongly inhibitory one.

It would appear that when mycelial growth is greatly inhibited as by T. viride, sporulation is also inhibited; but when less retardation of mycelial growth occurs then sporulation is stimulated, e.g. action of C. militaris. This might possibly be due to a hastening of physiological ageing of the hyphae concerned, i.e. diversion from vegetative to reproductive growth.

CONCLUSION.

When the overall effects of volatile metabolites from the test fungi on the five assay organisms are considered, it is seen that there are few species affecting more than one or two of the five. Fomes annosus was the only species tested which affected the growth of all. It inhibited the linear growth of all but R. sexualis, but stimulated sexual reproduction of this fungus.

All fungi previously reported as producing volatile metabolites with action on other fungi have also proved to affect the growth of some of the species assayed here. The phenomenon of production of volatile metabolites having effects on the growth of fungi appears to be scattered throughout all groups of fungi and no consistent patterns of effects are evident. However, there is some tendency for species showing action against one assay type to affect others also, though some assay species appear to be more resistant to the action of volatile substances than others.

The fact that different fungi can produce the same visible effect on the selected characters of the assay fungi does not, of course, necessarily imply that they all produce the same substance. Different substances could affect the characters in the same manner; or there could be a common chemical grouping present in all the substances.

Many of the species shown to produce volatile metabolites are known as plant pathogens, so that it may be surmised that in nature these substances may affect the resistance of the plant to fungal attack through changing the chemical composition of the host cells. However, the laboratory tests produced conditions so vastly different from those experienced in the field that no real comparisons of effects can be drawn.

A point of interest noted on examination of the assay cultures was the production of yellowish colouration in the C. globosum assay media during tests of T. viride and C. fumago. Both of these fungi inhibited linear growth of Chaetomium so that it is possible that colouration of the medium was due to a reaction of the Chaetomium to the volatile substances which involved excretion of a pigmented substance into the medium. Hence, inhibition of growth of Chaetomium in these cases might be postulated as being due to a diversion of metabolic processes as opposed to a simple retardation of the normal ones. However, the whole question of mode of action of volatile substances remains open and would provide opportunity for a further study.

PART 2

THE NATURE OF VOLATILE FUNGAL METABOLITES.

REVIEW OF LITERATURE:

Since the earliest days in the study of fungal organisms mycologists have been aware of the "musty" odours often associated with their cultures; but most have been content merely to note this fact and pass on to a morphological or other study. Indeed, many taxonomic diagnoses include a description of the characteristic odour of the fungus, e.g. E.M. Wakefield's description of Clitocybe odora with its "strong, fragrant smell of anise".

Many of these odours must be due to volatile organic substances produced by the fungus and liberated into the atmosphere. It is thus hard to tell why so much work should have been done on metabolic products secreted into the growth medium, whilst little has been attempted on volatile products other than Carbon dioxide. The answer probably lies in the many chemical problems associated with the collection and analysis of such products.

If one disregards Carbon dioxide, the volatile metabolic product of plants to which most attention has been given is ethylene. Much literature has appeared on this subject, but it is only recently that positive chemical identification of the substance has been made.

Knight and Crocker (1913) devised the "triple response of pea seedlings" as a method for detecting the presence of ethylene in an atmosphere, and this was used by Nickerson (1948) for identification of ethylene as a metabolic product of the fungi Blastomyces dermatidis

and Braziliensis and Histoplasma capsulatum. Young et al (1951) confirmed the supposition of earlier workers, who had used leaf epinasty tests as a measure of ethylene production by Penicillium digitatum. He did this by absorbing the gas in Mercuric perchlorate and reliberating with Lithium chloride or Hydrochloric acid.

However, the above methods are not chemically specific and full proof for identification of ethylene, as many olefines give a similar reaction. A specific identification was made by Spencer (1962). Whilst investigating the metabolism of sub-cellular particles of tomato fruits she employed the Mercuric perchlorate/Lithium chloride absorption/liberation method for collection of the gas in serum bottles, and then analysing this by Gas Chromatography.

Few reports of identification of other volatile fungal products are to hand:-

Hanna et al (1932) showed that the odour associated with spores of Tilletia levis, the stinking smut of wheat, was caused by trimethylamine; whilst Morgan (1948) isolated isobutyl acetate and methyl heptenone from cultures of Endoconidiophora coerulescens.

In the large volume of literature from the U.S.A. on the rusts there are several reports of volatile substances produced by uredospores of Puccinia graminis var. Tritici. Foeyth (1955) identified as trimethylethylene, the germination inhibitor produced by these uredospores; whilst Allen and French et al (both 1957) detected a volatile substance produced by uredospores which simulates

the action of the host plant in causing differentiation of structures similar to appressoria and vesicles. The latter authors later (1958) identified this substance as pelargonaldehyde, since it shows identical effects when supplied to the fungus.

Page (1959), when studying Pilobolus kleini, observed that in cultures contaminated with Mucor plumbeus there was a considerable increase in asexual reproduction of the Pilobolus. Further studies showed that this effect was transmitted over a distance of at least 1¼cm. and must have been due to a volatile substance, which proved to have similar properties to ammonia. Thus it was postulated that, when grown on asparagine, aspartic acid or sodium nitrate, Mucor plumbeus metabolises ammonia in low concentration.

All the above reports on production of volatile substances by fungi depend for identification on methods involving standard chemical techniques of absorption, distillation and other such reactions.

More recently the emphasis has been on physico-chemical methods for unknowns, using Mass Spectrometry and Gas Chromatography. McTeague et al (1959) used Mass Spectrometry for the identification of a volatile substance produced by mycelium of Agaricus campestris which stimulates spore germination of this fungus. They found it to be 2.3-dimethyl-1-pentene; but as no synthetic material was available were not able to verify that the pure substance had the same effect on spore germination and that the stimulatory action was not due to a combination of other factors.

Lockard and Kneebone (1962) also studied volatile substances evolved by mycelium of Agaricus campestris, but did not examine the biological effect of these. The method used by these workers was to introduce gas samples, taken directly from a stream of air passing through Agaricus cultures, into a Chromatograph incorporating a Flame Ionising Detector unit. Results published to date show no agreement with the findings from this department; i.e. compounds detected were ethyl alcohol, acetaldehyde, ethyl acetate, athylene and acetone.

Collins and Morgan (1962) made further studies on the metabolism of Ceratocystis (Endoconidiophora) spp., and showed by means of Gas Liquid Chromatography that these produced varying amounts of ethyl acetate, ethanol, isobutyl acetate, isobutanol, and propyl acetate.

EXPERIMENTAL.

The aim in this study has been to collect the volatile emanations from fungi *in toto* and to subject these to chemical analysis. By this means it was hoped to identify metabolites other than CO_2 which were suspected of inducing the observed biological effects.

The apparatus made available for analytical purposes by the courtesy of the Chemistry Department of the University was:-

A. Pye Argon Chromatograph.

B. Perkin Elmer Flame Ionising Detector Chromatographic Unit.

C. Mass Spectrometer.

On the advice of the chemists the most suitable method of analysis of unknown gases was taken to be Gas Liquid Chromatography, since Mass Spectrometry is primarily a confirmatory tool for the identification of substances known, or suspected, to be present in samples.

As the initial stimulus for this work came partly from the invitation of Dr. P.M. Joy to co-operate in the investigation of volatile metabolites of *Rhizopus sexualis*, all attempts in collection and analysis were made using this species.

A. ANALYSES USING THE PYE ARGON CHROMATOGRAPH.

Samples obtained from fungal cultures all contain a high proportion of water vapour which renders the chromatographic detector insensitive to other compounds. Hence, when making these analyses a refinement was added to the standard Chromatographic Unit. This was a Swoboda head for the separating column, which is a device holding back the entry of water vapour into the detector so that organic volatiles may pass through and be detected. - Appendix B1 contains a full description of the chromatographic methods.

1. 'Natural gases'.

The first attempts to analyse the volatile metabolites of R. sexualis were made by taking samples with a syringe from the head-space over cultures and injecting these directly into the Argon stream.

No positive results were obtained by this method with samples from the standard assay chamber of Part 1, from cultures on malt liquid and agar in 250 ml. Erlenmeyer flasks, or from those on malt liquid in Penicillin flasks.

2. Concentration of volatile metabolites.

When no results were obtained with samples taken directly from cultures, the next logical step towards analysis appeared to be

concentration of the volatile compounds to a level more easily detected.

(a). Continuous passage of air through culture vessels with trapping of effluent gases.

In their analysis of volatile metabolites produced by Agaricus campestris McTeague et al collected samples by passing a constant stream of air through the culture vessel for a period of some days, with continuous trapping of the effluent gases in a cooled receiver.

Hence, a similar method was used for collection of volatiles from Rhizopus growing on malt liquid medium in a series of Penicillin flasks. - See Fig. 1 of the appendix with accompanying description B2.

As an alternative to the above method of culturing in a series of flasks, a system of bulk culture was introduced. Fig. 2 of the appendix with description B3 shows the system of growth of Rhizopus in a 10 litre fermenter with continuous trapping of the effluent gases.

When R. sexualis is grown in the above type of vessel it adopts the pellet-like form taken up in liquid medium by many fungi under such conditions of aeration and agitation. Thus, experiments were carried out to determine whether or not there was also a change in physiological character. The results, given in appendix B4, showed that there was a significant increase in the numbers of zygospores produced in cultures of R. sexualis when exposed to an

airstream containing the effluent from deep cultures of Rhizopus. Hence, the fungus produces the 'active' metabolite in "deep" culture as well as on surface culture.

(b). Re-cycling of air through culture vessels.

A further modification of the deep culture system for producing a high concentration of volatile metabolites was made by re-cycling the same air through the culture vessel from a 250 litre gas holder for up to 10 days. - See Fig. 3 of appendix with accompanying description B5. Thus, with continual pumping of the same air through the culture, a considerable increase in concentration of the metabolic products was built up within the system; the drum providing a large sample of gases which could be used in chemical analysis.

Concentration of the volatile fungal metabolites by the above methods not only increased the concentration of complex organic substances but also those of CO_2 and water.

High concentrations of CO_2 do not inhibit detection of substances by the Pye Argon Unit, but water vapour renders the detector insensitive. Hence, dehydration of the samples was a major problem to be overcome before analysis could be attempted. In fact, with the continuous collection methods (a) above, the airstream became so saturated with water vapour as to block the tubes of the cold trap with ice.

Various methods of drying the gases were tried and these are

detailed as appendix B.6. The only procedure for removing water vapour from the system which proved effective and had no likely sources of loss of organic substances, was that involving passage of the gases through a tube packed in Drikold, thus causing most of the water to condense out.

Gas samples from which most of the water vapour had been removed were introduced into the chromatographic column using the Swoboda head to check the entry of any residual water.

No indication of the nature of the fungal volatiles present in any of the samples was given by these methods.

B. ANALYSES USING THE PERKIN ELMER FLAME IONISING DETECTOR UNIT.

Towards the end of the experimental period a Flame Ionising Detector became available for analyses. The great advantage of this unit is that its principle of detection depends on the combustion of samples in Helium, and so the presence of water vapour in samples does not hinder analysis. Hence, the need for drying procedures and attendant sources of loss of metabolites was obviated.

Samples of metabolites were taken directly from the headspace over surface cultures and from the drum reservoir of the re-cycling system. No positive evidence of their nature was determined.

C. ANALYSES USING THE MASS SPECTROMETER.

A number of test runs were made with Mass Spectrometer to confirm the opinion that this instrument was not suitable for analyses.

Some erratic and indistinct traces were obtained with samples from surface and deep culture, but these were too complex for analysis, and confused by the presence of large quantities of CO_2.

DISCUSSION.

In the initial period of this work, apparatus for identification of volatile substances was limited. Hence, much time was spent in evolving techniques for concentrating and drying the fungal metabolites.

However, biological evidence presented earlier of the continued production of the volatile substances in deep culture suggests that the methods of concentration of metabolites were successful and that failure to analyse compounds was due to some weakness in the analytical system. Separation of low concentration mixtures of a number of synthetic organic compounds by the F.I.D. suggested that the problem might not be insuperable with this unit.

Failure to record any positive results as to the nature of the volatile fungal metabolites may have been due to either loss of organic compounds in the drying and concentrating processes or to some inadequacy in the analytical systems.

When using the Swoboda head there was the remote possibility that the volatile metabolites may have been retained on the Glycol pre-column along with the water vapour and back-flushed out of the system before reaching the detector. It seems unlikely that this was so, since all the volatile substances sought would have a fairly fast elution time.

In fact, Lockard and Kneebone identified five volatile

substances from A. campestris by means of direct sampling of the airstream from mycelial cultures and subjecting them to F.I.D. analysis. They stated that they found no trace of the 2.3.dimethylpentene reported from this Department with Mass Spectrometer analysis. Conversely, I have found no trace of the compounds reported by these workers even although a synthetic mixture at low concentration was traced.

Conflicting evidence such as this merely shows further the difficulties involved in dealing with such small traces of metabolites.

PART 3

THE EFFECT OF KNOWN SUBSTANCES ON THE GROWTH OF CERTAIN FUNGI.

INTRODUCTION.

In the absence of guidance from the analytical work the choice of synthetic substances for test in this section was necessarily arbitrary. Two principles were considered as a basis for an experimental plan:-

1. Substances known to be produced by living cells.

Carbon dioxide. This substance which is the most common biological product was the first to be considered, and a substantial part of the following report deals with this gas.

Ethanol and Acetone.

These compounds are also well established as metabolic products and have been tested by other authors for effects on the growth of number of organisms. Hence, they have been included in this brief survey.

2. A small range of substituted olefines.

Since there are many reports of the biological activity of ethylene, a range of low boiling point olefines and similar substances was tested for effect on the assay species. Included in this test was a study of the different reactions of the fungi to methylation of an

olefine at different points, i.e. tests were made with

4. Methyl pentene.1.

4.Methyl.1.2.pentene

2.4.4.Tri methyl pentene.

The methods used for testing CO_2 were distinct from those used for the other compounds, hence the report is presented in two parts:-

A. Carbon dioxide.

B. Other organic substances.

A. CARBON DIOXIDE.

REVIEW OF LITERATURE.

There seems to be wide variation in tolerance of fungi to different concentrations of carbon dioxide. In general, concentrations of greater than 10% appear to inhibit growth of fungi to some degree. Brown (1922) reported the growth of Botrytis, Fusarium and Alternaria to be much reduced by 10% and 20% CO_2, the latter giving much greater reduction. However, in 1925, when studying the action of staling products of Sphaeropsis and Fusarium, he found that a CO_2 level of 5% allowed best growth due to its neutralising action on staling by ammonia. Barnet and Lilly (1955) showed the accumulation of CO_2 in Petri dishes to be the cause of reduction in sporulation of Choanephora cucurbitarum, whilst Abeygunawardena and Wood (1957) related reduced germination of sclerotia of Sclerotinium Rolfsii in upper layers of soil to accumulation of CO_2 in the soil.

Platz et al (1927) found that a number of plants stimulated the germination of chlamydospores of Ustilago zeae by production of CO_2. Under artificial conditions this was shown to be due to lowering of the pH of the medium by formation of carbonic acid. Concentrations of more than 15% CO_2 were inhibitory.

Brown (1922) stated that the effect on the growth of Botrytis cinerea produced by 2% CO_2 is small, whilst Rippel and Heilmann (1930) apparently contradicted this by reporting that concentrations of up to 1.2% CO_2 increased the dry weight of B. cinerea. However, one must distinguish here between Brown's comparison of growth at atmospheric

and higher concentrations of CO_2, and Rippel and Heilmann's experiments comparing growth in CO_2 free air with that containing a small percentage of CO_2. Barinova (1961) reported similar stimulation of growth of Aspergillus niger and Rhizopus nigricans with atmospheric CO_2 as opposed to CO_2 free air. She surmised as to the physiological action of this and showed that the stimulating action of CO_2 was not due to its fixation in acids of Kreb's cycle.

The study of the ecology of soil fungi has led to much discussion as to the reason for the characteristic zonation of fungi found. Bisby et al (1935) related the distribution of fungi in Manitoba soil profiles to differential oxygen tensions; but Burges and Fenton (1953) attributed the distribution to variation in CO_2 tensions at different levels. They showed that Penicillium nigricans, growing in the top 5cm. of soil, was inhibited by more than 5% CO_2 whilst Zygorrhyncus vuillemini at much greater depth tolerated 20%. Stover (1958) and Stover and Freiberg (1958) investigated the metabolism of another soil fungus, Fusarium oxysporum f. cubense, and found that 4% CO_2 was optimum for fixation of soil CO_2 in metabolic processes; although 2-25% frequently stimulated multiplication of this fungus. The observation was borne out by Bulit and Louvet (1960) who showed that Fusarium tolerated up to 23.5% CO_2 and could thus attack plants at greater soil depth than Sclerotinia minor whose growth was greatly depressed by more than 2% CO_2.

A further type of fungal habitat was studied by Thacker and

Good (1952) who measured the CO_2 concentrations found inside decaying tree trunks of sugar maple and found them to be of the order of 5-10%. They then demonstrated that fungi, e.g. Polyporus hirsutus and Corticium vellereum, which are common agents of decay, showed optimum growth on agar at CO_2 concentrations of 5-10% as found in the trees. Slight inhibition was recorded above 10% although some growth occurred with up to 35% CO_2 in the atmosphere.

Thus many fungi, notably soil organisms and some plant pathogens, seem to thrive in concentrations of CO_2 much greater than that experienced in ordinary atmospheric conditions of 0.03%.

EXPERIMENTAL.

1. Measurement of CO_2 concentrations within the assay chambers.

Growth of the test fungi in the assay chambers was expected to have increased the concentration of CO_2 within the apparatus, even allowing for some exchange of gases with the surrounding atmosphere at the junction of the two plates. Hence, experiments were carried out to obtain a measure of the CO_2 concentration produced in the tests.

Since no very accurate measurements were required, but merely an indication of the concentrations of CO_2 a colorimetric method was thought adequate:-

Ampoules of liquid indicator used in the British Oxygen Company Carbon Dioxide Analyser Unit were adapted for the purpose. Indication of CO_2 concentrations is given by these with a change in colouration of the liquid from magenta to straw colour as the CO_2 level rises from 0.3-5%.

Approximately 2mls. of the B.O.C. liquid was placed in a small dish and exposed to the atmosphere inside the basal half of an assay chamber for a period of 6 days. No colour change was noted in the fluid in any of the tests. Hence, it would appear that none of the fungi tested produced a concentration of CO_2 within the other half of the assay chamber greater than 0.3%. Confirmation of this was obtained by withdrawing 5ml. gas samples from the assay chambers with a syringe,

adding 2ml. of indicator fluid and shaking vigorously. Once again the fluid colouration indicated CO_2 concentrations of less than 0.3%.

2. A study of the effects of known concentrations of CO_2 on the growth of the test fungi.

The above experiments indicate that the concentration of CO_2 experienced by the assay fungi in the general survey was low. Hence, a range of concentrations of CO_2 was tested, including low values as well as those higher ones which a number of authors have found to affect the growth of fungi.

Concentrations tested (v/v) :

0.1%, 0.5%, 1.0%, 5.0%, 10.0%, 20.0%.

Method:

Desiccators of c.1.8 litre capacity were used as containers for the fungal cultures. A Petri dish base, inoculated with the assay fungus and covered with a cellophane membrane to ensure maximum exposure to the atmosphere, was placed in each desiccator. A calculated volume of air was then withdrawn from the desiccator under vacuum, and pressures equalised by allowing CO_2 from a cylinder to enter the vessel. - Basically the method of Kidd (1914).

Since the manometer used gave an accurate reading to only 0.1mm., concentrations of 0.5 and 0.1% CO_2 were achieved by first preparing a 1% mixture as above; after allowing of sufficient time to equilibriate the mixture, the vessel was evacuated to 0.5x and 0.1x atmospheric pressure respectively and air introduced until

atmospheric pressure was regained within the system.

Initial concentrations were not checked for each vessel since the Haldane Apparatus used for analysis required gas samples of approximately 10ml., and removal of this volume with subsequent reduction in pressure might have changed the growth pattern of the fungi. This further possible source of variation was not introduced, since a comparison was sought with the effects of fungal metabolites seen in the general survey.

Accuracy of the evacuation method in producing the required atmospheres was checked by first making a high concentration (10%) of CO_2 within the desiccators and reducing this by approximately 0.5 X, i.e. to 5% CO_2 which is a value easily measured with the Haldane Gas Analyser. The results of this test, Table A11 of the appendix, show that the mean concentration of CO_2 attained within the desiccators was 3.5% in place of the 5% expected. Thus, in making up the gas mixtures for subsequent tests a greater volume of CO_2 was introduced initially to allow for this error. Concentrations thus attained were checked by placing B.O.C. fluid in the desiccators and noting that the colour changes corresponded with those on the standard chart.

All tests in the effects of different concentrations of CO_2 were carried out in triplicate. Incubation was in the dark at room temperature of c.19°C.

RESULTS.

Growth of the five assay fungi in the various concentrations of CO_2 was recorded as for Part 1. However, only one measurement could be made for each test so that the period chosen was one seen by experience to give a representative record of the growth responses, i.e.:-

R. sexualis examined after 4 days.

B. cinerea " 5 "

A. niger, C. globosum, S. hirsutum examined after 8 days.

Full results are recorded as Appendix Tables A12 - A16, a summary of these being as follows:-

No increase in linear growth or sporulation was noted in any fungus with the CO_2 concentrations assayed; 0.1, 0.5 and 1.0% CO_2 had no apparent effects on any of the fungi, whilst concentrations $\geq$ 5% inhibited all, but to varying degrees.

DISCUSSION.

CO_2 concentrations of 5% produced reductions in linear growth of the assay fungi comparable with those produced by volatile metabolites of Trichoderma viride and Fomes annosus. However, this concentration did not increase zygospore formation in R. sexualis as did the metabolites of F. annosus; nor did tests with the indicator fluid indicate that this level of CO_2 was attained in the assay chambers.

Concentrations of 1%, 0.5% and 0.2% CO_2 had no effect on the growth of any of the assay fungi, and hence the concentrations thought to be produced by the test fungi in the culture chambers would be ineffective in giving the results observed on Part 1.

Growth responses of the 5 assay species may thus be attributed to the action of more complex organic substances produced by the test fungi rather than to the action of CO_2. Hence more accurate measurements of the actual concentrations of CO_2 produced in the assay chamber was not thought to be justified.

Durbin (1959), investigating the effects of high concentrations of CO_2 on the growth of several soil fungi, reported no morphological differences produced at 20% CO_2, but merely varying degrees of inhibition. The results obtained here agree with this and indicate a wide degree of toleration to the different concentrations assayed. R. sexualis was the most sensitive, being completely inhibited at 20% CO_2 as was

B. cinerea. Brown (1922) did not report complete inhibition of this fungus, but was in agreement that inhibition at 20% was much greater than that at 10% CO_2.

Stereum hirsutum was much more tolerant to high concentrations of CO_2 than any of the other organisms tested, and this may be related to its natural environment as a wood-rotting fungus, a type which Thacker and Good reported to favour concentrations of CO_2 of up to 10%.

B. SOME ORGANIC SUBSTANCES OF LOW BOILING POINT.

REVIEW OF LITERATURE.

It has been established by a number of authors that a variety of simple organic compounds which are inhibitory to the growth of fungi when supplied at high concentration, are capable of stimulating growth when at low concentration.

Duggar (1901) showed that traces of ethyl alcohol stimulated germination of Aspergillus spores whilst Latham (1905), by means of inadequately replicated experimants, demonstrated that chloroform at a concentration of 0.3c.c. per litre increased growth of Sterigmatocystis (Aspergillus) niger. Decreases were noticed at concentrations of 0.5 - 0.6 c.c. per litre whilst 1 c.c. per litre completely inhibited all growth.

In 1922 Brown studied the effects of volatile substances arising from a number of plant tissues on several fungi including Botrytis cinerea, and showed that similar effects were produced by some essential oils, and esters of ethyl alcohol. In particular, ethyl acetate stimulated germination of Botrytis spores when supplied at a dilution of one drop in 50c.c., but was inhibitory at high concentration.

Tomkins (1932) made a thorough study of the effects of a number of volatile substances on the growth of moulds, notably Trichoderma lignorum. Aqueous solutions at dilutions of 0-3% of acetone, chloroform, ether, ethyl alcohol, amyl formate and the lower aldehydes were assayed. Growth of all fungi tested was depressed by

these concentrations, whilst more than 3% ethyl alcohol was said to be sufficient to inhibit growth completely at 25°C.

Nutman and Roberts (1962) surveyed the effects of high dilutions, i.e. 5 - 0.1 p.p.m., of sixteen fungicides on the germination of spores of Colletotrichum coffeanum and Hemileia vastatrix. These compounds, e.g. phenyl mercuric acetate, are highly toxic to the fungi when applied at moderate concentration but at very low concentration are stimulatory to germination. This agrees with the findings of Brown on the stimulatory effect of low concentrations of some substances.

EXPERIMENTAL.

The organic substances tested for action on the growth of the five assay fungi were:-

Ethanol	
Acetone	
Cyclohexene	
1.Octene	
4.Methylpentene.1.	(4.M.P.1)
4.Methyl.1.2.pentene	(4.M.1.2.P.)
2.4.4.Trimethylpentene	(2.4.4.T.M.P.)
2.2.Dimethylbutane	(2.2.D.M.B.)

i.e. some highly volatile compounds which have been reported as biological products or, in the case of the olefines, substances similar in structure to those.

Dilutions of the volatile liquids were made using water for the ethanol and acetone mixtures; and for the others, pure paraffin liquid - a substance exerting no partial pressure of itself. Vapour pressures assayed were those obtained over 25ml. aliquots of the mixtures at dilutions of the volatile liquids of:-

1%, 0.1% and 0.01%.

The method of testing for effects of these on the growth of the assay fungi was essentially that used in the survey of the biological effects in Part 1. - The double Petri dish chamber was employed, with the bottom half containing the dilution of the volatile liquid in place of a test fungus. All tests were made in triplicate and incubated, as before, at 20°C in the dark. Controls were set up

containing 25ml. of the appropriate diluent in place of the volatile mixture.

Measurements of growth of the assay fungi were made as in Part 1, i.e. Rhizopus sexualis

Mean colony diameter and representative numbers of zygospores per plate recorded after 4 days.

Chaetomium globosum

Mean colony diameter recorded after 8 days and perithecial production after 10 days.

Stereum hirsutum

Mean colony diameter recorded after 8 days.

Aspergillus niger

Mean colony diameter and sporulation recorded after 10 days.

Botrytis cinerea

Mean colony diameter recorded after 5 days and sporulation after 7 days.

These results are recorded in full as Tables A17 - A21 of the appendix, and in graph form as figs. 12 - 17.

Fig. 12.

Linear growth of Rhizopus sexualis in presence of volatile substances.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Volatile substances tested, from left to right:-

1%, 0.1% 0.01%, 0.00%(control) Ethanol

" Acetone

" Cyclohexene

" 1.Octene

" 4.Methylpentene.1.

" 4.Methyl.1.2.pentene

" 2.4.4.Trimethylpentene

" 2.2.Dimethylbutane

Fig.12

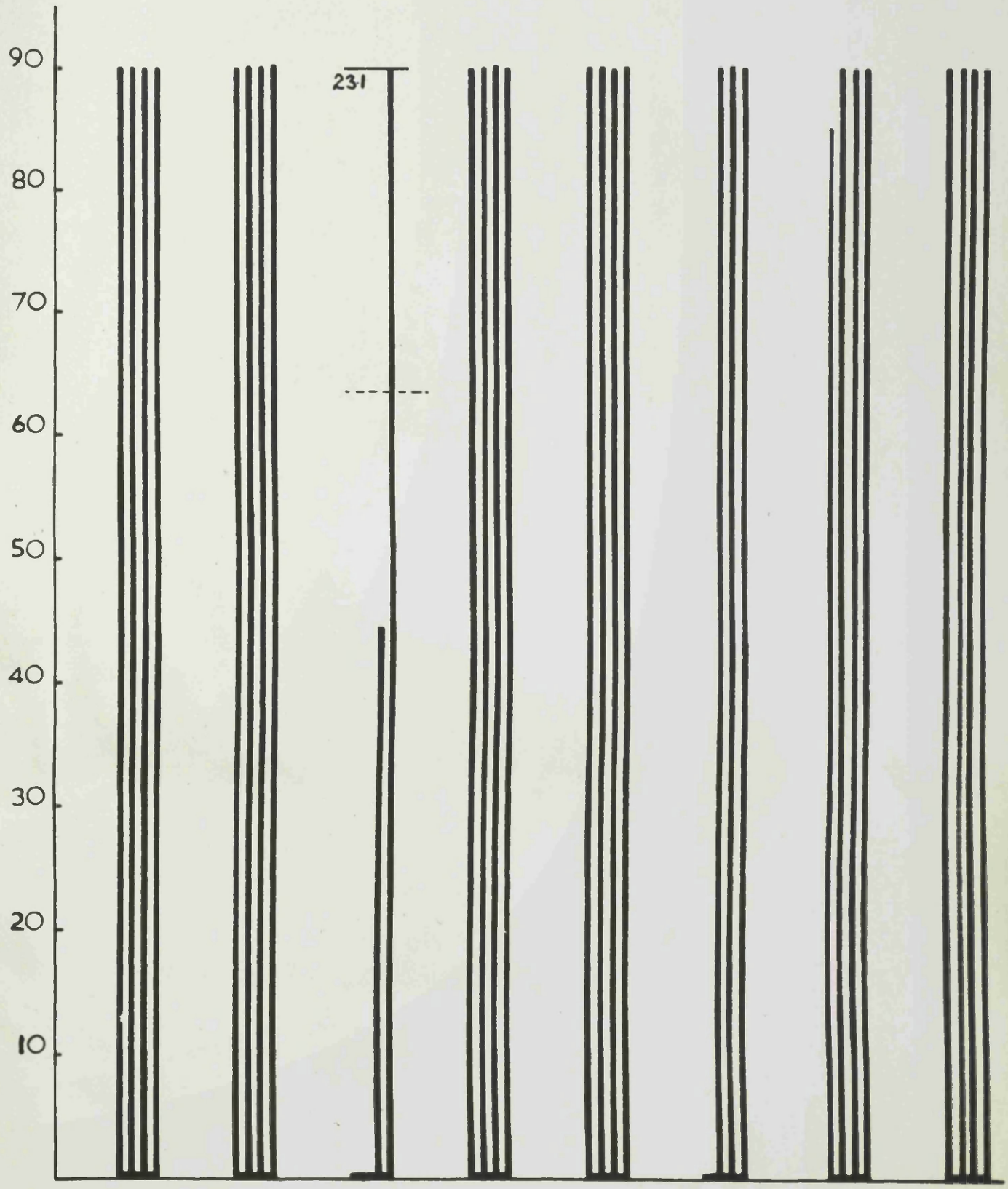


Fig. 13.

Zygospore formation of Rhizopus sexualis in presence of volatile substances.

Vertical axis - Mean representative number of zygospores per plate

Horizontal axis - Volatile substances tested, from left to right:-

1%, 0.1% 0.01%, 0.00%(control)	Ethanol
"	Acetone
"	Cyclohexene
"	1.Octene
"	4.Methylpentene.1.
"	4.Methyl.1.2.pentene
"	2.4.4.Trimethylpentene
"	2.2.Dimethylbutane

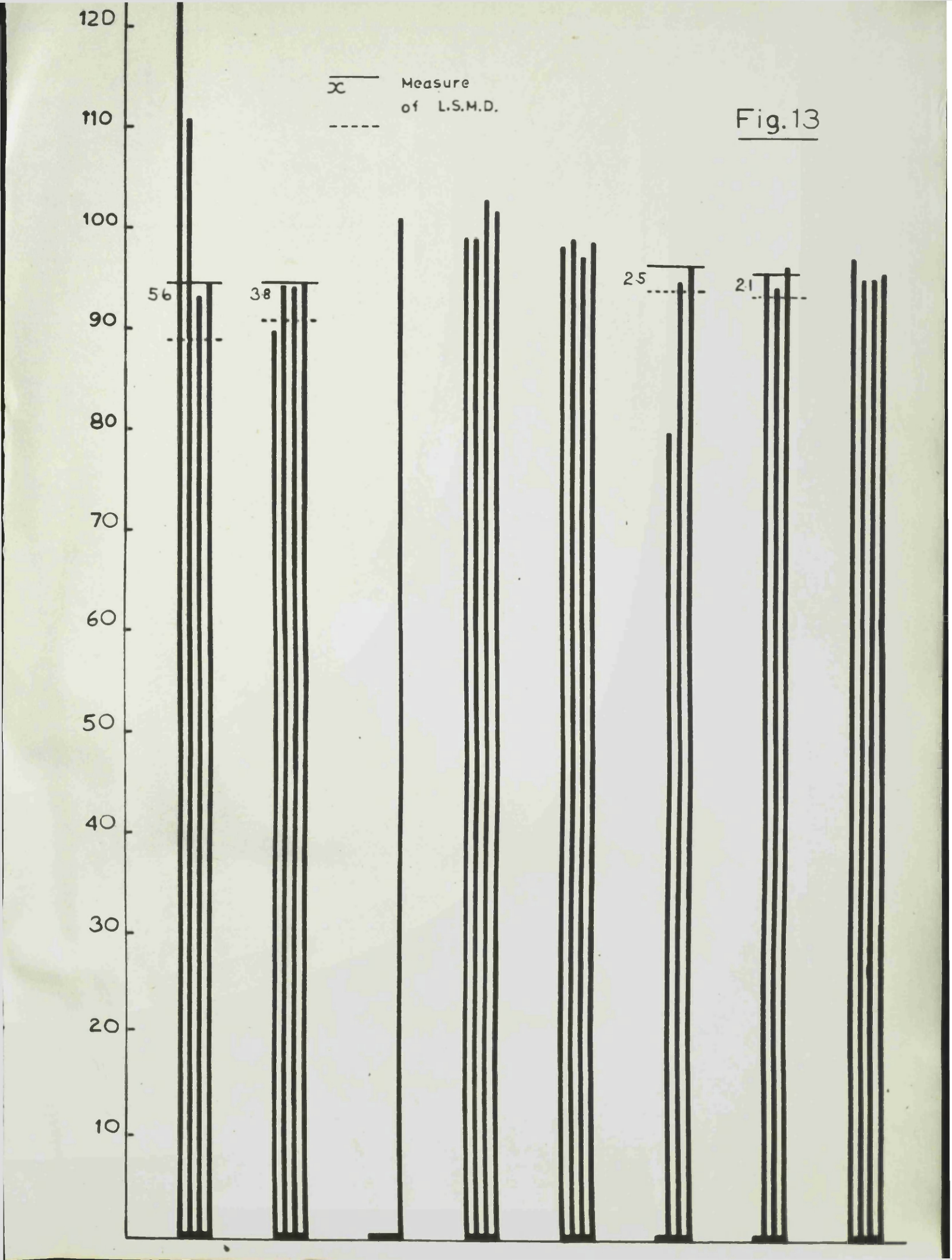


Fig. 13

Fig. 14.

Linear growth of Chaetomium globosum in presence of volatile substances.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Volatile substances tested, from left to right:-

1%, 0.1%, 0.01%, 0.00%(control)	Ethanol
"	Acetone
"	Cyclohexene
"	1.Octene
"	4.Methylpentene.1.
"	4.Methyl.1.2.pentene
"	2.4.4.Trimethylpentene
"	2.2.Dimethylbutane

Fig.14.

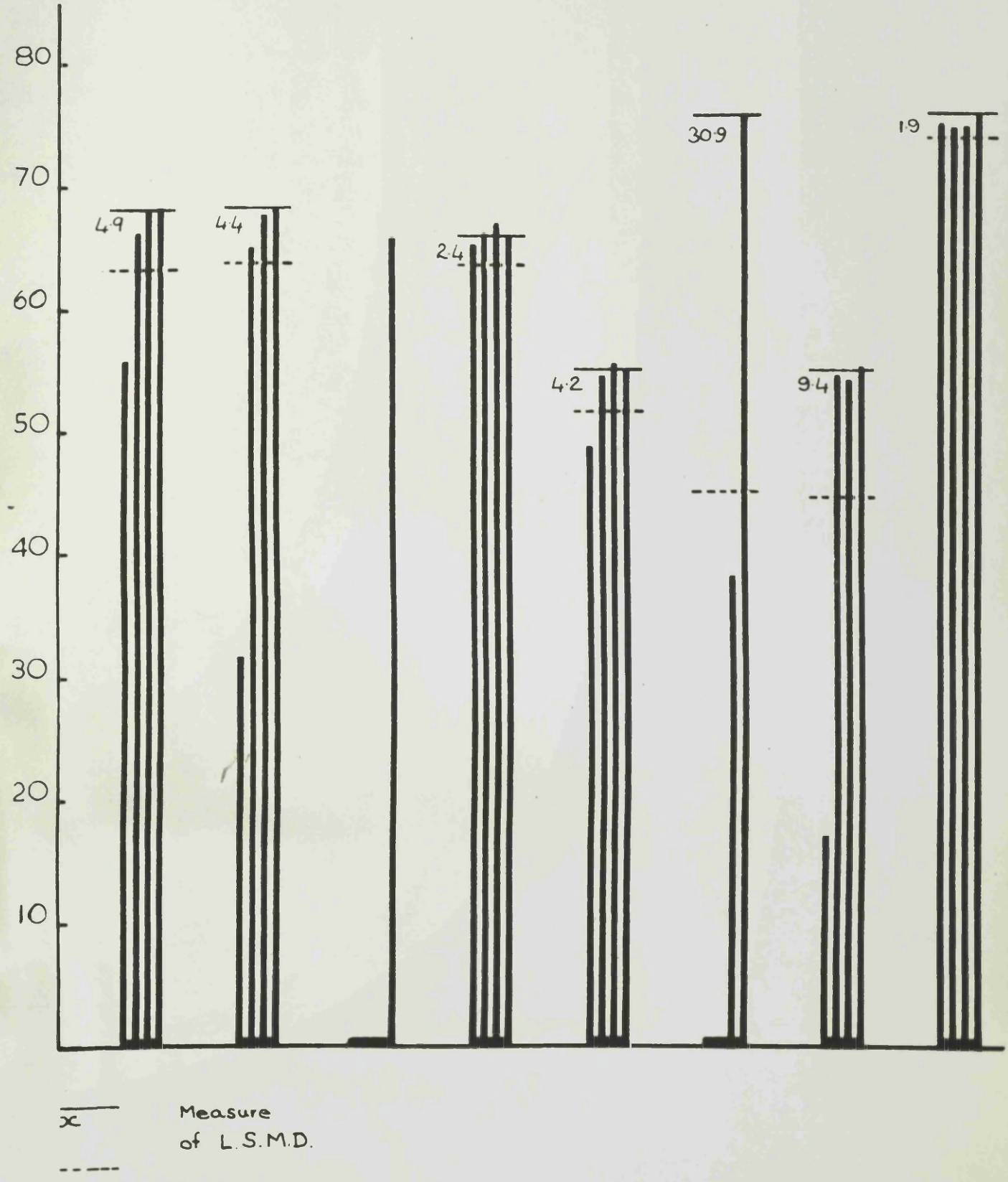


Fig. 15.

Linear growth of Stereum hirsutum in presence of volatile substances.

Vertical axis: - Mean colony diameter, mm.

Horizontal axis - Volatile substances tested, from left to right:-

1%, 0.1%, 0.01%, 0.00%(control)	Ethanol
"	Acetone
"	Cyclohexene
"	1.Octene
"	4.Methylpentene.1.
"	4.Methyl.1.2.pentene
"	2.4.4.Trimethylpentene
"	2.2.Dimethylbutane

Fig.15.

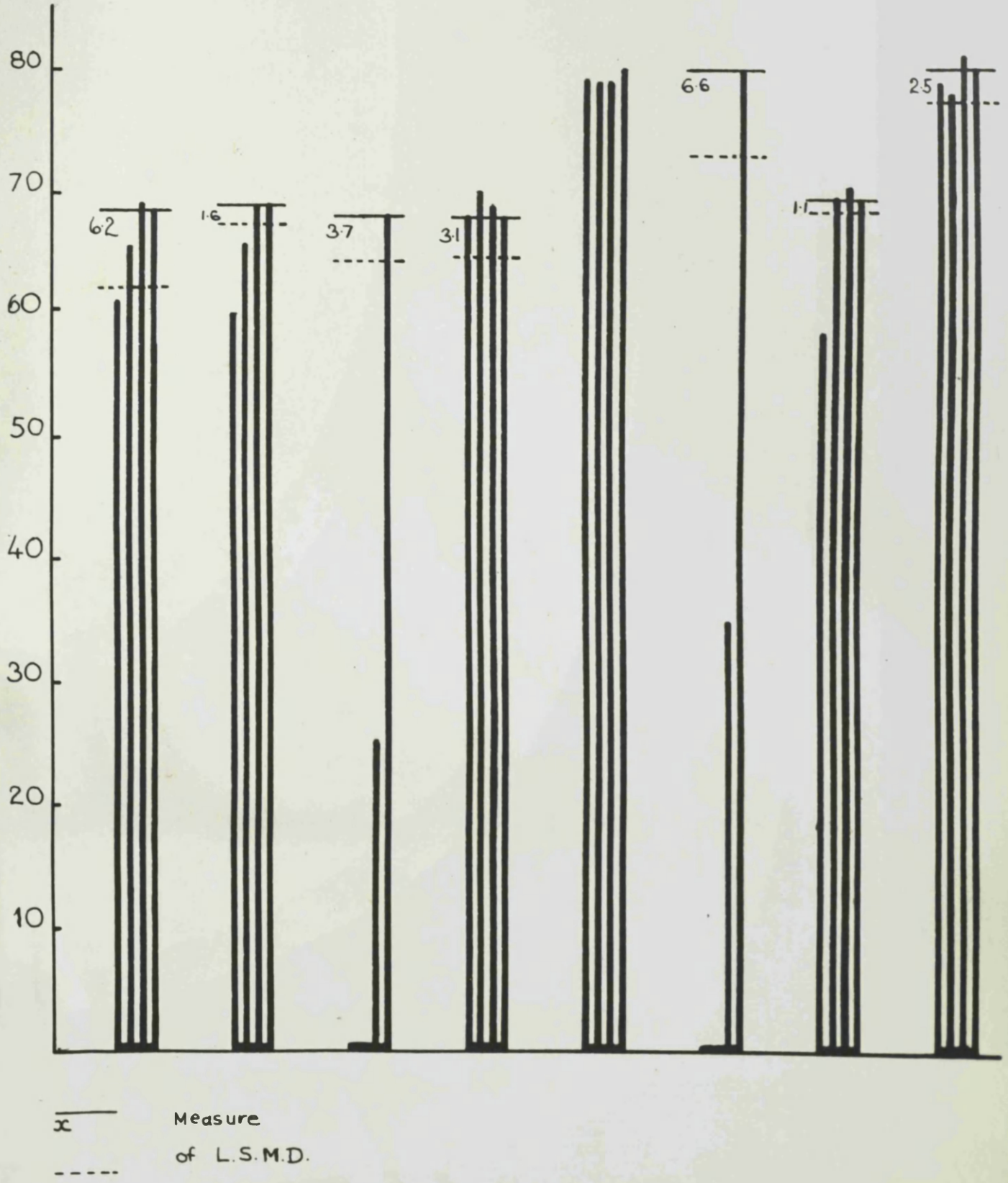


Fig. 16.

Linear growth of Aspergillus niger in presence of volatile substances.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Volatile substances tested, from left to right:-

1%, 0.1%, 0.01%, 0.00%(control) Ethanol

" Acetone

" Cyclohexene

" 1.Octene

" 4.Methylpentene.1.

" 4.Methyl.1.2.pentene

" 2.4.4.Trimethylpentene

" 2.2,Dimethylbutane

Fig.16.

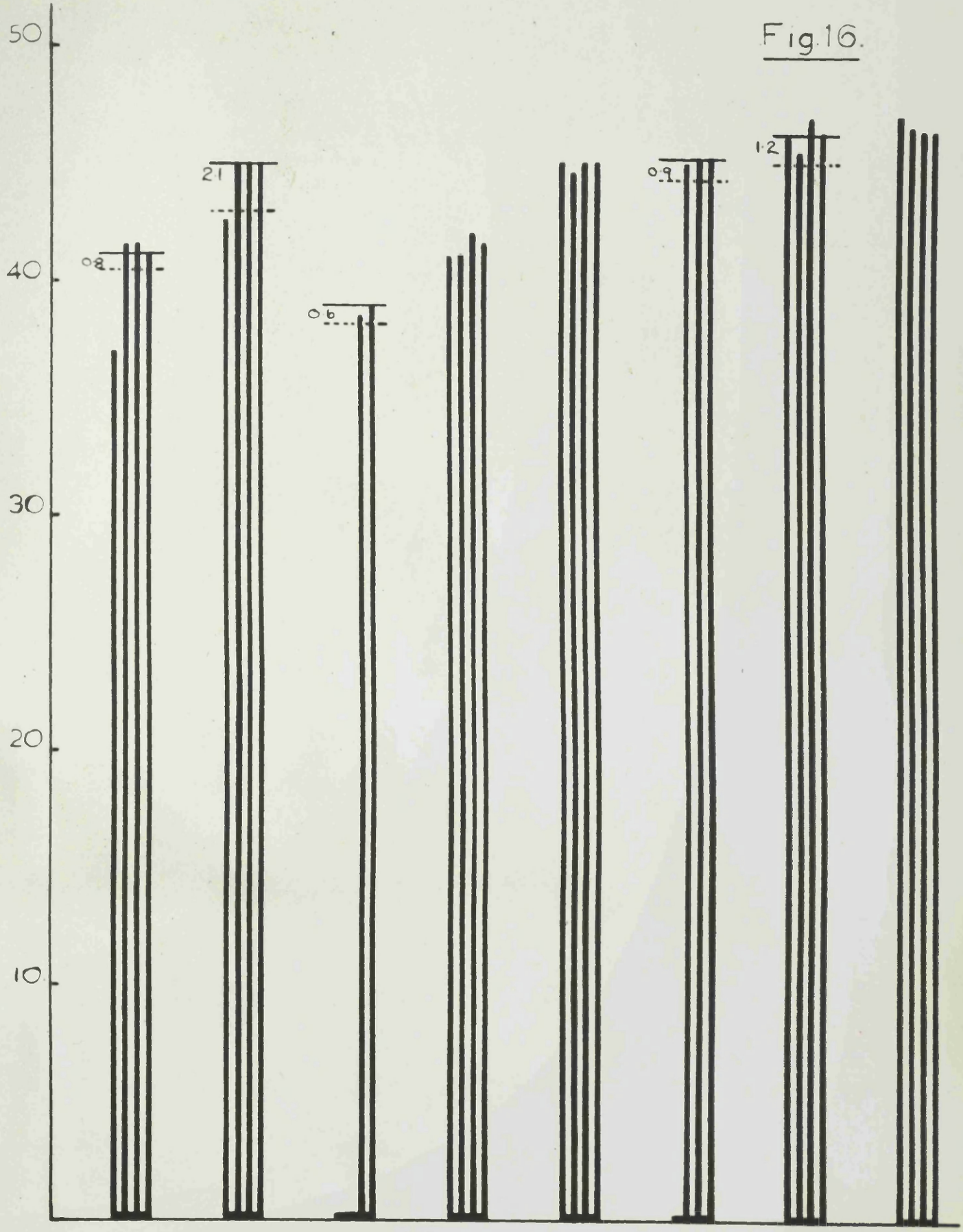


Fig. 17.

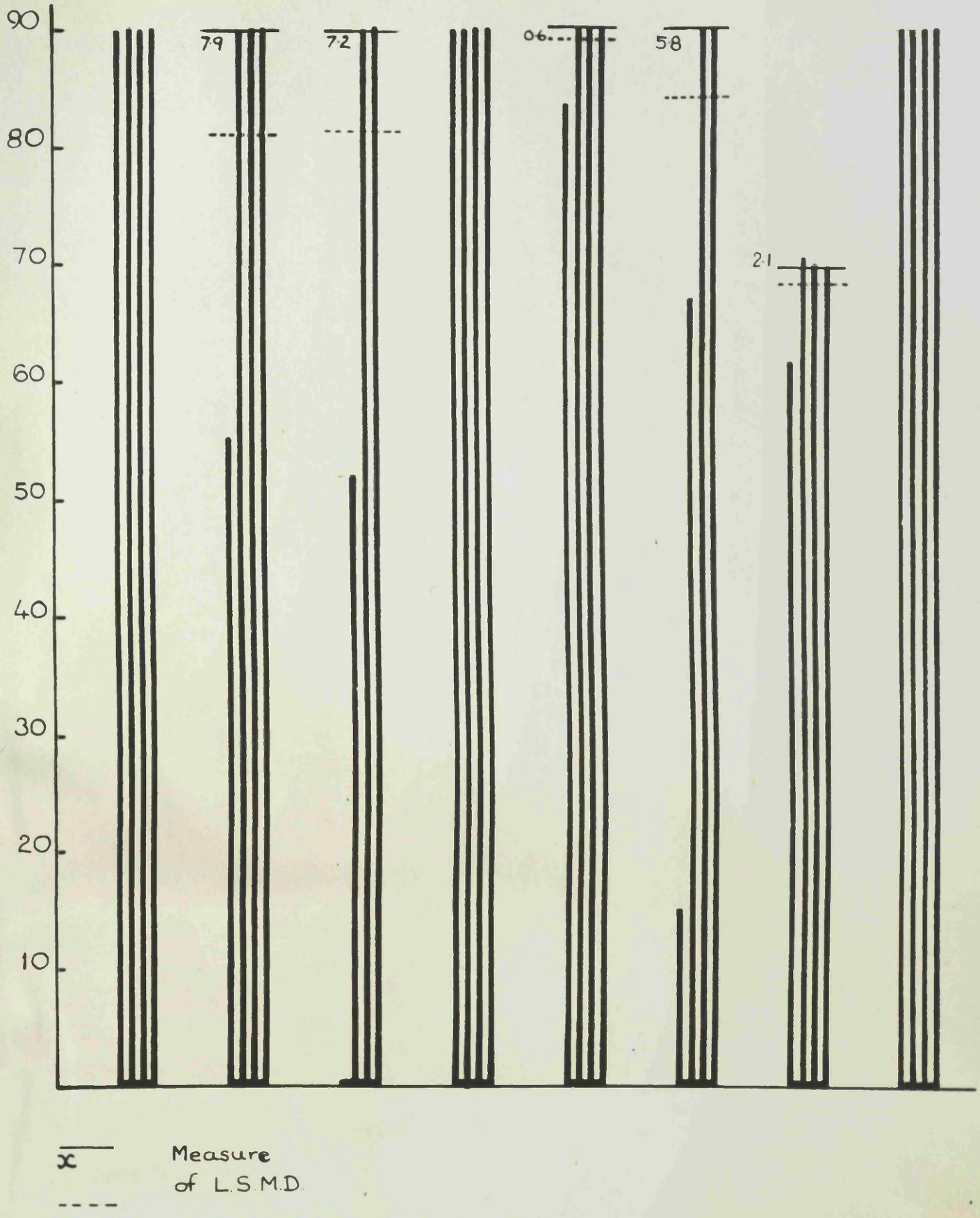
Linear growth of Botrytis cinerea in presence of volatile substances.

Vertical axis - Mean colony diameter, mm.

Horizontal axis - Volatile substances tested, from left to right:-

1%, 0.1%, 0.01%, 0.00%(control)	Ethanol
"	Acetone
"	Cyclohexene
"	1.Octene
"	4.Methylpentene.1.
"	4.Methyl.1.2.pentene
"	2.4.4.Trimethylpentene
"	2.2.Dimethylbutane

Fig. 17



SUMMARY OF RESULTS.

R. sexualis.

Linear growth as measured by colony diameter was not affected by ethanol, acetone, 1.octene, 4MP1 or 2.2DMB; although 1% acetone appeared to enhance the production of aerial mycelium. No substance increased linear growth, whilst cyclohexene inhibited growth completely at 1% and 0.1%, and by c.45% at concentration of 0.01%. 4M.1.2.P. inhibited growth only at 1%, whilst 2.4.4.TMP gave c.10% inhibition at 1%.

Zygospore formation was not affected by 1.Octene, 4.MP.1, or 2.2.DMB at the dilutions tested. Increased production was brought about only by ethanol, at dilutions of 1% and 0.1%.

In most other cases zygospore production was directly proportional to amount of vegetative mycelium produced, except in the cases of 0.01% cyclohexene, where a very dense central zone of sexual spores was formed; and 0.1% 4M.1.2.P, in which there was a decrease in numbers of zygospores unrelated to colony diameter.

C. globosum.

Linear growth was not affected by 1.Octene or 2.2.DMB at the concentrations tested. No increases in colony diameter were evident by the action of any volatile. Cyclohexene inhibited all growth at

the three concentrations used, whilst 4.M.1.2.P gave complete inhibition at 1% and 0.1% with 50% inhibition at 0.01%. Acetone, 4.MP.1 and 2.4.4.TMP all inhibited to some degree at a concentration of 1%. - In the case of the latter there was a marked lag period before the inoculum began to grow, i.e. no growth occurred until after the 4th day.

Fruit body formation was not affected by ethanol, 1.Octene or 2.2.DMB. 1% acetone was the only compound which increases numbers of perithecia formed, giving a dense central zone. Otherwise formation of perithecia was proportional to colony diameter.

S. hirsutum.

Colony diameter was not affected by any concentrations of 1.Octene, 4.MP.1 or 2.2.DMB. No increases in linear growth were recorded. Cyclohexene and 4.M.1.2.P inhibited growth completely at 1% and 0.1%, whilst 0.01% gave c.55% inhibition in both cases. Concentrations of 0.01% of these volatiles also delayed any growth of the inoculum until after the 4th day of the assays. Ethanol and acetone depressed growth at concentrations of 1% and 0.1%, whilst 1% 2,4,4,TMP had a similar effect.

A. niger.

1.Octene, 4.MP.1, 2.4.4.TMP and 2.2.DMB had no effect on linear growth of this fungus.

No volatile substance increased colony diameter.

Cyclohexene at 1% and 0.1% completely inhibited all growth, as did 1% of 4.M.1.2.P. Ethanol and acetone at 1% produced some inhibition of linear growth.

Sporulation was not affected by any concentrations of ethanol, acetone, 1.Octene, 4.MP.1, 2.4.4.TMP or 2.2.DMB.

No increased spore production was apparent, nor was any decrease seen except in so far as all growth was suppressed by some concentrations of cyclohexene and 4.M.1.2.P.

B. cinerea.

Linear growth patterns were not affected by ethanol, 1.Octene or 2.2.DMB.

No increase in colony diameter was observed with any of the three concentrations of all substances tested.

1% cyclohexene was the only compound tested which inhibited all growth completely, whilst 0.1% of this decreased linear growth by about 40%. 4.M.1.2.P allowed only slight growth at 1% and also gave some decrease at 0.1%; whilst acetone and 4.MP.1 also decreased linear growth.

Sporulation was not affected by any concentrations of ethanol or 2.2.DMB.

1% acetone and 0.1% cyclohexene and 4.M.1.2.P increased

sporulation by c.25%. Conidial heads were widespread over the plates as opposed to the peripheral production in the controls.

0.1% 1.Octene gave a decrease in sporulation of c.25% without apparently affecting linear growth. Otherwise, sporulation was decreased proportionately to colony diameter.

DISCUSSION.

Overall, patterns of effects on the five assay species were similar to previous tests, with Stereum being the most resistant and Botrytis the most susceptible to the effects of volatile substances.

The olefine 1.Octene and paraffin 2.2.DMB were the only compounds having no effect on the linear growth of any assay species. They affected sporulation only in Botrytis, where a decrease was noted.

As noted in the general survey, inhibition of linear growth was in many cases accompanied by increase in sporulation, which might be interpreted as physiological ageing of the fungus.

Cyclohexene and 4.M.1.2.P were the only substances inducing strong inhibition of growth in all species. Some inhibition of most species was brought about by concentrations of 1% of the majority of volatiles. The exception was ethanol which, although inhibiting linear growth of C. globosum, S. hirsutum and A. niger, increased zygospore formation in R. sexualis without a corresponding increase in colony diameter.

Duggar and Tomkins both reported inhibition of fungal growth at the concentrations tested here, whereas slight traces of ethanol were stimulatory. Hence, once again sexual reproduction in Rhizopus reacted in a different manner from all other aspects of growth studied. Concentrations of ethanol higher than 1% were not tested, but it is possible that higher concentrations would be inhibitory to zygospore

formation also.

The inhibitory actions of the majority of volatiles tested are merely reductions in the general rate of linear growth. These are thus in accordance with the findings of Tomkins on the effects of acetone and ethanol on the growth of Trichoderma lignorum, i.e. that growth was as constant as, but slower than the controls. However, 2.4.4.T.M.P. on Chaetomium, and 4.M.1.2.P. and cyclohexene on Stereum induced a lag period before growth commenced. This is similar to the effect of the higher aldehydes on Trichoderma.

A pointer to the specificity of reaction of biological material to 'active' compounds is seen from the fact that 4.MP.1 had little effect on the total growth of all species whilst 4.M.1.2.P was strongly inhibitory to most and 2.4.4.TMP was intermediary in effect. Thus the substitution of a second methyl group seems to render the pentene more toxic, whilst a third one modifies the effect.

In general, little correlation could be deduced between the effects of various concentrations of these known substances and those seen by action of volatile fungal metabolites. However, although no direct comparison can be made, it is evident that low concentrations of organic substances such as must be produced by the fungi can have effect on the growth of the assay organisms.

GENERAL CONCLUSION.

The general survey of a number of fungal species made in Part 1 of this work indicates that the production of volatile fungal metabolites is widespread, and that these may affect various aspects of growth of fungi. Approximately 30% of the total number of species tested showed some action against at least one of the assay fungi, but the number affecting more than two of these was small. In general the trend was for inhibitory rather than stimulatory activity. It was not possible to deduce any obvious taxonomic relationships between those fungi shown to produce 'active' metabolites.

Delicate techniques were used in analysis of the volatile metabolites, and the fact that these produced no clearly identifiable data is thought to indicate that the metabolites were active in very low concentrations. The level of activity is probably similar to that of the growth substances of the higher plants.

In the tests of synthetic metabolites it was found that concentrations of more than 5% CO_2 inhibited the growth of the assay fungi to varying degrees. However, no evidence was seen that any of the range of fungal species tested produced these concentrations of CO_2 within the assay chambers. Hence it was assumed that CO_2 production could not account for all the reactions which were observed. Tests of concentrations of 0.01 to 1% of ethanol, acetone and a number of the

lower olefines showed that these all affected the growth of the assay species to some extent; but no parallels could be drawn between the action of these substances and those of the test fungi.

It seemed logical in this research project to use the one test species, R. sexualis, in the investigation of techniques for analysis of fungal volatiles. However, now that these methods have been perfected from the biological standpoint, it would be of interest to widen the study and analyse the metabolites shown to be produced by a range of fungi. Any chemical similarities or differences between these could then be defined and possibly help in the elucidation of the mode of action of volatile substances in the growth of fungi.

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Choanephora circinans (Nag. & Kawa.) Hesselt. & Benj.	C.M.I. 74,524.
Cunninghamella echinulata Thaxt.	C.M.I. 25,327.
Mortierella ramanniana (Moller) Linn.	*C.V.S.
Mucor hiemalis + Wehm.	C.M.I. 21,216.
Mucor hiemalis - Wehm.	C.M.I. 21,217.
Mucor plumbeus Bon.	C.M.I. 14,741.
Piptocephalis cylindrica Bain.	C.M.I. 17,745(a).
Pythium ultimum Trow.	Dr. Hadley, 1961.
Rhizopus sexualis (Smith) Callen.	Dr. Joy, 1960.
Rhizopus stolonifer + (Ehrenb. ex Fr.)	C.M.I. 57,761.
Rhizopus stolonifer - (Ehrenb. ex Fr.)	C.M.I. 57,762.
Zygorhyncus moelleri Vuill.	C.M.I. 47,187.
ASCOMYCETES.	
Byssochlamys fulva Olliver & Smith.	C.M.I. 58,422.
Ceratocystis coerulescens (Münch) Bakshi.	C.M.I. 32,391.
Chaetomium globosum Kunze ex Fr.	Dr. Elliott, 1956.
Chromocrea spinulosa (Fuckel) Petch ex Brooks & Math.	C.M.I. 39,915.
Cordyceps militaris (L.) Link.	Dr. Hutchinson, 1960.
Cordyceps ophioglossoides	" 1960.
Gibberella fujikuroi (Sawada) Wollenw.	Boots Research Lab. 917.
Gymnoascus subumbrinus A.L.Smith & Ramsb.	C.M.I. 41,112.
Nectria haematococca Berk & Br.	C.M.I. 76,761.
Ophiobolus graminis Sacc.	Dr. Elliott, 1962.
Pericystis apis Maasen ex Claussen	C.M.I. 35,650.
Pleospora androsaces Fuckel.	Dr. Elliott, 1959.
Pyronema domesticum (Sow ex Fr.) Sacc.	C.M.I. 57,472.
Saccharomyces cerevisiae Meyen ex Hansen.	Grey Owl Res. Stn., 1960.
Shizosaccharomyces octosporus Beijer.	C.M.I. 23,657.
Sordaria fimicola (Rob.) Ces. & de Not.	Dr. Elliott, 1960.

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Agaricus campestris L. ex Fr.	Pinkerton, 1960.
Armillaria mellea (Vahl. ex Fries) Kummer.	C.M.I. 86,070.
Fomes annosus (Fr.) Cooke.	For. Prod. Res. Lab., 1958.
Merulius lacrymans (Jacq.) Fr.	Dr. Hutchinson, 1961.
Polyporus sulphureus (Bull.) Fr.	For. Prod. Res. Lab., 1958.
Stereum hirsutum (Willd.) Fr.	Dr. Hadley, 1959.

FUNGI IMPERFECTI.

Acremoniella atra (Corda) Sacc.	C.M.I. 80,523.
Alternaria sp. (tenuis group).	C.M.I. 72,726.
Aspergillus fumigatus Fresen.	Lond. School of Trop.Med., 1960.
Aspergillus niger van Tiegh.	Dr. Hood, 1957.
Aspergillus terreus Thom.	Glaxo Labs. Ltd., 1958.
Botrytis allii Munn.	C.M.I. 61,987.
Botrytis cinerea Pers ex Fr.	R.K.S. Wood, 1953.
Botrytis fabae Sardina.	C.M.I. 50,374.
Botrytis spectabilis Harz.	C.M.I. 60,069.
Botrytis tulipae Lind.	C.M.I. 76,834.
Caldariomyces fumago (Woron)	C.V.S.
Carpenteles sp.	Dr. Gentles, 1959.
Cephalosporium cretatum	
Cladosporium herbarum (Pers) Link ex Fr.	C.M.I. 49,630.
Colletotrichum lindemuthianum (Sacc. & Magn.) Bri. & Cav.	C.M.I. 63,364.
Curvularia lunata (Wakker) Boedijn.	C.M.I. 77,020,
Fusarium culmorum (W.G. Smith) Sacc.	C.M.I. 35,099.
Gliochladium roseum Bain.	C.M.I. 59,284.
Hansfordia grisella (Sacc.) Hughes.	C.M.I. 20,743.
Helminthosporium sativum Link ex Fr.	C.M.I. 21,191.
Penicillium claviforme Bain.	Mus.Nat.Hist. Paris, 1958.
Penicillium digitatum Sacc.	C.M.I. 39,748.
Pullularia pullulans (de Bary) Berkh.	C.M.I. 70,103.
Rhodotorula rubra (Demne) Lodder.	C.M.I. 38,784.
Thielaviopsis basicola (Berk.&Br.) Ferr.	Glaxo Labs. Ltd., 1958.
Tilachlidium nigrum	Dr. Hutchinson, 1953.
Trichoderma koningi Oudem.	C.M.I. 73,022.
Trichoderma viride Pers ex Fr.	Dr. Hadley, 1957.
Trichothecium roseum (Pers.) Link ex Fr.	R.W. Richards, 1961.
Verticillium psalliotae Treschaw	C.M.I. 90,861,
Verticillium sp.	Dr. Hood, 1959.

+C.M.I. = Commonwealth Mycological Institute.

*C.V.S. = Centraalbureau Voor Schimmelcultures.

APPENDIX A.

TABLE A1.

Assessment of accuracy in recording zygospore numbers in R. sexualis.

Plate No.	Replicate No.	No. of zygospores per unit area.	Total/5 fields.
8	1	17 18 21 17 15	88
	2	17 17 19 17 17	87
	3	18 16 18 19 19	90
1	1	16 17 16 21 17	87
	2	17 16 17 16 19	87
	3	14 17 21 17 18	85
3	1	19 18 17 18 19	91
	2	22 15 21 19 21	98
	3	20 17 19 17 17	90
10	1	16 18 18 19 18	89
	2	17 16 21 15 18	87
	3	17 20 16 21 17	91
2	1	19 15 16 18 17	85
	2	16 15 20 16 19	88
	3	18 16 20 20 14	88
7	1	18 22 16 18 19	93
	2	21 17 18 15 20	91
	3	20 17 20 18 20	95
9	1	15 18 17 17 16	93
	2	16 20 19 17 19	91
	3	17 17 19 20 18	91
6	1	19 17 19 19 16	90
	2	17 18 20 17 17	88
	3	14 18 19 16 20	87
5	1	17 18 20 17 17	89
	2	19 18 16 18 17	88
	3	16 19 18 17 18	88
4	1	16 19 20 18 17	90
	2	16 18 17 20 17	88
	3	18 17 17 18 20	90

TABLE A2.

The effect of metabolites of R. sexualis on zygospore production of the same fungus as assay organism.

a. Malt peptone medium.

Plate No.	Number of zygospores per unit area: Test fungus present	Rep.Tot. per plate	Test fungus absent	Rep.Tot. per plate
1.	47 48 44 45 46	230	35 32 36 37 34	174
2.	45 42 44 46 46	223	38 32 34 36 37	177
3.	41 42 44 45 43	215	39 34 35 36 34	178
4.	48 41 44 43 47	223	35 34 37 33 34	175
5.	44 43 42 45 44	218	37 33 34 35 36	175
6.	41 47 45 46 44	223	34 33 35 35 33	170
7.	42 42 41 46 45	221	33 35 34 35 35	172
8.	46 44 47 45 43	225	34 35 33 36 37	176
9.	43 41 44 42 46	216	35 36 37 35 37	180
10.	44 41 45 43 46	219	33 34 35 34 35	171
Total		2213		1748

Between tests variance ratio F_9^1 = 540, i.e. significantly greater than 5.1 required for 0.05% level.

TABLE A3.

The effect of metabolites of R. sexualis on zygospore production of the same fungus as assay organism.

b. Malt medium.

	Number of zygospores per unit area			
Plate No.	Test fungus present	Rep.Tot. per plate	Test fungus absent	Rep.Tot. per plate
1.	25 28 26 25 26	130	18 20 20 22 19	99
2.	23 28 29 24 27	131	20 18 19 19 22	98
3.	25 28 26 24 27	140	21 20 20 20 18	99
4.	24 28 25 25 27	139	18 20 18 19 20	95
5.	24 24 27 24 27	126	21 20 20 18 19	98
6.	25 24 28 25 26	128	19 19 20 19 23	100
7.	26 24 27 28 26	131	19 20 20 21 20	100
8.	27 26 26 27 25	131	20 18 17 21 21	97
9.	25 27 24 25 25	126	18 21 21 20 18	98
10.	25 28 26 25 25	129	19 18 20 20 20	97
Total		1311		981

Between tests variance ratio F_9^1 = 8.4, i.e. significantly greater than 5.1 required for 0.05% level.

TABLE A4.

Effect of volatile metabolites on the growth of fungi.

ASSAY SPECIES - Rhizopus sexualis

Expt. No.	Test species	Representative no. of zygos./plate after 4 days.			Mean
1.	Control	119	107	130	115.3
	Chaetomium globosum	108	124	115	115.6
2.	Control	107	99	92	99.3
	Agaricus campestris	110	112	117	113
3.	Control	79	84	80	81
	Rhizopus sexualis	129	116	123	122.6
	Botrytis cinerea	118	103	110	110.3
4.	Control	103	97	93	97.6
	Polyporus sulphureus	109	98	86	97.6
	Caldariomyces fumago	97	98	93	96
5.	Control	89	87	88	88
	Mucor plumbeus	119	92	120	110.3
	Saccharomyces cerevisiae	94	86	88	89.3
6.	Control	90	91	91	90.6
	Pyronema domesticum	87	93	95	91.6
	Trichothecium roseum	88	90	88	88.6
	Penicillium digitatum	85	88	90	87.6
7.	Control	100	98	103	100.3
	Trichoderma viride	72	74	74	73.3
	Penicillium claviforme	92	93	88	91
	Aspergillus fumigatus	95	97	107	97.6
	Sordaria fimicola	100	100	103	101
	Ceratocystis coerulescens	127	110	118	118.6
8.	Control	95	91	96	94
	Aspergillus terreus	92	95	95	94
	Thielaviopsis basicola	113	113	117	114.3
	Gibberella frujikuroi	115	110	111	112
	Cladosporium herbarum	93	94	95	94
9.	Control	99	94	94	95.6
	Mucor hiemalis +	107	98	100	99.6
	Mucor hiemalis -	99	94	104	99
	Pythium ultimum	93	97	104	98
	Plecospora androgyna	101	101	98	100
	Piptocephalis cylindrospora	100	100	94	98
	Shizosaccharomyces octosporus	98	95	102	98.3
	Helminthosporium sativum	113	114	119	115.3
	Fusarium culmorum	100	94	97	97
	Verticillium sp.	98	97	93	96
	Alternaria tenuis	98	95	100	97.6
	Pericystis apis	99	100	99	99.3
10.	Control	101	96	98	98.3
	Stereum hirsutum	97	98	100	98.3
	Tilachlidium nigrum	101	100	100	100.3
	Byssochlamys fulva	100	95	100	98.3
	Ophiobolus graminis	99	100	101	100.0
	Carpenteles sp.	99	96	98	97.6
	Armillaria mellea	87	86	87	86.6
	Cephalosporium cretatum	100	98	100	99.3
	Verticillium psalliotae	101	101	100	100.6
11.	Control	95	94	98	95.6
	Botrytis tulipae	97	94	95	96
	Botrytis spectabilis	112	118	114	114.6
	Botrytis fabae	97	96	96	96.3
	Choanephora circinans	98	99	96	97.6
	Curvularia lunata	98	95	94	95.6
	Trichoderma koningi	81	82	82	81.6
	Rhodotorula rubra	96	96	98	96.6
	Acremoniella atra	79	86	87	84
12.	Control	95	97	97	96.3
	Colletotrichum lindemuthianum	101	100	99	100
	Cunninghamella echinulata	100	102	98	100
	Nectria haematococca	96	96	95	95.6
13.	Control	94	94	95	94.3
	Hansfordia grisella	94	94	93	93.6
	Cordyceps militaris	121	120	126	122.3
	Rhizopus stolonifer -	96	94	95	95
14.	Control	93	96	95	94.8
	Fomes annosus	124	126	125	125
	Botrytis allii	99	98	97	98
	Abaidia spinosa	102	107	101	103.3
	Cordyceps ophioglossoides	95	96	96	95.6
	Rhizopus stolonifer +	99	99	101	99.6
	Pullularia pullulans	98	95	96	96.3
15.	Control	93	96	95	94.6
	Aspergillus niger	100	93	97	96.6
	Merulius lacrymans	97	99	98	98.0
16.	Control	123	122	123	122.6
	Mortierella rumaniana	124	124	125	124.3
	Zygorhyncus moelleri	123	123	123	123.0

TABLE A5.

Effect of volatile metabolites on the growth of fungi.

ASSAY SPECIES - Chaetomium globosum

Expt. No.	Test species	Colony diameter after 8 days			Mean	Perithecial formation after 10 days
1.	Control	59	59	59	59	3+
	Chaetomium globosum	55	59	59	57.6	3+
	Botrytis cinerea	59	59	59	59	3+
	Saccharomyces cerevisiae	54	30	45	43	+
2.	Control	65	64	66	65	3+
	Mucor plumbeus	65	67	66	66	2+
	Aspergillus niger	66	63	64	64.3	3+
3.	Control	53	50	52	51.6	3+
	Zygorhyncus moelleri	50	50	51	50.3	3+
	Tilachlidium nigrum	50	50	51	50.3	3+
	Agaricus campestris	53	52	52	52.3	3+
4.	Control	56	56	56	56	3+
	Cordyceps ophioglossoides	61	61	61	61	3+
	Cladosporium herbarum	57	56	55	56	3+
	Stereum hirsutum	57	60	61	59.3	3+
	Sordaria fimicola	57	58	61	59.3	3+
	Aspergillus terreus	54	58	56	56	3+
	Gibberella frujikuroi	56	60	59	58.3	3+
	Carpenteles sp.	65	64	64	64.3	3+
	Pericystis apis	57	58	58	57.6	3+
	Polyporus sulphureus	56	56	56	56	3+
	Ceratocystis coerulescens	57	53	53	54.3	+
	Pyronema domesticum	55	55	58	56	3+
	Penicillium claviforme	56	60	56	57.3	3+
5.	Control	67	68	66	67	3+
	Colletotrichum lindemuthianum	62	64	57	61	3+
	Helminthosporium sativum	65	65	66	65.3	3+
	Alternaria tenuis	66	66	66	66	3+
	Fusarium culmorum	67	66	66	66.3	3+
	Byssochlamys fulva	60	60	55	58.8	3+
	Ophiobolus graminis	65	70	65	66.6	3+
	Verticillium psalliotae	66	66	64	65.3	3+
6.	Control	60	64	60	61.3	3+
	Aspergillus fumigatus	61	60	60	60.3	3+
	Acremoniella atra	63	63	62	62.6	3+
7.	Control	64	62	63	63	3+
	Choanephora circinans	64	63	64	63.6	3+
	Rhodotorula rubra	63	63	63	63	3+
	Trichoderma koningi	62	63	64	63	3+
	Schizosaccharomyces octosporus	59	60	58	59	3+
	Fomes annosus	44	46	45	45	2+
8.	Control	55	55	51	53.6	3+
	Pleospora androgyna	55	54	53	54	3+
	Botrytis allii	46	50	50	48.6	3+
	Rhizopus stolonifer +	57	56	55	56	3+
	Mucor hiemalis +	56	56	56	56	3+
	Mucor hiemalis -	53	49	54	52	3+
	Cephalosporium cretatum	48	52	46	48.6	3+
	Pythium ultimum	52	53	49	51.3	3+
	Cunninghamella echinulata	54	52	53	53	3+
9.	Control	62	63	60	61.6	3+
	Trichoderma viride	47	46	49	47.3	3+
	Botrytis spectabilis	63	66	63	64	3+
	Botrytis tulipae	63	61	58	60.6	3+
	Armillaria mellea	60	64	63	62.3	3+
	Verticillium sp.	63	64	62	63	3+
	Penicillium digitatum	63	65	63	63.6	3+
	Hansfordia griselia	59	60	59	59.3	3+
	Curvularia lunata	62	62	57	60.3	3+
	Merulius lacrymans	60	57	61	59.3	3+
	Nectria haematococca	64	63	57	61.3	3+
10.	Control	53	52	53	52.6	3+
	Caldariomyces fumago	50	50	50	50	3+
	Trichothecium roseum	53	53	53	53	3+
11.	Control	53	51	53	52.3	3+
	Rhizopus sexualis	53	53	53	53	3+
	Thielaviopsis basicola	54	50	53	52.3	3+
	Pullularia pullulans	52	53	54	53	3+
	Absidia spinosa	49	48	50	49	3+
	Piptocephalis cylindrospora	53	55	52	53.3	3+
12.	Control	68	68	67	67.6	3+
	Mortierella ramanniana	67	67	68	67.3	3+
13.	Control	55	55	57	55.6	3+
	Cordyceps militaris	56	53	56	55.0	3+
	Rhizopus stolonifer -	57	54	55	55.3	3+
	Botrytis fabae	56	56	55	55.6	3+

TABLE A6.

Effect of volatile metabolites on the growth of fungi.

ASSAY SPECIES - Stereum hirsutum

Expt. No.	Test species	Colony diameter after 8 days			Mean
1.	Control	74	73	73	73.3
	Carpenteles sp.	72	72	71	71.6
	Shizosaccharomyces octosporus	70	70	69	69.6
	Aspergillus terreus	70	71	70	70.3
	Verticillium sp.	72	72	72	72
	Fusarium culmorum	72	73	73	72.6
	Zygorhyncus moelleri	72	72	72	72
	Pythium ultimum	75	75	75	75
	Cunninghamella echinulata	70	71	71	70.6
	Gibberella fujikuroi	72	72	72	72
	Cladosporium herbarum	72	72	72	72
	Penicillium digitatum	74	73	72	73
	Thlachlidium nigrum	67	68	67	67.3
	Mucor plumbeus	71	71	72	71.3
2.	Control	73	74	73	73.3
	Helminthosporium sativum	73	73	73	73
	Saccharomyces cerevisiae	68	68	67	67.6
	Sordaria fimicola	74	73	73	73.3
	Stereum hirsutum	74	74	73	73.6
	Absidia spinosa	74	73	73	73.3
	Polyporus sulphureus	74	74	74	74
	Aspergillus fumigatus	72	72	72	72
	Alternaria tenuis	73	72	73	72.6
	Caldariomyces fumago	74	73	74	73.6
	Cordyceps ophioglossoides	73	73	73	73
	Pyronema domesticum	72	73	72	72.3
	Ceratocystis coerulescens	75	74	74	74.3
3.	Control	76	76	77	76.3
	Botrytis allii	76	76	73	75
	Botrytis tulipae	75	76	75	75.3
	Botrytis spectabilis	76	80	78	78
	Botrytis fabae	78	80	75	79.6
	Hansfordia grisella	75	76	76	75.6
	Pullularia pullulans	77	74	76	75.6
	Trichoderma koningi	76	77	76	76.3
	Acremoniella atra	77	77	77	77
	Mucor hiemalis +	78	77	76	77
	Mucor hiemalis -	78	77	78	77.6
4.	Control	80	80	80	80
	Verticillium psalliotae	80	80	80	80
	Penicillium claviforme	80	80	80	80
	Byssochlamys fulva	80	85	80	81.6
	Rhodotorula rubra	80	80	80	80
	Choanephora circinans	80	81	80	80.3
5.	Control	61	61	64	62
	Fomes annosus	22	57	30	36.3
	Pericystis apis	60	61	63	61.3
	Nectria haematococca	60	60	63	61
6.	Control	75	75	75	75
	Aspergillus niger	75	75	76	75.3
	Rhizopus sexualis	74	74	75	74.3
	Pleospora androgyna	76	74	74	74.6
	Chaetomium globosum	75	75	74	74.6
	Cordyceps militaris	76	77	77	76.3
	Cephalosporium cretatum	75	75	74	74.6
	Rhizopus stolonifer +	75	74	74	74.3
	Rhizopus stolonifer -	73	73	74	73.3
7.	Control	78	77	78	77.6
	Trichoderma viride	76	73	76	75
	Ophiobolus graminis	78	78	78	78
8.	Control	74	74	75	74.3
	Merulius lacrymans	75	75	74	74.6
	Curvularia lunata	73	73	74	73.3
	Colletotrichum lindemuthianum	74	74	74	74
	Trichothecium roseum	74	74	75	74.3
9.	Control	64	64	64	64
	Agaricus campestris	63	64	64	63.6
	Thielaviopsis basicola	64	64	64	64
	Piptocephalis cylindrospora	64	64	63	63.6
	Botrytis cinerea	63	64	64	63.6
10.	Control	68	68	68	68
	Armillaria mellea	68	69	67	68
	Mortierella ramanniana	69	68	68	68.3

TABLE A7.

Effect of volatile metabolites on the growth of fungi.

ASSAY SPECIES - Aspergillus niger.

Expt. No.	Test species	Colony diameter after 10 days			Mean	Sporulation after 10 days
1.	Control	55	54	54	54.3	3+
	Trichoderma viride	54	54	53	53.6	"
	Agaricus campestris	55	55	55	55	"
	Penicillium digitatum	53	54	54	53.6	"
	Caldariomyces fumago	55	54	54	54.3	"
2.	Control	53	53	53	53	"
	Aspergillus niger	54	54	53	53.6	"
	Mucor plumbeus	52	53	54	53	"
	Tilachlidium nigrum	53	53	54	53.3	"
	Absidia spinosa	53	53	53	53	"
	Chaetomium globosum	54	54	54	54	"
	Stereum hirsutum	53	54	54	53.6	"
	Polyporus sulphureus	54	54	54	54	"
3.	Control	46	46	46	46	"
	Mucor hiemalis +	46	46	46	46	"
	Mucor hiemalis -	46	47	47	46.6	"
	Verticillium sp.	47	47	47	47	"
	Helminthosporium sativum	45	45	45	45	2+
	Shizosaccharomyces octosporus	41	41	41	41	3+
	Rhizopus stolonifer -	47	47	47	47	"
	Rhizopus stolonifer +	47	47	47	47	"
	Sordaria fimicola	47	46	47	46.6	"
	Periaystis apis	47	47	47	47	"
	Pythium ultimum	46	46	46	46	"
4.	Control	46	45	46	45.6	"
	Aspergillus fumigatus	46	46	46	46	"
	Ceratocystis coerulescens	47	47	46	46.6	2+
	Thielaviopsis basicola	46	45	45	45.3	3+
	Aspergillus terreus	46	46	45	45.6	"
	Gliocladium fujikuroi	46	47	47	46.6	"
	Cladosporium herbarum	44	45	45	44.6	2+
	Cunninghamella echinulata	45	45	45	45	3+
	Penicillium claviforme	46	46	46	46	"
	Pyronema domesticum	46	46	45	45.6	"
5.	Control	45	45	46	45.3	"
	Armillaria mellea	46	45	45	45.3	"
	Fomes annosus	30	31	28	29.6	2+
	Cephalosporium cretatum	44	45	45	44.6	3+
	Verticillium psalliotae	44	44	44	44	"
	Byssochlamys fulva	45	45	45	45	3+
	Ophiobolus graminis	46	46	48	46.6	"
	Carpenteles sp.	45	45	45	45	"
	Cordyceps militaris	45	45	45	45	"
	Piptocephalis cylindrospora	45	45	45	45	"
	Cordyceps ophioglossoides	45	45	46	45.3	"
6.	Control	45	45	45	45	"
	Botrytis spectabilis	45	45	46	45.3	"
	Botrytis tulipae	45	45	44	44.6	"
	Pullularia pullulans	45	45	45	45	"
	Rhodotorula rubra	43	44	44	43.6	"
	Botrytis fabae	44	44	45	44.3	"
	Botrytis allii	46	46	46	46	"
	Rhizopus sexualis	44	45	45	44.6	"
	Trichoderma koningi	44	45	44	44.3	"
	Acremoniella atra	44	45	45	44.6	"
7.	Control	44	45	44	44.3	"
	Colletotrichum lindemuthianum	44	45	45	44.6	"
	Choanephora circinans	45	44	44	44.3	"
	Curvularia lunata	45	44	46	45	"
	Nectria haematococca	44	45	44	44.3	"
	Trichothecium roseum	45	46	44	45	"
	Saccharomyces cerevisiae	41	41	40	40.6	2+
8.	Control	40	40	40	40	3+
	Zygorhyncus moelleri	40	41	40	40.3	"
9.	Control	41	38	37	38.3	"
	Hansfordia grisella	40	36	36	37.3	"
	Pleospora didrogyna	40	37	41	39.3	"
10.	Control	46	46	47	46.3	"
	Fusarium culmorum	46	47	47	46.6	"
	Merulius lacrymans	46	46	47	46.3	"
	Botrytis cinerea	48	47	46	47.0	"
11.	Control	50	50	50	50	"
	Alternaria tenuis	49	50	50	49.6	"
12.	Control	46	45	45	45.3	"
	Mortierella ramaniana	46	45	45	45.3	"

TABLE A8.

Effect of volatile metabolites on the growth of fungi.

ASSAY SPECIES - Botrytis cinerea.

Expt. No.	Test species	Colony diameter after 5 days			Mean	Sporulation after 7 days
1.	Control	90	90	90	90	3+ peripheral
	Saccharomyces cerevisiae	80	82	77	79.6	3+ widespread
	Chaetomium globosum	90	90	88	89.3	3+ peripheral
	Botrytis cinerea	90	90	90	90	"
	Agaricus campestris	90	90	90	90	"
2.	Control	80	80	81	80.3	"
	Penicillium digitatum	86	86	86	86	"
	Trichothecium roseum	83	82	83	82.6	"
	Mucor plumbeus	83	83	84	83.3	"
	Pyronema domesticum	79	80	80	79.6	"
3.	Control	77	77	77	77	"
	Ceratocystis coerulescens	71	72	71	71.3	"
	Aspergillus terreus	76	76	78	76.6	"
	Cunninghamella echinulata	71	71	66	69.6	"
	Thielaviopsis basicola	68	70	69	69	"
	Gibberella fujikuroi	71	71	71	71	"
4.	Control	77	77	77	77	"
	Trichoderma viride	36	35	35	35.3	NIL
	Cordyceps ophioglossoides	75	74	75	74.6	2+ peripheral
	Penicillium claviforme	76	76	76	76	3+ peripheral
	Sordaria fimicola	73	73	73	73	"
	Polyporus sulphureus	68	68	68	68	"
	Pericystis apis	73	73	73	73	"
	Aspergillus fumigatus	77	73	74	74.6	"
5.	Control	85	86	85	85.3	"
	Byssochlamys fulva	84	85	83	84	"
	Pythium ultimum	86	85	85	85.3	"
	Abaidia spinosa	87	87	86	86.6	"
	Stereum hirsutum	80	79	78	79	"
	Carpenteles sp.	85	85	85	85	"
	Fusarium culmorum	83	82	83	82.6	"
	Helminthosporium sativum	84	84	86	83.6	"
	Verticillium sp.	83	84	83	83.3	"
	Rhizopus sexualis	86	83	85	84.6	"
	Alternaria tenuis	84	84	86	84.6	"
6.	Control	80	80	82	80.6	"
	Botrytis fabae	82	83	81	82	"
	Botrytis tulipae	80	80	81	80.3	"
	Botrytis spectabilis	82	80	81	81	"
	Fomes annosus	68	77	77	74	"
	Botrytis allii	80	80	80	80	"
	Acremoniella atra	90	90	90	90	3+ widespread
	Verticillium psalliotae	83	84	86	84.3	3+ peripheral
7.	Control	90	90	90	90	"
	Rhodotorula rubra		"		"	"
	Trichoderma koningi		"		"	4+ widespread
	Pullularia pullulans		"		"	3+ peripheral
	Mucor hiemalis +		"		"	"
	Mucor hiemalis -		"		"	"
	Rhizopus stolonifer +		"		"	"
	Rhizopus stolonifer -		"		"	"
8.	Control	74	75	75	74.6	"
	Ophiobolus graminis	70	72	70	70.6	"
	Nectria haematococca	70	72	72	71.3	"
9.	Control	90	90	90	90	"
	Armillaria mellea	75	74	84	77.6	3+ widespread
	Colletotrichum lindemuthianum	90	90	90	90	3+ peripheral
	Hansfordia grisella	90	90	90	90	"
	Cordyceps militaris	76	72	73	73.6	3+ widespread
10.	Control	90	90	90	90	3+ peripheral
	Curvularia lunata		"		"	"
	Choanephora circinans		"		"	"
11.	Control	90	90	90	90	"
	Aspergillus niger		"		"	"
	Zygorhyncus moelleri		"		"	"
	Tilachlidium nigrum		"		"	"
12.	Control	76	77	76	76.6	"
	Schizosaccharomyces octosporus	65	68	70	67.8	2+ widespread
13.	Control	90	90	90	90	3+ peripheral
	Merulius lacrymans	90	90	90	90	3+ "
	Cladosporium herbarum	90	90	90	90	"
14.	Control	90	90	90	90	3+ "
	Piptocephalis cylindrospora	90	90	90	90	"
	Mortierella ramanniana	90	90	90	90	"
15.	Control	90	90	90	90	3+ "
	Cephalosporium cretatum	90	90	90	90	"
	Caldariomyces fumago	90	90	90	90	"
	Pleospora androgyna	90	90	90	90	"

Note: 90mm. represents total cover of Petri dish, but for sake of consistency this amount of growth at 5 days was recorded, any increase or decrease in colony diameter being monitored over the preceding period.

TABLE A9.

Effect of volatile metabolites of test fungi on growth of assay spp.

(a). Linear growth. - Figures indicate mean increase or decrease of three assay cultures, over controls.

Assay species	R.sexualis	C.globosum		S.hirsutum		A. niger		B.cinerea	
Test species		Mean	LSMD	Mean	LSMD	Mean	LSMD	Mean	LSMD
Absidia spinosa		-3.3	2.2	0	1.0	0	0.9	+1.3	2.6
Choanephora circinans		+0.6	1.5	+0.3	2.5	0	1.4	0	-
Cunninghamella echinulata		-0.6	3.6	-1.7	1.0	-0.6	-	-7.4	2.2
Mortierella ramanniana	No	-0.3	-	+0.3	-	0	-	0	-
Mucor hiemalis +		+2.4	3.6	+0.7	2.8	0	0.6	0	-
M. hiemalis -		-1.6	3.6	+1.3	2.8	+0.6	0.6	0	-
Mucor plumbeus		+1.0	2.4	-2.0	1.0	0	0.9	+3.0	1.1
Piptocephalis cylindrospora		+1.0	2.2	-0.4	-	-0.3	1.4	0	-
Pythium ultimum		-2.3	3.6	+1.7	1.0	0	0.6	0	2.6
Rhizopus sexualis		+0.7	2.2	-0.7	1.2	-0.4	1.4	-0.7	2.6
R. stolonifer +		+2.4	3.6	-0.7	1.2	+1.0	0.6	0	-
R. stolonifer -	deviation	-0.3	3.6	-1.7	1.2	+1.0	0.6	0	-
Zygorhyncus moelleri		-1.3	1.8	-1.3	1.0	+0.3	-	0	-
Byssochlamys fulva		-8.2	3.5	+1.6	2.5	-0.3	1.4	-1.3	2.6
Ceratocystis coerulescens		-1.7	2.4	+1.0	1.0	+1.0	-	-5.7	2.2
Chaetomium globosum		-1.4 2.4	1.1 1.9	-0.4	1.2	+1.0	0.9	-0.7	1.9
Cordyceps militaris		-0.6	-	+1.3	1.2	-0.3	1.4	-6.4	4.6
C. ophioglossoides		+5.0	2.4	-0.3	1.0	0	1.4	-2.2	1.2
Gibberella fujikuroi		+2.3	2.4	-1.3	1.0	+1.0	-	-6.0	2.2
Nectria haematococca		+2.0	3.7	-1.0	14.8	0	1.4	-3.3	3.1
Ophiobolus graminis		-0.4	3.5	-0.4	2.1	+1.3	1.4	-4.0	3.1
Pericystis apis	from	+1.6	2.4	-0.7	14.8	+1.0	0.6	-4.0	1.2
Pleospora androgyna		+0.4	3.6	-0.4	1.2	+1.0	-	0	-
Pyronema domesticum		0	2.4	-1.0	1.0	0	-	-0.7	1.1
Saccharomyces cerevisiae		-16.0	1.1	-5.7	1.0	-3.7	1.4	-10.4	1.9
Schizosaccharomyces octosporus		-4.0	1.5	-3.7	1.0	-5.0	0.6	-8.8	1.9
Sordaria fimicola		+1.3	2.4	0	1.0	+0.6	0.6	-4.0	1.2
Agaricus campestris		+0.7	1.8	-0.4	14.8	+0.7	1.1	0	1.9
Armillaria mellea		+0.7	3.7	0	-	0	1.4	-12.4	4.6
Fomes annosus		-18.0	1.5	-25.7	14.8	-15.7	1.4	-6.6	3.4
Merulius lacrymans		0	3.7	+0.3	-	0	-	0	-
Polyporus sulphureus		0	2.4	+0.7	1.0	+1.0	0.9	-9.0	1.2
Stereum hirsutum	controls	+3.3	2.4	+0.3	1.0	+0.6	0.9	-6.3	2.6
Acremoniella atra		+1.3	2.9	+0.7	2.8	-0.4	1.4	+9.4	3.4
Alternaria tenuis		-1.0	3.5	-0.7	1.0	-0.4	-	+0.7	2.6
Aspergillus fumigatus		-1.0	2.9	-1.3	1.0	+0.4	-	-2.4	1.2
A. niger		-0.7	2.2	+0.3	1.2	+0.6	0.9	0	-
A. terreus		0	2.4	-3.0	1.0	0	-	-0.4	2.2
Botrytis allii		-5.0	3.6	-1.3	2.8	+1.0	1.4	-0.6	3.4
B. cinerea		0 0	1.1 1.9	-0.4	-	+0.7	-	0	1.9
B. fabae		0	-	+1.7	2.8	-0.7	1.4	+1.4	3.4
B. spectabilis		+2.4	3.7	+1.7	2.8	+0.3	1.4	+0.4	3.4
B. tulipae		-1.0	3.7	-1.0	2.8	-0.4	1.4	-0.3	3.4
Caldariomyces fumago		-2.6	0.8	+0.3	1.0	0	1.1	0	-
Carpenteles sp.		+8.3	2.4	-1.7	1.0	-0.3	1.4	-0.3	2.6
Cephalosporium cretatum		-5.0	3.6	-0.4	1.2	-0.7	1.4	0	-
Cladosporium herbarum		0	2.4	-1.3	1.0	-1.0	-	0	-
Colletotrichum lindemuthianum		-6.0	3.5	-0.3	-	+0.3	1.4	0	4.6
Curvularia lunata		+1.0	3.7	-1.0	-	+0.7	1.4	0	-
Fusarium culmorum		-0.7	3.5	-0.7	1.0	+0.3	-	-2.7	2.6
Hansfordia grisella		-2.3	3.7	-0.7	2.8	-1.0	-	0	4.6
Helminthosporium sativum		-1.7	3.5	-0.3	1.0	-1.0	0.6	-1.7	2.6
Penicillium claviforme		+1.3	2.4	0	2.5	+0.4	-	-1.0	1.2
P. digitatum		+2.0	3.7	-0.3	1.0	-0.7	1.1	+5.7	1.1
Pullularia pullulans		+0.7	2.2	-0.7	2.8	0	1.4	0	-
Rhodoturula rubra		0	1.5	0	2.5	-1.4	1.4	0	-
Thielaviopsis basicola		0	2.2	0	-	-0.3	-	-8.0	2.2
Tilachlidium nigrum		-1.3	1.8	-6.0	1.0	+0.3	0.9	0	-
Trichoderma koningi		0	1.5	0	2.8	-0.7	1.4	0	-
T. viride		-14.3	3.7	-2.6	2.1	-0.7	1.4	-41.7	1.2
Trichothecium roseum		+0.4	0.8	0	-	+0.7	1.4	+2.3	1.1
Verticillium psalliotae		-1.7	3.5	0	2.5	-1.3	1.4	+3.7	3.4
Verticillium sp.		+1.4	3.7	-1.3	1.0	+1.0	0.6	-2.0	2.6

TABLE A10.

Effect of volatile metabolites of test fungi on growth of assay spp.

(b). Sporulation. - Mean increase or decrease of three assay cultures, over controls.

Assay species	R.sexualis		C.globosum	S.hirsutum	A. niger	B.cinerea
Test species	Mean	LSMD				
Absidia spinosa	+9.5	3.2	No deviation		No deviation	No deviation
Choanephora circinans	+2.0	3.1	"		"	"
Cunninghamella echinulata	+3.7	2.4	"		"	"
Mortierella ramanniana	+1.7	-	"		"	"
Mucor hiemalis +	+4.0	5.3	"	Growth	"	"
M. hiemalis -	+3.4	5.3	"		"	"
M. plumbeus	+22.3	18.6	- 25%		"	"
Piptocephalis cylindrospora	+12.4	5.3	No deviation		"	"
Pythium ultimum	+2.4	5.3	"	in	"	"
Rhizopus sexualis	+41.6	27.2	"		"	"
R. stolonifer +	+4.8	3.2	"		"	"
R. stolonifer -	+0.7	11.8	"	all	"	"
Zygorhyncus moelleri	+0.4	-	"		"	"
Byssochlamys fulva	0	3.0	"	cases	"	"
Ceratocystis coerulescens	+18.3	7.1	Complete inhibition		- 25%	"
Chaetomium globosum	+0.3	-	No deviation		No deviation	"
Cordyceps militaris	+28.0	11.8	"	purely	"	+ 25%
C. ophioglossoides	+0.8	3.2	"		"	- 25%
Gibberella fujikuroi	+18.0	3.8	"		"	No deviation
Nectria haematococca	-0.7	2.4	"		"	"
Ophiobolus graminis	+1.7	3.0	"	vegetative	"	"
Pericystis apis	+3.7	5.3	"		"	"
Pleospora androgyna	+4.4	5.3	"		"	"
Pyronema domesticum	+1.0	2.7	"		"	"
Saccharomyces cerevisiae	+1.3	18.6	- 25%		- 25%	+ 25%
Schizosaccharomyces octosporus	+2.7	5.3	No deviation		No deviation	- 25%
Sordaria fimicola	+0.7	7.1	"		"	No deviation
Agaricus campestris	+13.7	13.1	"		"	"
Armillaria mellea	-11.7	3.0	"		"	"
Fomes annosus	+30.2	3.2	- 25%		- 25%	+ 25%
Merulius lacrymans	+3.4	3.8	No deviation		No deviation	No deviation
Polyporus sulphureus	0	-	"		"	"
Stereum hirsutum	0	3.0	"		"	"
Acremoniella atra	-11.6	3.1	"		"	"
Alternaria tenuis	+2.0	5.3	"		"	"
Aspergillus fumigatus	-2.7	7.1	"		"	"
A. niger	+2.0	3.8	"		"	"
A. terreus	0	3.8	"		"	"
Botrytis allii	+3.2	3.2	"		"	"
B. cinerea	+29.2	27.2	"		"	"
B. fabae	+1.0	3.1	"		"	"
B. spectabilis	+19.0	3.1	"		"	"
B. tulipae	+0.4	3.1	"		"	"
Caldariomyces fumago	-1.6	-	"		"	"
Carpenteles sp.	-0.7	3.0	"		"	"
Cephalosporium cretatum	+1.0	3.0	"		"	"
Cladosporium herbarum	0	3.8	"		- 25%	"
Colletotrichum lindemuthianum	+3.7	2.4	"		No deviation	"
Curvularia lunata	0	3.1	"		"	"
Fusarium culmorum	+1.4	5.3	"		"	"
Hansfordia grisella	-0.7	11.8	"		"	"
Helminthosporium sativum	+19.7	5.3	"		- 25%	"
Penicillium claviforme	-9.3	7. 1	"		No deviation	"
P. digitatum	-3.0	2.7	"		"	"
Pullularia pullulans	+1.5	3.2	"		"	"
Rhodotorula rubra	+1.0	3.1	"		"	"
Thielaviopsis basicola	+20.3	3.8	"		"	"
Tilachlidium nigrum	+2.0	3.0	"		"	+ 25%
Trichoderma koningi	-14.0	3.1	"		"	Complete inhibition
T. viride	-27.0	7.1	"		"	No deviation
Trichothecium roseum	-2.0	2.7	"		"	"
Verticillium psalliotae	+2.3	3.0	"		"	"
Verticillium sp.	+0.4	5.3	"		"	"

Table A11

Efficiency of evacuation method in producing required concentrations of CO_2 within desiccators.

Initial conc. of CO_2 supplied.	Conc. of CO_2 after evacuation to X 0.5 atmospheric pressure.	Mean final conc.
10%	3.5%	
10%	4.0%	
10%	3.3%	
10%	3.5%	
10%	4.0%	3.56%
10%	3.2%	
10%	3.9%	
10%	3.1%	

TABLE A12.

Effect of CO_2 on Rhizopus sexualis.

CO_2 concentration	Mean colony diameter after 4 days-mm.			Representative zygospore counts per plate after 4 days		
		Mean	LSMD		Mean	LSMD
20%	Nil Nil Nil	Nil		Nil Nil Nil	Nil	
0.5%	90 90 90	90	100	94 95 100	96.3	
0.1%	90 90 90	90	100	94 92 100	96.3	
0.03% (control)	90 90 90	90	100 -	95 104 96	98.3	-
10%	36 35 35	35.3	40	Nil Nil Nil		
5%	75 72 74	73.6	81	Very few, immature		
1%	90 90 90	90	100	96 95 96	95.6	
0.03% (control)	90 90 90	90	100 1.6	96 98	97.0	-

TABLE A13.

Effect of CO_2 on Chaetomium globosum.

CO_2 concentration	Mean colony diameter after 8 days-mm.			Sporulation after 8 days
		Mean	LSMD	
20%	4	8.6	11	Nil
	13			Nil
	9			Nil
10%	20	21.3	43	Nil
	21			Nil
	23			Nil
5%	31	31.6	64	Nil
	28			Nil
	36			Nil
0.03% (control)	52	49.3	6.5	+
	46			+
	50			+
1%	54	53	100	+
	53			+
	52			+
0.5%	53	53.6	100	+
	54			+
	54			+
0.1%	53	53.3	100	+
	53			+
	54			+
0.03% (control)	54	53.3	-	+
	53			+
	53			+

TABLE A14.

Effect of CO_2 on Stereum hirsutum.

CO_2 concentration	Mean colony diameter after 8 days-mm.		
		Mean	LSMD
20%	49 50 60	53	69
0.5%	74 75 75	74.6	100
0.1%	75 75 76	75.3	100
0.03% (control)	77 76 76	76.3	5.5
10%	75 74 76	75.0	90
5%	78 82 78	79.3	96
1%	82 83 81	82.0	100
0.03%	82 83 83	82.6	2.6

TABLE A15.

Effect of CO_2 on Aspergillus niger.

CO_2 concentration	Mean colony diameter after 8 days-mm.	Mean	LSMD	Sporulation after 8 days
20%	12 12 11	11.6	46	Nil Nil Nil
10%	19 18 19	18.6	74	+ + +
5%	21 20 21	20.6	80	+ + +
0.03% (control)	25 25 25	25	100 1.1	3+ 3+ 3+
1%	18 19 18	18.5	100	2+ 2+ 2+
0.5%	19 18 18	18.5	100	2+ 2+ 2+
0.1%	18 19 18	18.5	100	2+ 2+ 2+
0.03% (control)	19 18 18	18.5	100	2+ 2+ 2+

TABLE A16.

Effect of CO_2 on Botrytis cinerea.

CO_2 concentration	Mean colony diameter after 5 days-mm.			Sporulation after 5 days
		Mean	LSMD	
20%	Nil Nil Nil	Nil		Nil Nil Nil
0.5%	90 90 90	90	10	3+ peripheral 3+ peripheral 3+ peripheral
0.1%	90 90 90	90	10	3+ peripheral 3+ peripheral 3+ peripheral
0.03% (control)	90 90 90	90	10 -	3+ peripheral 3+ peripheral 3+ peripheral
10%	43 43 44	43.6	63	Nil Nil Nil
5%	61 60 59	60	87	+ immature + immature + immature
1%	68 70 68	68.6	100	2+ 2+ 2+
0.03% (control)	68 70 69	69	1.6	2+ 2+ 2+

TABLE A17. **Effect of volatile substances on growth of assay organisms.** (Note: Colony diameter of 90+ represents a culture which has covered a 90mm. Petri dish. Observations made up to 4 days growth showed no increased growth of assay fungus over controls.)

ASSAY FUNGUS - Rhizopus sexualis, growth after 4 days.

Volatile tested	Concentration of volatile substance.													
	Control			0.01%			0.1%			1.0%			L.S.M.D.	
	Col. diam.	Mean	Zygo-spore Nos.	Mean	Col. diam.	Mean	Zygo-spore Nos.	Mean	Col. diam.	Mean	Zygo-spore Nos.	Mean	Col. diam.	Zygo-spore Nos.
Ethanol	All		93		All		96		All		113		All		127		-	5.6
			96	94.3			92	93.3			108	110.6			119	124.6		
	90+		94		90+		92		90+		111		90+		128			
Acetone	All		95		All		92		All		95		All		92		-	3.8
			94	94.6			97	94.3			94	94.6			86	89.6		
	90+		95		90+		94		90+		94		90+		91			
Cyclohexene	All		97		49		Cent. zone		Nil		Nil		Nil		Nil		11.5	-
			106	101	35	44.6	only approx.											
	90+		100		60		35/field											
1.Octene	All		97		All		107		All		103		All		98		-	-
			106	101			99	102.6			95	99			100	99.3		
	90+		100		90+		102		90+		97		90+		100			
4.Methylpentene.1	All		99		All		97		All		98		All		97		-	-
			99	98.6			99	97			101	99			101	98.3		
	90+		98		90+		95		90+		98		90+		97			
4.Methyl.1.2.pentene	All		94		All		94		All		81						-	2.5
	90+		97	96.3			96	94.6			79	79.6	Nil		Nil			
			98		90+		94		90+		79							
2.4.4.Trimethylpentene	All		97		All		96		All		96		80		All immat.		2.2	2.1
			96	96.3			92	94.3			96	95.6	85	82.3	app. 95/field			
	90+		96		90+		95		90+		95		82					
2.2.Dimethylbutane	All		95		All		96		All		96		All		98		-	-
			96	95.6			95	95			95	95			98	97.3		
	90+		96		90+		94		90+		94		90+		96			

TABLE #18. **Effect of volatile substances on growth of assay organisms.**

ASSAY FUNGUS - Chaetomium globosum; colony diameter after 8 days, sporulation 10 days.

Volatile tested	Concentration of volatile substance.												
	Control			0.01%			0.1%			1.0%			
	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	L.S.M.D.
Ethanol	68 69 68	68.3	All 3+	68 68 68	68	All 3+	67 64 67	66	All 3+	57 50 60	55.6	All 3+	4.9
Acetone	68 69 68	68.3	All 3+	66 69 68	67.6	All 3+	65 65 65	65	All 3+	37 29 29	31.6	All 4+, very central	4.4
Cyclohexene	66 66 65	65.6	All 3+	0 0 0	0	Nil	0 0 0	0	Nil	0 0 0	0	Nil	-
1.Octene	66 66 66	66	All 3+	67 67 67	67	All 3+	64 68 67	66.3	All 3+	67 64 65	65.3	All	2.4
4.Methylpentene.1	54 56 55	55	All 3+	55 56 55	55.3	All 3+	56 54 54	54.6	All 3+	48 48 50	48.6	All 2+	4.2
4.Methyl.1.2.pentene	75 77 76	76	All 3+	52 63 0	38.3	All 3+ very central	0 0 0	0	Nil	0 0 0	0	Nil	30.9
2.4.4.Trimethylpentene	54 56 55	55	All 3+	54 54 55	54.3	All 3+	54 56 54	54.6	All 3+	18 17 0	11.6	Nil	9.4
2.2.Dimethylbutane	75 77 76	76	All 3+	74 75 75	74.6	All 3+	75 76 73	74.6	All 3+	76 74 76	75.3	All 3+	1.9

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TABLE A19. Effect of volatile substances on growth of assay organisms.

ASSAY FUNGUS - Stereum hirsutum, growth after 8 days.

Volatile tested	Concentration of volatile substance								
	Control		0.01%		0.1%		1.0%		
	Colony diam.	Mean	Colony diam.	Mean	Colony diam.	Mean	Colony diam.	Mean	L.S.M.D.
Ethanol	68 69 69	68.6	69 69 69	69	65 66 66	65.6	60 63 60	61	6.2
Acetone	69 69 69	69	69 70 69	69.3	66 67 64	65.6	59 60 60	59.6	1.6
Cyclohexene	68 68 68	68	30 23 23	25.3	0 0 0	0	0 0 0	0	3.7
1.Octene	68 68 68	68	68 72 68	69.2	72 71 68	70.3	68 68 68	68	3.1
4.Methylpentene.1	80 80 80	80	80 77 80	79	80 80 77	79	78 80 80	79.3	-
4.Methyl.1.2.pentene	80 80 80	80	37 41 27	35	0 0 0	0	0 0 0	0	6.6
2.4.4.Trimethylpentene	69 69 69	69	70 72 70	70.6	69 70 70	69.6	60 58 58	58.6	1.1
2.2.Dimethylbutane	80 80 80	80	83 80 80	81	80 77 77	78	80 80 77	79	2.5

TABLE A20. Effect of volatile substances on growth of assay organisms.

ASSAY FUNGUS - Aspergillus niger, growth after 10 days.

Volatile tested	Concentration of volatile substance.												
	Control			0.01%			0.1%			1.0%			
	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	L.S.M.D.
Ethanol	42	41.3	All	42	41.6	All	41	41.6	All	37	37	All	0.8
	41			42			42			37			
	41		3+	41		3+	42		3+	37		3+	
Acetone	45	45	All	45	45	All	45	45	All	42	42.6	All	2.0
	45			45			45			43			
	45		3+	45		3+	45		3+	42		3+	
Cyclohexane	39	39	All	39	38.6	All	0	0	Nil	0	0	Nil	0.6
	39			38			0			0			
	39		3+	39		3+	0			0			
1.Octene	41	41.6	All	43	42	All	40	41	All	40	41	All	-
	42			41			43			40			
	41		3+	42		3+	40		3+	43		3+	
4.Methylpentene.1	45	45	All	45	45	All	45	44.6	All	45	45	All	-
	45			45			44			45			
	45		3+	45		3+	45		3+	45		3+	
4.Methyl.1.2.pentene	43	45.3	All	45	45.3	All	45	45	All	0	0	Nil	0.9
	45			46			45			0			
	46		3+	45		3+	45		3+	0			
2.4.4.Trimethylpentene	46	46.3	All	47	47	All	46	45.6	All	46	46	All	1.2
	46			47			46			47			
	47		3+	47		3+	45		3+	45		3+	
2.2.Dimethylbutane	46	46.3	All	47	46.3	All	47	46.6	All	47	47	All	-
	47			46			47			47			
	46		3+	46		3+	46		3+	47		3+	

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TABLE A21. Effect of volatile substances on growth of assay organisms.

ASSAY FUNGUS - Botrytis cinerea.
(Col. diam. 5 days, Sporln. 7 days)

Volatile tested	Concentration of volatile substance.												
	Control			0.01%			0.1%			1.0%			
	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	Col. diam.	Mean	Sporln.	L.S.M.D.
Ethanol	All 90+		All 4+ p.	All 90+		All 4+ p.	All 90+		All 4+ p.	All 90+		All 4+ p.	-
Acetone	All 90+		All 2+ w.	All 90+		All 2+ w.	All 90+		All 2+ w.	60 60 45	55	All 4+ c.	7.9
Cyclohexane	All 90+		All 3+ p.	All 90+		All 3+ c.	53 67 66	62	All 4+ c.	0 0 0	0	Nil	7.2
1.Octene	All 90+		All 3+ p.	All 90+		All 2+ p.	All 90+		All 90+ p.	All 90+		Nil	-
4.Methylpentene.1	All 90+		All 2+ p.	All 90+		All 2+	All 90+		All 2+ p.	83 84 84	83.6	All 2+ w.	0.6
4.Methyl.1.2.pentene	All 90+		All 3+ p.	All 90+		All 3+ w.	78 76 78	77.3	All 4+ w.	17 20 8	15	Nil	5.8
2.4.4.Trimethylpentene	68 70 70	69.3	All 3+ p.	70 70 70	70	All 3+ p.	72 70 70	70.6	All 3+ p.	62 63 60	61.6	All + w	2.1
2.2.Dimethylbutane	All 90+		All 4+ p.	All 90+		All 4+ p.	All 90+		All 4+ p.	All 90+		All 4+ p.	-

Note: Colony diameter of 90+ represents a culture which has covered a 90mm. Petri dish. Observations made up to 5 days growth showed no increased growth of the fungus over controls. p = sporulation peripheral; w = widespread; c = central.

APPENDIX B.

Methods employed in collection and analysis of volatile fungal metabolites.

B.1. PYE ARGON CHROMATOGRAPH.

Gas or liquid mixtures injected into the Argon carrier gas of the Chromatographic apparatus are separated by passage through a column packed with a material which shows greater affinity for some organic substances than others. Hence, elution times for the different components of a mixture varies. These components then pass to the ionising detector at different times and reaction to their presence is recorded over a period.

Packing for the main column may be made using a variety of organic compounds according to the type of substances to be analysed. Almost any organic compound may be used as the stationary phase when coated on the support material, Celite. The type used depends on the group of substances for which separation is required.

Since the identity of the fungal metabolites was unknown, the stationary phase used for column packing in the first attempts at analysis was Apiezon L. This was the packing material most commonly used in the Chemistry Department and is good for general separations and for separation of esters and hydrocarbons. The maximum temperature for operation of this material is high, i.e. 200-225°C. Hence, when no identification was made using APL, then Squalane, Polyethylene glycol and Carbowax packings were used. These give separation of alcohols, amines, esters and hydrocarbons at temperatures up to 125°C.

It was thought that the metabolites, which are volatile at room temperature, might have passed through the columns too quickly for separation at high temperatures. Thus, in later analyses, tests were made at room temperature using Squalane, Polyethylene glycol, Carbowax and Activated Charcoal columns. This latter substance gives separation of unsaturated hydrocarbons at low temperatures.

Swoboda head to column.

The problem of elimination of water was overcome by the acquisition of a Swoboda Head or short pre-column, for the Chromatograph. In essence this is a device whereby any unwanted gases in a sample may be backflushed out of the system before they have time to pass to the main column. This is effected by first determining the time taken for the 'foreign' substances to pass through the precolumn when packed with an adsorbent identical with that of the main column; and then repacking with a substance which will delay the elution time and allow of back-flushing of the unwanted gases when the main flow of carrier gas is reversed at the calculated time.

In these analyses, the unwanted gas being water vapour, the pre-column was packed with 20% Glycerol which obstructs the passage of water and facilitates the operation of the backflushing device.

Sources of sample for analysis.

1. Direct sampling from the headspace above cultures.

2 and 5 ml. samples of the atmosphere above cultures on malt agar in 250 ml. conical flasks, on liquid malt medium in Penicillium flasks, and in the standard biological assay chamber, were removed with a syringe and injected into the Chromatograph.

2. Direct sampling from drum reservoir.

2ml samples were removed from the reservoir using a syringe inserted through the connective rubber tubing and allowing the gases to be forced into the syringe by pressure from the circulatory pump. These were then introduced directly into the gas stream of the Chromatograph.

3. Apiezon L or Carbowax packing.

A portion of the gas stream from the drum reservoir of the closed culture system was passed, after drying with Drikold packing, through a short length of tubing filled with either of these two adsorbent substances. They were then placed in the main Chromatographic column along with additional material of the same type. Any volatile substances so adsorbed would then pass into the detector on heating of the column.

4. Cooled receiver.

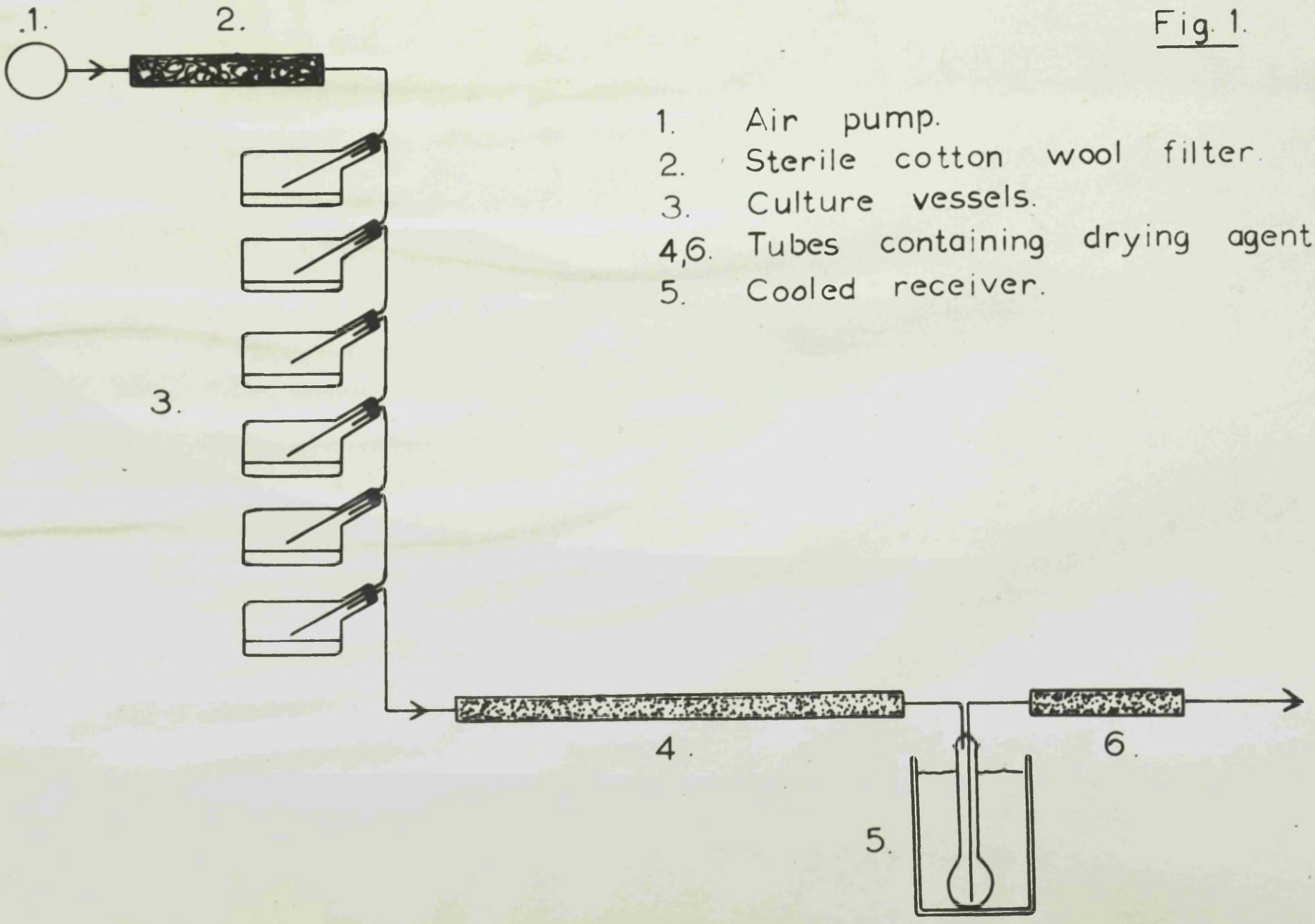
The cold trap used for obtaining effluents from deep culture vessels was attached to the Chromatographic column and brought to room temperature, thus allowing any volatile organic substances present to pass into the column for elution to the detector on heating.

PERKIN ELMER FLAME IONISING DETECTOR.

The column used with this detector was a 150ft. Golay capillary column coated with polypropylene glycol. Operating temperature was 50°C.

Samples for analysis were collected by methods 1. and 2. above, i.e. directly from the surface and deep cultures.

Fig. 1.



1. Air pump.
2. Sterile cotton wool filter.
3. Culture vessels.
4,6. Tubes containing drying agent.
5. Cooled receiver.

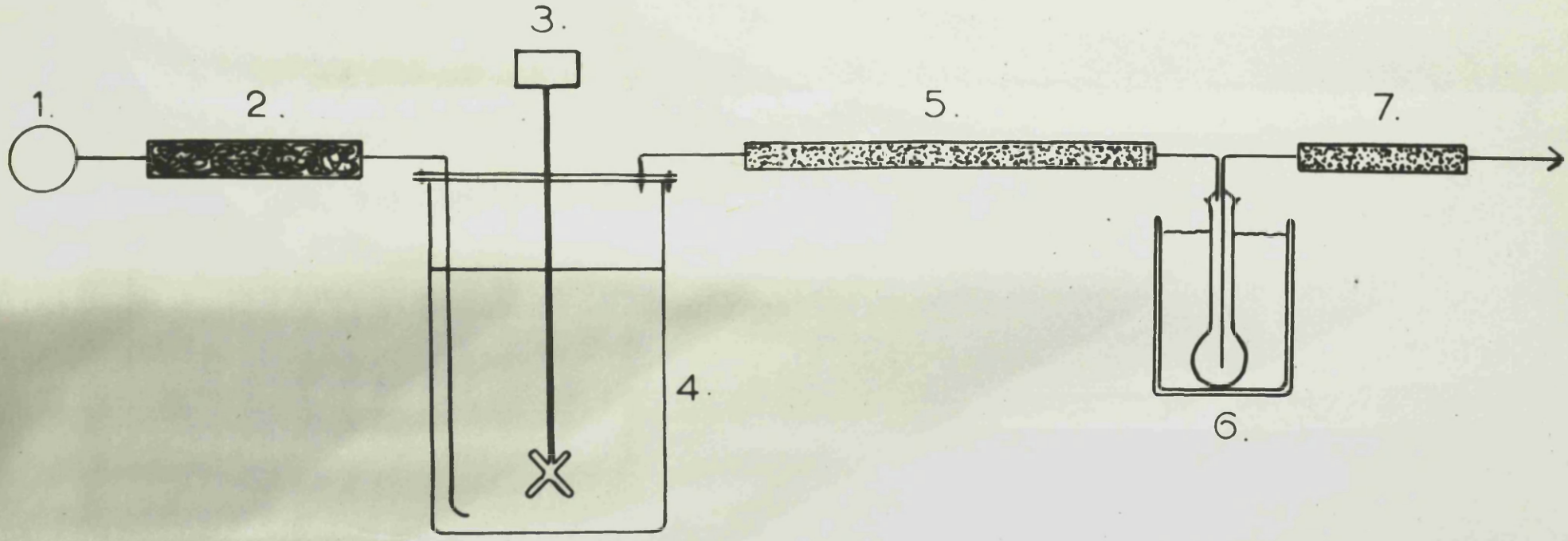
B.2.

Continuous trapping of effluent gases from surface culture.(Fig.1)

The fungus was grown in a series of six Penicillin flasks each containing 250ml. liquid medium of the following composition:-

'Glaxo' malt extract	30gm.
'Difco' bactopeptone	5gm.
Distilled water	1000 ml.

After inoculation with a suspension of zygospores in distilled water, these were incubated at 20°C for a period of three days. A constant stream of air was then pumped at a rate of approx. 15 litres per hour into the flasks, thence through a phosphorus pentoxide drying tube, into a long-necked 100ml. flask immersed in liquid Nitrogen which acted as a collecting vessel.



1. Air pump.
2. Sterile cotton wool filter.
3. Stirring unit.
4. Culture vessel containing 7 L. medium.
5,7. Tubes containing drying agent.
6. Cooled receiver.

Fig. 2

B.3.

Concentration of metabolites by deep culture.(Fig.2)

The vessel contained 7 litres of malt/peptone medium as formulated previously. This was inoculated with a suspension of zygospores and incubated at the culture room temperature of 26°C for three days, with constant aeration from a simple air pump. When a vigorously growing culture was obtained the airstream from it was passed, over a period of 2-5 days, through a Drikold tube to dry the gases, and thence into a cooled receiver.

B.4.

Action of volatile metabolites from deep cultures of R. sexualis on zygospore formation in young cultures of the same fungus.

A fermenting vessel was set up as in Fig.2. When the culture was growing vigorously a second vessel was connected to the culture one, and the effluent air allowed to pass through. In this second vessel were stacked ten Petri dishes each containing 25ml. malt/peptone medium, inoculated centrally with R. sexualis. The lids of the dishes were slightly raised by insertion of portions of sterile rubber tubing in order to make the atmosphere within the dishes and containing vessel as uniform as possible.

A similar vessel, also containing ten cultures of R. sexualis was connected to a fermenter containing only sterile malt/peptone medium.

After five days growth at a temperature of 26°C counts of zygospores were made in both sets of plates as described in Part 1. (See Table B.4 following.)

TABLE B.4.

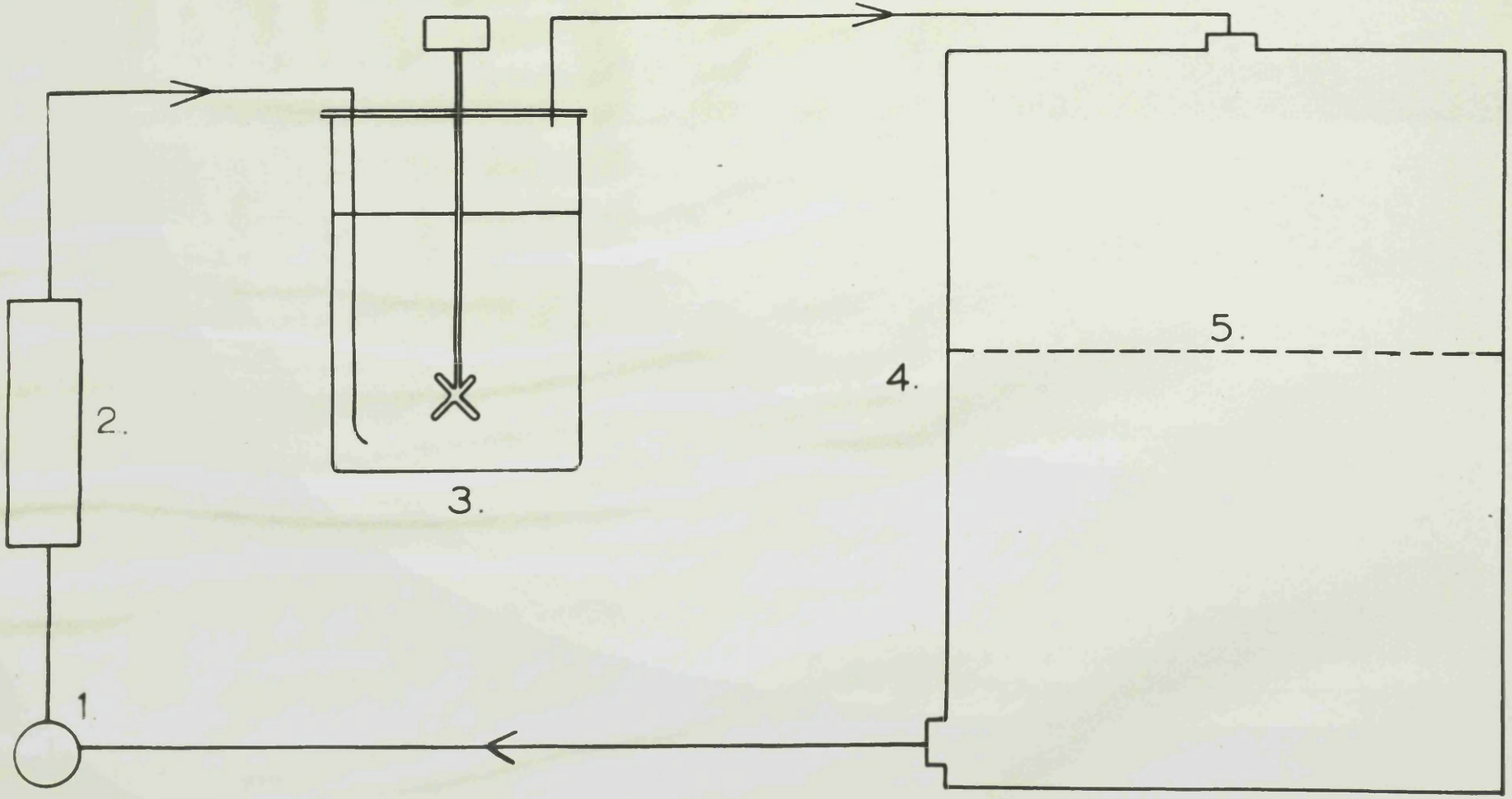
The effect of gas streams from deep culture vessels on zygospore formation in R. sexualis.

	No. of zygospores per unit area											
Plate no.	Gas stream from deep culture of R. sexualis					Rep. Tot.	Gas stream from sterile medium.					Rep. Tot.
1	28	27	28	29	26	138	17	16	13	14	16	76
2	26	27	31	29	28	141	14	18	17	16	15	80
3	28	28	32	30	28	146	21	17	18	20	18	94
4	32	28	25	26	32	143	14	22	17	19	16	88
5	29	33	30	26	27	145	22	23	18	20	25	108
6	30	29	31	33	35	158	21	25	23	21	19	109
7	31	32	34	27	34	158	25	22	24	20	25	116
8	32	27	29	27	28	143	29	23	30	20	26	130
9	31	34	26	27	30	148	22	28	25	24	21	120
10	27	28	26	27	33	141	21	20	19	20	19	99

Statistical analysis of the above results gave variance ratios:-

Between tests F_9^1 = 83.8 (5.1 required for 5% significance)

Between plates F_9^9 = 2.1 (3.25 ")



1. Circulating pump.
2. Sterile cotton wool filter.
3. Culture vessel.
4. 250 L. gas reservoir
5. Muslin baffle.

Fig. 3

B.5.

Deep culture with recycling of air.(Fig.3)

As a further measure towards increasing the concentration of metabolites produced, a system of recycling the air passing through the culture vessel as described in B.3. was introduced.

A 250 litre galvanised iron drum was made airtight to act as gas reservoir and fitted with narrow bore inlet and outlet tubes. Muslin stretched across the diameter of the drum acted as a baffle to give greater circulation of the gases within the drum.

B.6. REMOVAL OF WATER VAPOUR.

Methods used:

(i). Phosphorous pentoxide and Calcium chloride.

The first substances to be tried were these conventional ones. When packed in 150 x 5cm. diameter tubes both proved ineffective in removing much water vapour from the air stream under conditions of such high humidity, and were thus discarded.

(ii). Molecular sieves (B.D.H. Type 4A).

Approximately 150gm. of these packed in a tube 30 x 4mm. proved efficient in that icing of the cold trap was prevented, but analysis of the flask contents gave no positive results. Thus, since molecular sieves have a very high affinity for adsorption of low molecular weight substances, it seemed likely that the organic substances sought had also been adsorbed.

(iii). Physical dehydration.

To avoid difficulties of adsorption of organic compounds it was decided to investigate the possibility of using a strictly physical method for removal water vapour from the airstream, i.e. cooling the air to a temperature below the dew point in order to condense the water. Two methods were tried:-

(a). Ethylene glycol bath.

The gas stream was passed through a flask immersed in ethylene glycol refrigerated to -10°C. This temperature proved to be insufficiently low to condense out the water vapour, but was the minimum attainable with existing refrigeration apparatus.

(b). Drikold packing.

A temperature of approximately -25°C was maintained in a tube 150 x 5mm., surrounded by Drikold. Gases passed through this were freed from a large proportion of their water vapour, so that subsequent trapping in liquid Nitrogen could be attempted.