The Influence of Certain Fungi on the Sporulation of Melanospora destruens Shear and of Some Other Ascomycetes.

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With Plate V.

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1. HISTORICAL.

THE first record of the action of one organism in stimulating the sporulation of another is that of Molliard (9) in 1903. He noticed that a species of Ascobolus, growing on carrot, produced apothecia when the cultures were contaminated by a bacterium, but did not fruit in pure cultures.

A similar effect was noted by Sartory (14, 15, 16). He claimed that the presence of bacteria was necessary for the sporulation of a yeast and for the production of the perithecia of a species of Aspergillus. The stimulating effect of the particular bacterium was shown more clearly on certain media than on others.

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In 1909, Heald and Pool (5) published the first record of the stimulatory effect of one fungus on the sporulation of another. They worked with *Melanospora pampeane* Speg., which gave only a scanty white mycelium when grown in pure culture, but which produced perithecia in abundance when grown in mixed culture with *Fusarium moniliforme*. Similar stimulatory effects were produced by *F. culmorum*, *Basisporium gallarum* and an undetermined species of Fusarium, but not by another undetermined species of Fusarium. The mycelial growth produced by *B. gallarum* or *F. moniliforme* was removed, and the media were sterilized at 110° C. and inoculated with *M. pampeane*. Perithecia were formed within a few days on media prepared from young cultures of Basisporium or Fusarium, but growth of the Melanospora was inhibited in media prepared from older cultures. Heald and Pool concluded that certain chemical compounds are necessary for the development of the perithecia of *M. pampeane*, and that these compounds are regularly produced by *B. gallarum* and *F. moniliforme* and, possibly, by other fungi.

Zeller and Schmitz (20), working with mixed cultures, showed that the usual effect of one organism on another was a mutual inhibition of growth, before or after actual contact of the mycelia took place. Only in a few cases did one fungus colony grow over another, and very rarely was any actual stimulation of growth observed. In some cases, however, the sporulation of one organism was stimulated by the presence of another. Thus there was an increase in the number and size of conidiophore heads of *Aspergillus Sydowi* when in contact with *Merulius pinastri* and of *A. niger* when in contact with *A. glaucus*. Zeller and Schmitz state that they found no definite relation between the acidity produced by these fungi and their ability to inhibit or stimulate the growth and sporulation of other fungi.

Porter (12), experimenting with a number of bacteria and fungi, found evidence of growth changes and antagonistic action occurring in mixed cultures. He observed that the action of one organism on another stimulated sporulation along the line of contact between the colonies. In many cases malformation also occurred. With a species of Helminthosporium he was able to obtain somewhat similar effects, on both sporulation and malformation, by the use of certain poisons.

McCormick (8) found that no perithecia were formed in monoconidial cultures of *Thielavia basicola* Zopf, whereas when ascospores were used as inocula perithecia were formed. Mono-conidial cultures, however, readily produced perithecia when grown in mixed cultures with *Cladosporium fulvum*, *Aspergillus umbrosus*, *A. glaucus*, *Eurotium amstelodami* and *Fusicladium pirinum*.

In contrast to the results of Heald and Pool (l.c.), McCormick was unable to demonstrate any activity in fungal extracts which had been

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heated to 110° C. for twenty minutes, but extracts of active fungi sterilized by passage through a Berkefeld filter did stimulate perithecial formation.

Wilson (19) stated that, in the presence of certain fungi, *Venturia inaequalis* formed perithecia more abundantly in a zone near the periphery of the contaminating colony. A mycelial extract of *Penicillium* sp. gave a similar result. Autoclaving diminished the activity of the extract.

The effects of colonies of *P. glaucum* and of extracts of this fungus on the growth and sporulation of a number of other fungi were described by Sibilia (17). The Penicillium extract markedly stimulated conidial production in *Microcera coccophila*, but the latter did not produce conidia when grown in mixed culture with *P. glaucum*. Vegetative growth was stimulated in a number of other fungi.

All the papers mentioned above describe the stimulatory effect of one organism on the sporulation of another. Nadson and Jolkevitch (11), however, describe the antagonism between *Spicaria purpurogenes* and *Saccharomyces cerevisiae* and state that, when these two organisms are grown together, the yeast is killed and the formation of conidia by Spicaria is completely inhibited.

Gwynne-Vaughan (4), in a review of the question of sex and nutrition in the fungi, cited the case of *Humaria granulata*, a heterothallic form in which, although both strains produced well-grown female organs, ascogenous hyphae were not formed unless both + and — strains were present. The behaviour of this fungus led Gwynne-Vaughan to seek an explanation of heterothallism which was not based on a difference in sex but on a difference of metabolism of the one strain brought about by the presence of the second strain.

Wineland (18), finding that two strains of *F. moniliforme*, neither of which produced the perfect stage in pure culture, did so when mated in the same culture tube, suggested that these facts might be interpreted as a case of normal heterothallism, or that alternatively they might be accounted for by the chemical stimulation of one strain by the other.

Working with strains of Glomerella, Egerton (3) showed that, although both so-called + and — strains of this fungus could form fertile perithecia in pure culture, they did so more readily when in contact with one another. This suggests a form of nutritive heterothallism or chemical stimulation.

Moreau and Moruzi (10) claimed that neither the union of nuclei nor the contact of hyphae of + and — strains of *Neurospora sitophila* was necessary for the production of fertile perithecia. They grew two strains of this fungus (neither of which produced fertile perithecia in pure culture, but which did so when grown together) in opposite ends of a U-tube containing culture medium. After some time fertile perithecia were produced at one end of the tube. Since it was claimed that no mycelium was present in the central part of the tube, these authors concluded that the production

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of perithecia was due to the diffusion of some substance through the culture medium from one colony to the other.[1]

Das Gupta (2) reported that two infertile saltants of *Cytosporina ludibunda* produced pycnidia when the mycelia were allowed to intermingle. These saltants were strikingly unlike in morphological character. Das Gupta thought it unlikely that the difference between them was sexual in nature, and concluded that the formation of pycnidia when the two strains intermingled was a form of 'nutritive heterothallism'.

2. Experimental Methods.

A strain of *Melanospora destruens* Shear, which was isolated from a diseased apple in the Plant Pathological Laboratory of the Imperial College of Science,[2] showed a striking behaviour in that, while it formed perithecia in pure culture on certain media, it did so more freely when certain contaminating organisms were also present.

Stock cultures of the strain of *M. destruens* were maintained in tubes of 1·5 per cent. malt agar at laboratory temperature. In order to reduce the risk of the cultures becoming sterile, perithecia were included in the inoculum at each successive reculturing.

Petri-dish cultures, incubated at 25° C. were used for the study of perithecial formation. The methods of experiment were generally of the type usual in mycological work. Special methods were occasionally used and will be described in the appropriate places in the text.

It was necessary to devise a method of measuring perithecial frequency on any given plate. The perithecia of *M. destruens* have light coloured walls but black spores, so that when mature they appear black and are readily visible to the naked eye. In any plate which had been inoculated at the centre the density of perithecia varied somewhat from one part to another, usually increasing from the centre outwards. Plates of standard size (11 cm. diameter) were always used. These were divided into ten equal sectors by drawing ten equidistant radii on the bottom of the plate, the first radius being drawn at random. The number of perithecia per microscopic field (at a magnification of 60) was determined for four points on each radius, these points being at arbitrary distances from the centre. Thus the figure given for perithecial frequency for each plate is the average of forty determinations. In each experiment the number of plates was replicated at least five, and often ten, times.

The number of perithecia per unit area on any particular medium

[1] An attempt was made to repeat this experiment during the present work. This was unsuccessful, since even with a depth of 5 cm. of medium in the U-tube the hyphae grew through from one side to the other.

[2] Isolated by Dr. A. S. Horne and identified by Mr. Mason of the Imperial Mycological Institute.

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varied with the depth of medium in the plate. Accordingly, throughout the investigation, each plate of standard size received approximately 50 c.c., so that variations in perithecial frequency due to differences in depth of medium were avoided.

As time went on, sporulation of the Melanospora became progressively less free, so that the values for perithecial frequency on a standard medium are not comparable throughout this paper. Values given in any one Table are strictly comparable.

3. Factors Influencing Sporulation in Pure Cultures of *M. Destruens*.

(a) *Nature of the Nutrient Medium.*

In testing the effects of various contaminating organisms on sporulation of *M. destruens* it was desirable to avoid media which were either entirely unsuitable for the formation of perithecia, or on which they formed in such abundance that any further increase in their number would be difficult to detect. A suitable medium for this purpose would be one on which fruiting occurred rather sparingly.

Table I.

	Medium.	Perithecial frequency.
1.	3 % Malt	8·5
2.	Coons' solution	2·8
3.	Glucose 0·5 %, peptone 0·35 %	6·8
4.	Glucose 1 %, peptone 0·2 %	5·2
5.	Maltose 0·5 %, peptone 0·35 %	3·8
6.	Glucose 0·5 %, potassium nitrate 0·35 %	2·4
7.	Glucose 0·5 %, potassium nitrate 0·2 %	2·2
8.	Glucose 0·5 %, asparagin 0·35 %	2·1
9.	Glucose 0·5 %, asparagin 0·2 %	0·4
10.	Glucose 0·5 %, ammonium nitrate 0·35 %	0
11.	Glucose 0·5 %, ammonium tartrate 0·35 %	0

The number of perithecia per unit area, produced in fourteen days on a variety of media, is illustrated in Table I. The synthetic media 3—10 contained the following basal salt mixture:—KH_2PO_4, 0·125—0·175 per cent.; $MgSO_4$, 0·075 per cent., with 1·5 per cent. agar.

The medium 6 (which will afterwards be referred to as (Medium A), on which moderate perithecial formation took place was chosen for further work. The formula of Medium A is: glucose, 5 gm.; KNO_3 3·5 gm.; KH_2PO_4, 1·75 gm.; $MgSO_4$, 0·75 gm.; agar, 15 gm.; water, 1 litre.

Table I shows that the medium most favourable to perithecial formation among those tested was malt agar. One of the glucose peptone media was almost as good. Of the sources of nitrogen tested peptone was the best and salts of ammonia the worst. These results were amply confirmed by further experiments.

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The number of perithecia formed on the full Medium A was compared with the number formed on a series of media from which a single constituent was lacking. This experiment was repeated several times, but variation in perithecial frequencies between individual plates of the same batch was sufficiently great seriously to diminish the significance of some of the results. The following results, however, were significant. The absence of phosphate materially reduced the amount of growth of the Melanospora and no perithecia were formed. Growth was less seriously reduced by the absence of nitrogen,

TABLE II.

Medium used.	Perithecial frequency. 6 days after inoculation.	14 days after inoculation.	27 days after inoculation.
A	0	0·22	2·4
0·6 A	0	1·3	2·6
0.2 A	0·6	1·8	2·0
Plain agar	0	0·06	0·4
A (0·6 G)	0	1·4	2·4
A (0·2 G)	1·7	2·26	2·3
A (0 G)	0·32	0·4	0·6
A (0·6 N)	0	0·2	2·3
A (0·2 N)	0	0·2	2·1
A (0 N)	0	0	0
A (0·6 P)	0	0·2	2·2
A (0·2 P)	0	0·18	2·3
A (0 P)	0	0	0

but sporulation was almost completely inhibited, a few scattered perithecia being formed about seven weeks after inoculation of the plates. The absence of magnesium sulphate did not significantly decrease either growth or perithecial frequency. Fruiting was actually hastened, by about four days, by absence of glucose, but growth was poor, and after three weeks there were normally more perithecia on Medium A than on that medium lacking glucose.

The Melanospora was grown on Medium A and on dilutions of that medium to 0·6 and 0·2 of the original strength (given as 0·6 A and 0·2 A in Table II) and on plain agar. It was also grown on a series of modifications of Medium A in which the concentration of glucose was reduced to 0·6 and 0·2 of that in the standard medium, the other constituents remaining unaltered [given as A (0·6 G) and A (0·2 G) in the Table] and on Medium A lacking glucose [A (0 G)]. Media in which the concentration of potassium nitrate and potassium dihydrogen phosphate were similarly reduced to 0·6 and 0·2 of that in the standard medium, or were lacking, were also used [given as A (0·6 N), A (0·2 N), A (0 N), A (0·6 P), A (0·2 P), A (0 P) in the Table]. The results of this experiment are given in Table II, the figures referring to perithecial frequency.

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The results obtained by growing the fungus on a series of dilutions of Medium A and on a series of media in which the glucose content was varied were thus very similar. Dilution of the medium to 0·6 of its original strength or reduction of the glucose content to 0·6 of that in Medium A both led to an earlier production of perithecia. A similar reduction of the amount of nitrate or phosphate did not hasten fruiting, reduction of either of these to 0·2 of their original concentration having no significant effect. It is thus suggested that the stimulatory effects on sporulation of diluting the standard medium are largely due to the reduction in glucose content.

These results were confirmed by further experiments, and the fact was established that the actual concentrations of glucose and nitrogen present had a greater effect on sporulation than did the ratio of these, since with the same C/N ratio, widely different numbers of perithecia were formed. A similar statement was made by Robinson (13) for apothecial formation in *Pyronema confluens*.

The hydrogen-ion concentration of the medium had no marked effect on perithecial frequency within wide limits. Malic acid or sodium bicarbonate was added in varying quantities to the normal Medium A (pH 6·2) to give a range of pH from 3·5 to 8·6. All the media on which the Melanospora developed became slightly more acid during the experiment. Over the range of initial pH 4·8 to 7·6 there was no marked difference, either in mycelial development or perithecial formation. Outside these limits mycelial growth was reduced considerably, and only a few scattered perithecia were formed.

Krause (6) also found that, within wide limits, pH had no influence on the fruiting of seven species of Hypocreaceae, including a species of Melanospora.

(b) *Transference from One Medium to Another.*

Leonian (7), who made a detailed study of the factors promoting pycnidium formation in the Sphaeropsidales, stated that sudden transference from a concentrated to a dilute medium led to increased sporulation in some species, but had the opposite effect in others. He worked chiefly with liquid cultures.

In the present investigation the method adopted for transferring the fungus from one medium to another was to cut out a segment of agar medium (of standard size) in advance of a growing colony, and to replace it by a medium of different concentration. Petri dishes of Medium A were inoculated at the centre with *M. destruens*, and segments of the medium were cut out and replaced by media of strength 0·75 A, 0·5 A, 0·25 A, 0·1 A, 0·01 A or plain agar. Perithecia developed at the periphery of the segment, where a more dilute medium had been inserted, and this

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zone of perithecia slowly spread inwards towards the centre. A typical culture plate from this series is illustrated in Pl. V, Fig. 3. Sporulation was greatest when the medium was replaced by 0·1 A, 0·01 A, or plain agar, and diminished with increasing concentration of the replacing medium. The perithecia began to form in the replaced segment within ten days from the time of inoculation, but later were scattered over the whole plate.

Transference of the fungus from Medium A to a weaker medium thus stimulated an earlier production of perithecia. This result is comparable with Claussen's well-known experiment with *P. confluens* (1).

(c) *Temperature.*

The rate of growth of *M. destruens* rose to a distinct optimum at a temperature of approximately 25° C. The optimum temperature for sporulation was also in the neighbourhood of 25° C., but the number of perithecia formed was remarkably uniform over the range 15–35° C.

(d) *Light.*

The effect of light was tested by exposing plates on the laboratory bench side by side with others wrapped in black paper. There was no significant difference in the number of perithecia formed, but those produced in continuous darkness were approximately 50 per cent. smaller.

(e) *Mechanical Barrier to Growth.*

When *M. destruens* was grown on a standard-sized plate of Medium A, perithecia developed first at the edge of the plate; and, at a later stage, were usually less numerous at the centre, than towards the edges (see Pl. V, Fig. 2). To test whether sporulation was stimulated by the presence of a mechanical barrier to growth, the fungus was grown on plates 4, 7, 9, 11, 14, and 20 cm. diameter. Perithecia were produced in all after two weeks. They arose first at the margin of the colonies, and extended towards the centre. In the smallest plate the growing margin of the culture reached the edge in four days, but no perithecia were formed until ten days later. In the largest plate they were visible on the fourteenth day, although the growing colony only extended part way across the plate. Sporulation was thus not induced by contact with the edge of the plate, but presumably first took place in the young parts of a colony of a suitable age.

Attempts to stimulate sporulation by cutting or rubbing the mycelium were unsuccessful.

Robinson (l.c.) was able to stimulate the formation of the apothecia of *P. confluens* by the use of various chemical checks to growth. Attempts to stimulate sporulation of the Melanospora by similar methods failed.

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4. The Influence of Certain Fungal Colonies on the Fruiting of *M. Destruens* and of Certain other Ascomycetes.

It has already been stated that *M. destruens* produced perithecia more readily in the presence of certain other organisms than in pure culture. Thirty species of fungi, together with two unidentified species of bacteria were tested in this connexion. Plates of Medium A were inoculated with the Melanospora, and with one of the other organisms, the two inocula being placed about 4 cm. apart.

Some of the organisms used as contaminants, viz. *Fusarium fructigenum*, *F. bulbigenum*, *Penicillium* sp., *Botrytis cinerea*, *Monilia fructigena*, and two unidentified species of bacteria caused a distinct ring of perithecia to be formed round their margins. Pl. V, Figs. 4 and 5 illustrate this type of effect.

With a second group of contaminants, *Sclerotinia trifoliorum*, *Gloeosporium fructigenum*, *Helminthosporium* sp., *Hormodendron* sp., and *Stagonospora Curtisii*, there was a definite zone of clear medium between the two colonies, across which neither fungus was able to pass. Perithecia were formed along the margins of the Melanospora colony in a distinct ring. Pl. V, Figs. 6, 7, and 8 show this type of behaviour.

The hyphae of a third type of contaminant, viz. *Phytophthora erythroseptica*, *Rhizopus nigricans*, *Absidia glauca*, *Blakeslea trispora*, *Sphaeropsis Malorum*, *Colletotrichum linicolum*, *Fusarium culmorum*, *Botrytis Allii*, and *Ascochyta Pisi*, intermingled with those of the Melanospora colony, the perithecia of the latter being formed on or below the surface of the contaminant colony. (See Pl. V, Fig. 9).

The fungi mentioned above caused an earlier production of perithecia by *M. destruens* when planted on plates of Medium A at the same time as the Melanospora was planted. Others, however, showed no stimulatory effect under these conditions, Certain fungi of this type, viz. *Mucor hiemalis* + and − strains, *Cunninghamella elegans* and *Pythium de Baryanum*, caused the formation of a distinct ring of perithecia round their margins if planted two to three days after the Melanospora. Others, viz. *Penicillium* sp., *Pestalozzia Hartigii*, *Rhizoctonia Solani*, *Sclerotinia cinerea*, and *Trichoderma* sp. produced a similar effect if planted one to two days before the Melanospora. Some of the organisms listed above as stimulating sporulation when planted simultaneously with *M. destruens*, failed to produce this effect if planted at a different time. Thus there is seen to be a very delicate balance between the Melanospora and the 'contaminant' which is upset if either be allowed to develop sufficiently rapidly to prevent normal growth of the other. If the contaminant used be either a rapidly growing organism, such as *M. hiemalis* or *C. elegans*, or be given sufficient start before the plate is inoculated with *M. destruens*, the latter is unable

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to develop normally, and formation of perithecia is checked or actually inhibited. If, however, the contaminating organism be slow growing, or if the plate be inoculated with Melanospora some days before inoculation with the contaminant, then growth of the latter is insufficient to have any significant effect on the former.

Two of the fungi tested, viz. *Sporodinia grandis* and *Basidiobolus ranarum* produced negligible growth on Medium A. These also stimulated fruiting of *M. destruens* if plates of weak malt agar were used.

The effects of some of these fungi on certain other Ascomycetes were also investigated. *Fusarium fructigenum* and *Botrytis cinerea* were selected as suitable contaminants, and their effects were tested on eight species of Ascomycetes, viz. *Sordaria fimicola*, *Sordaria* sp., *Philocopra* sp., *Rosellinia necatrix*, *Chaetomium cochlioides*, *Ascobolus denudatus*, *A. viridulis* and *A. Leveillei*. Plates of Medium A were inoculated at the same time with the two fungi. The effects of the contaminants were not the same in every case, three different types of response being shown.

It has already been recorded that the presence of a colony of *F. fructigenum* or *B. cinerea* caused the formation of a distinct ring of perithecia by *M. destruens*. A similar response was shown by *Sordaria* sp., *S. fimicola*, *A. denudatus*, *A. viridulis*, and *A. Leveillei*. For example, perithecia of the *Sordaria* sp. were not formed in pure culture on Medium A until twenty-six days after inoculation, but a distinct ring of perithecia was formed round a colony of *F. fructigenum* six days after inoculation.

R. necatrix and *Philocopra* sp. fruited in both control and 'contaminated' plates. There was no concentration of perithecia round the edges of the contaminant colony, but they were formed earlier, and in greater numbers in the presence of the latter than in pure culture.

The strain of *C. cochlioides* used formed perithecia with equal rapidity, and in approximately equal quantity in both contaminated and pure cultures. This fungus was then tested in mixed culture with *M. destruens* when the sporulation of the latter was strikingly stimulated.

5. The Effects of Previously 'Staled' Media.[1]

Botrytis cinerea, *Fusarium fructigenum*, *Gloeosporium fructigenum*, *Sclerotinia trifoliorum*, *Monilia fructigena*, *Cunninghamella elegans*, *Penicillium* sp. and *Helminthosporium* sp. were grown in conical flasks containing Medium A without agar. In the first few experiments the liquid was decanted off after ten to fourteen days, and a strong agar solution was added to it so that the mixture contained 1·5 per cent. agar and set when cool. Segments were cut from plates of Medium A, and were replaced by

[1] In this paper a 'staled' medium means one in which a fungus has grown, irrespective of whether it has reached the stage at which growth is checked.

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the agar medium prepared from the 'staled' liquid. The plates were then inoculated at the centre with *M. destruens*. In some cases the Melanospora was planted four days before the replacement of the segment took place. These experiments were in general vitiated by the fact that spores of the staling agent were carried over with the staled medium. The effects produced were, however, very distinct, and where the Melanospora was planted some days before the replacement of the segment perithecia were produced earlier than could be accounted for by the development of colonies from the spores carried over in the medium.

Tests showed that the effects of the staled media were not significantly reduced by autoclaving for fifteen minutes at 115° C. In the later experiments, therefore, the staled liquid was strained through muslin, powdered agar was added, and the medium was autoclaved. In some cases tests were made by the replaced segment method, in others plates were poured with the staled medium and inoculated at the centre with *M. destruens*. The latter method had the advantage that quantitative estimates of perithecial frequency could be made by the method described above.

Certain of the fungi which it was desired to test did not grow well in liquid Medium A. These fungi were grown on plates of agar Medium A and, after a suitable interval, the medium was melted, strained, and reautoclaved. These two methods of preparation of staled media produced comparable results.

(a) *Replaced Segment Method.*

Sporulation of *M. destruens* was stimulated when the replaced segment consisted of medium staled by any of the fungi used in these experiments. If the staled medium were prepared from a young culture of any of the fungi tested the resulting effect was similar to that produced by replacing a portion of Medium A with a weaker medium. See p. 331 and Pl. V, Fig. 3.) In this case fruiting began at the outer margin of the inserted segment and progressed inwards. If, however, the inserted medium were prepared from older cultures, the perithecia were formed in a zone along the line of junction of the two media (Pl. V, Fig. 10). This zone then spread outwards or, in extreme cases, growth of the Melanospora was checked and perithecia were limited to the zone of junction of the media.

Similar results were obtained by growing the Melanospora in a small Petri dish containing Medium A, placed inside a larger one containing a staled medium, after the manner of Claussen's experiment (l.c.).

(b) *The Use of Whole Plates of Staled Medium.*

When plates poured with a staled medium prepared from fairly young cultures of any of the eight fungi listed above (p. 334) were inoculated with *M. destruens*, perithecia were formed more rapidly and more abundantly

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than on Medium A. The intensity of this effect varied with the fungus used as staling agent.

Medium A on which *M. destruens* had been growing was autoclaved and reinoculated with the fungus. A slight, but significant, increase in perithecial frequency was observed. This increase was greater when the original cultures had already fruited before the medium was reautoclaved than when younger cultures were used.

TABLE III.

Days after inoculation.	Medium A.	Media staled by *B. cinerea*.		
		7 days.	18 days.	28 days.
6	0	0	1·0	0·3
13	0	2·2	2·3	0·7
21	2·2	3·0	3·4	0·7

TABLE IV.

Medium used.	Perithecal frequency.	
	6 days after inoculation.	20 days after inoculation.
A	0	0[1]
$A_B \times 4$	4·2	5·0
$A_B \times 3$	2·2	3·9
$A_B \times 2$	1·8	3·5
A_B	1·0	2·0
$A_B \div 2$	0·8	0·9
$A_B \div 10$	0	0·5
$A_B \div 30$	0	0
$A_F \times 3$	1·0	2·0
$A_F \times 2$	0·6	1·3
A_F	0·5	1·0
$A_F \div 2$	0·3	0·8
$A_F \div 10$	0	0.3
$A_F \div 30$	0	0

The fruiting of the eight species of Ascomycetes listed above (p. 334), with the exception of Chaetomium, was quicker and more dense on plates of staled medium prepared in the usual manner on Medium A. Chaetomium fruited freely on fresh or staled medium.

The age of the culture from which the staled medium was prepared influenced the amount of stimulation of perithecial production by *M. destruens*. In a typical experiment flask cultures of *B. cinerea* in liquid Medium A were set up and allowed to grow at laboratory temperature for 7, 18, and 28 days respectively. The staled medium was then prepared in the usual way. The results, which are set out in Table III show that media prepared from 7 or 18 days old cultures of Botrytis had a stimulatory effect on the formation of the perithecia of *M. destruens*, but that those prepared from old cultures reduced the final number produced. Similar results were obtained with other staling agents. Accordingly staled media were always prepared from fortnight old cultures, unless otherwise stated.

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Sporulation of the species of Sordaria and Rosellinia was even more readily checked in staled media from old cultures than that of Melanospora.

The effects of concentration or dilution of the staled media were investigated. The liquid in which the staling agent had been growing was strained and either concentrated over a water bath or diluted with distilled water to give a range of concentrations. The results of a typical experiment with media staled by *B. cinerea* or *F. fructigenum* are given in Table IV and show that, within the limits of the experiment, the amount of stimulation of perithecial production runs parallel with the concentration of the staled medium. In the table A_B means Medium A staled by Botrytis, A_F means Medium A staled by Fusarium.

A culture solution which has been staled by the growth of a fungus differs from the original in containing substances produced by the fungus and in having a reduced amount of the food substances which were present at the beginning. It was of interest, therefore, to determine how far the stimulating effect of the staled solutions, described above, was effected by the complete or partial restoration of some or all of the original constituents. Table V illustrates such an experiment. By the symbol $A_B + 0{\cdot}2$ A is meant that to the solution staled by Botrytis the constituents of Medium A were added in such an amount that, apart from any residue present in the staled medium, the concentration in the mixture was one-fifth that of the fresh medium; and similarly for the other mixtures included in the Table. The amount of glucose present in the staled medium A_B was estimated as 0·22 per cent., i.e. approximately two-fifths of that originally present.

The results set out in the first six lines of the Table show that the addition of all the constituents of Medium A increases the stimulatory effect up to a point, but that further additions sharply diminish it. Nevertheless the Medium $A_B + A$, which obviously contains more of each food substance than does Medium A, is more favourable than the latter for perithecial development.

The addition of glucose alone to the staled medium gives results closely similar to those obtained when all the constituents are added. The same trend is shown where nitrate or acid-phosphate is added, but with these a higher concentration, in relation to that originally present, is necessary for the suppression of the stimulatory effect. The general result is, therefore, that the stimulating effect of a staled medium on perithecial production is removed by the addition of a sufficient amount of any of the food substances, but that a total concentration which is unfavourable in the fresh medium does not suppress perithecial formation in a staled one.

Similar results were obtained when medium staled by *F. fructigenum* was used. The effects of adding glucose to various other staled media were

[1] At the time of this experiment *M. destruens* did not form perithecia on Medium A until about 28 days after inoculation.

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tested by the replaced segment method and were in good agreement with those described above for media staled by *B. cinerea.*

The foregoing results suggest that the staled liquids contained certain substances which stimulated perithecial formation and certain others consisting partly at least of unused residues of the original medium, which acted

TABLE V.

Medium used.	Perithecial frequency. 6 days after inoculation.	20 days after inoculation.
A	0	0
A_B	1·0	2·0
A_B + 0·2 A	1·75	2·9
A_B + 0·4 A	0·85	2·5
A_B + A	0	1·0
A_B + 2 A	0	0
A_B + 0·2 G	1·9	2·65
A_B + 0·4 G	0·75	2·4
A_B + G	0	1·3
A_B + 2 G	0	0
A_B + 0·2 N	1·5	2·5
A_B + 0·4 N	1·85	2·6
A_B + N	2·7	2·9
A_B + 2 N	0	0
A_B + 0·2 P	0·8	2·3
A_B + 0·4 P	0·85	2·6
A_B + P	1·4	3·0
A_B + 2 P	0	0

A_B = medium staled by Botrytis; G, N, and P represent respectively the amount of glucose, KNO_3 and KH_2PO_4, present in medium A.

TABLE VI.

Staling agent.	Days after inoculation.	Perithecial frequency. Medium A.	Staled medium.	Ether-insoluble fraction.	Ether-soluble fraction.	Recombined fractions.
Helminthosporium sp. (staled 14 days)	9	0	1·7	6·5	0·1	—
	12	0	1·8	12·5	0·1	—
Cunninghamella elegans (staled 14 days)	8	0	0·6	10·7	0·7	0·5
	15	2·0	2·9	12·3	1·1	2·8
Fusarium fructigenum (staled 21 days)	15	2·1	No growth	9·0	No growth	No growth
Botrytis cinerea (staled 14 days)	11	0	2·3	4·0	0·5	2·4
Penicillium sp. (staled 14 days)	11	0	1·75	2·1	0·75	—

in the opposite direction. Attempts were made to separate some of these substances by fractionation with ether. For this purpose liquid media, staled by certain fungi, were shaken up in acid solution with two volumes of ether. After separation of the layers and removal of ether, agar was added to each fraction and the usual tests were made by both the standard methods. Illustrations of the results obtained, by the segment method are

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given in Pl. V, Figs. 10, 11, and 12. Perithecial frequencies obtained in a typical experiment with the whole-plate method are summarized in Table VI.

Clearly the ether-insoluble fraction gave a much more pronounced stimulatory effect than did the original staled medium. The effect of the ether-soluble fraction was, in general, comparable with that of dilution of the medium, viz. an earlier production of perithecia, but a lower final perithecial frequency. The increased activity of the ether-insoluble part was not due to the effects of traces of ether, since if the two fractions were recombined the resulting effects were similar to those produced by the use of the original staled medium.

Sporulation of the species of Sordaria and Rosellinia was also greater in the ether-insolube fraction than in the staled medium, and the ether-insoluble fraction in some instances stimulated sporulation even when the staled medium itself inhibited growth and sporulation.

6. Discussion.

The effect of one organism or of its products upon the sporulation of another may be considered to arise from a variety of causes, such as mechanical or chemical interference with growth, change in the reaction or in the osmotic pressure of the medium, reduction in concentration of food substances or production of substances which induce or favour sporulation. The discussion of the factors responsible will now be taken up in the light of the foregoing results.

When *M. destruens* is grown in Petri dishes of about the ordinary size used in culture work, sporulation begins some little time after the fungal colony has reached the edge of the plate. Mechanical interference with growth might thus be suggested as an inducing factor. There is, however, no connexion, as the experiments with large plates quoted above (p. 332) have shown. Under any given set of conditions sporulation begins after a certain time interval, independently of whether the culture has reached the edge of the plate or not. Moreover mechanical interference cannot explain the increased sporulation on certain staled media.

The association of sporulation with a chemical check to growth is suggested by such appearances as are illustrated in Pl. V, Fig. 6, and Pl. V, Figs. 7 and 8, where a zone of clear medium separates the 'contaminant' colony from that of the Melanospora, perithecia being formed on the margin of the latter. Here again the two effects are not necessarily correlated. Experiments described above have clearly shown that sporulation may occur on media prepared from young or, in certain instances, from old cultures of the staling organism. Whereas media prepared from old cultures reduce the rate of growth of the Melanospora, those prepared from younger cultures do not, and usually give a growth rate of the fungus

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greater than that on the unaltered medium. The only effect of growth-retarding or inhibiting fungal products on sporulation is to determine where, under certain experimental conditions, the formation of the perithecia occurs (as in Pl. V, Fig. 10), or, when such substances are present in large amount, to suppress perithecial formation altogether (see Table VI).

The acidity of the various preparations which influence sporulation is, within wide limits, of no importance. Adjustment of various staled liquids to the pH of the original medium does not reduce the stimulating effect, and the fungus sporulates freely over a wide range of initial pH.

Changes in concentration of medium have a distinct effect upon sporulation, but not in any direct relation to osmotic pressure. Thus increased concentration of the fresh medium, above a certain point, suppresses sporulation (Table II), while increased concentration of certain staled media increases it (Table IV). The addition of various food substances, and especially of glucose, to staled media reduces or suppresses their spore-promoting quality (Table V). Conversely dilution of a fresh medium (Table II), or transference from one medium to a more dilute one, as when the culture of Melanospora is allowed to grow from Medium A on to a segment of diluted agar (Pl. V, Fig. 3), stimulates sporulation. It is clear from these results that, while the concentrations of the various media have a marked effect on sporulation, the effect is not simply due to osmotic relationships. The latter point was also proved directly in an experiment in which osmotically equivalent amounts of sodium chloride, potassium nitrate, or magnesium sulphate were added to a staled medium, with widely different effects on the amount of sporulation.

It has already been mentioned that dilution of the fresh medium increases sporulation, and it has been shown above that reduction of concentration of certain of the food substances in the original medium has a similar effect. Such a reduction in concentration of food substances must necessarily have been brought about in the staled media by the organism acting as staling agent. This in itself is insufficient to account for the stimulatory effects of staled media, since an increase in sporulation far greater than the maximum brought about by dilution of the fresh medium was observed in certain cases. Moreover increased concentration of the staled medium led to increased sporulation.

The results of the present paper and of a further one in course of preparation are readily interpreted on the hypothesis that many fungi produce a substance or substances which stimulate sporulation. In the culture solution in which a fungus has grown there would, therefore, be three sets of substances, viz. the stimulatory substances referred to, staling products which tend to slow down growth, and unused constituents of the original medium. It is known from the work described above that the last of these, and especially glucose, tend to negative the action of

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the stimulatory factor. The effect of staling substances would be to depress the activity of the Melanospora if they were present in sufficient quantity, i.e. if the cultures from which the media were prepared were old or if the 'contaminant' fungus were of a strongly staling character. By the interplay of the three factors indicated the behaviour of the Melanospora in mixed culture and on staled media, as described above, can be explained. Thus, in mixed cultures, it was noted (p. 333) that there was a delicate balance between the Melanospora and the contaminant which was upset if either colony were allowed to develop at the expense of the other. It may be assumed that slow growing organisms are only able to influence the sporulation of Melanospora if given time to develop sufficiently to enable an effective quantity of the spore promoting substances to be formed. Strongly staling organisms, however, are liable to produce sufficient staling substances to inhibit perithecial formation if they have been growing for some time before contact with the Melanospora colony takes place.

The response of the fungus to certain staled media can also be explained by a consideration of the interplay of the three sets of substances mentioned above. The favourable effects of an increased concentration of the staled media can be attributed to an increased concentration of the stimulatory substances, while the poor effects of media prepared from old cultures (Table III) can be explained by the presence of staling substances. The increased stimulation afforded by the ether-insoluble fraction of a staled medium (Table VI) is then explained by the removal of these substances by the ether. Since the fractionation with ether was carried out in acid solution it is probable that organic acids would be the substances in question. The further consideration of the nature of the active principle will be given in a later paper.

The active principle, it will be noted, is produced by Melanospora itself (p 336) but not so rapidly or in such large quantity as it is produced by certain other fungi. On this account, presumably, a beneficial effect on sporulation is shown in the presence of certain other organisms or of their products.

7. Summary of Results.

1. The formation of perithecia by *M. destruens* is markedly influenced by the composition of the nutrient media used. Details of the effects of various constituents are given in the text.

2. Transference of the fungus from one medium to a weaker one stimulates perithecial formation.

3. Temperature, light, pH concentration of the medium, mechanical checks to growth have, within wide limits, little effect on sporulation.

4. Sporulation of *M. destruens* and some other Ascomycetes is stimulated by the presence in the culture plates of certain other organisms or of

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products derived from the latter. The activity in this respect of various 'staled' solutions increases with the degree of concentration but is diminished by the addition of a sufficient amount of various food substances.

5. Particularly active stimulation of fruiting is brought about by the ether-insoluble fraction of 'staled' solutions.

6. It is concluded that the intensity of sporulation in a 'staled' medium is determined by a combination of three factors, viz. reduction in food concentration by the organism used as 'staling agent', the production by the organism of inhibitory substances, and the production of a substance or substances which stimulate perithecial formation and which are formed but slowly by *M. destruens* itself.

The writers wish to express their thanks to Professor W. Brown for suggesting this investigation and for his interest and direction during its progress.

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EXPLANATION OF PLATE V.

Illustrating the paper by Dr. Asthana and Dr Hawker on 'The Influence of Certain Fungi on the Sporulation of *Melanospora destruens* Shear and of Some Other Ascomycetes'.

Perithecia, where present, can be seen with the aid of a hand lens as black dots. In Figs. 4–9 the *Melanospora* inoculum is the lower one in each case.

The Petri dishes used were 10·5 cm. in diameter.

Fig. 1. *M. destruens* on Medium A, 10 days after inoculation. No perithecia formed.

Fig. 2. *M. destruens* on Medium A, 16 days after inoculation. Numerous perithecia formed.

Fig. 3. *M. destruens*, 10 days after inoculation, original medium in plate = Medium A, medium in segment = 0·1 strength of Medium A.

Fig. 4. *M. destruens* and *Penicillium* sp. on Medium A, 7 days after inoculation. Note ring of perithecia.

Fig. 5. *M. destruens* and *Monilia fructigena* on Medium A, 12 days after inoculation. Note ring of perithecia.

Fig. 6. *M. destruens* and *Sclerotinia trifoliorum* on Medium A, 12 days after inoculation. Note clear zone between colonies.

Fig. 7. *M. destruens* and *Gloeosporium fructigenum* on Medium A, 12 days after inoculation. Note clear zone between colonies.

Fig. 8. *M. destruens* and *Helminthosporium* sp. on Medium A, 12 days after inoculation. Note clear zone between colonies.

Fig. 9. *M. destruens* and *Sphaeropsis malorum* on Medium A, 12 days after inoculation.

Fig. 10. *M. destruens*, 10 days after inoculation. Original medium in plate = Medium A. Medium in segment = medium stained by *Penicillium* sp. A few perithecia can be seen along line of junction of the segment.

Fig. 11. *M. destruens*, 10 days after inoculation. Original medium in plate = Medium A. Medium in segment = ether-insoluble part of medium stained by *Penicillium* sp. Numerous perithecia can be seen along line of junction of the segment.

Fig. 12. *M. destruens*, 10 days after inoculation. Original medium in plate = Medium A. Medium in segment = ether-soluble part of medium stained by *Penicillium* sp. Perithecia can be seen at periphery of segment (cf. Fig. 3). The dark patch along the line of junction is here due to dark hyphae and not to perithecia as in Fig. 11.

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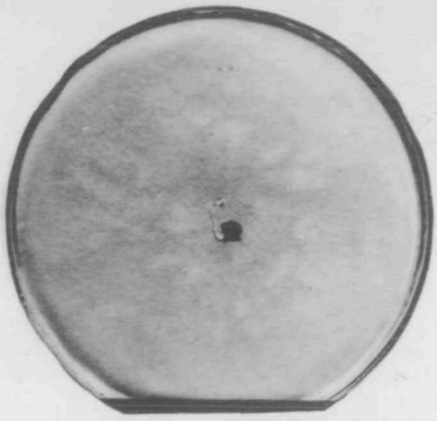

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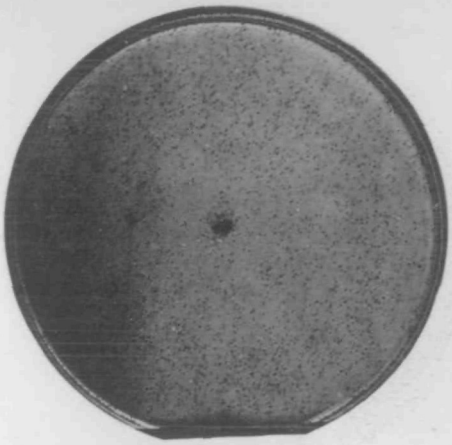

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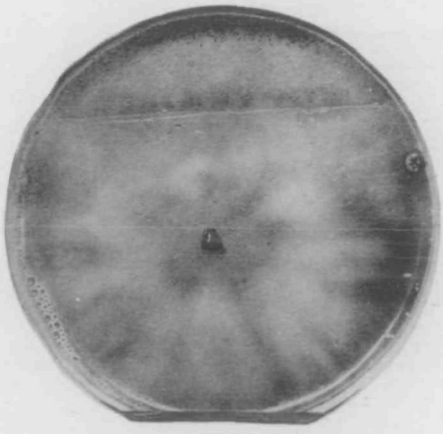

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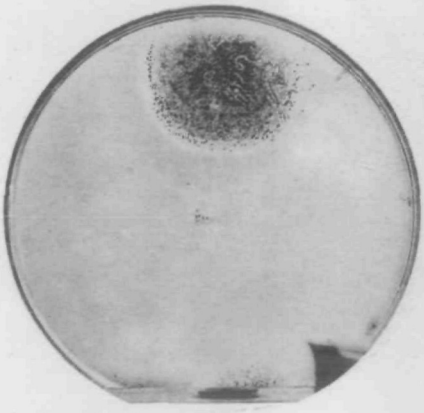

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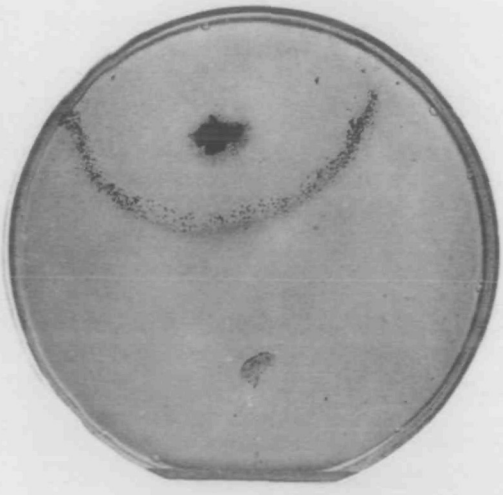

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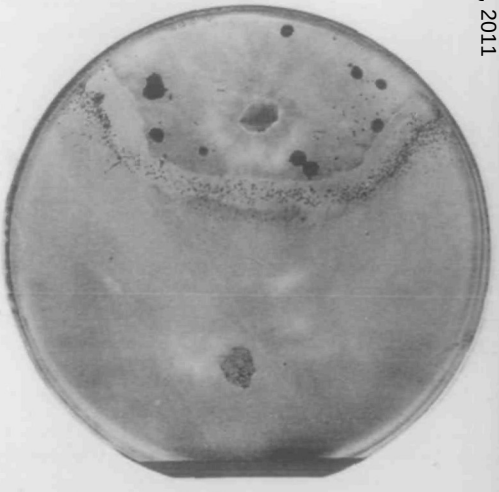

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ASTHANA AND HAWKER — MELANOSPORA.

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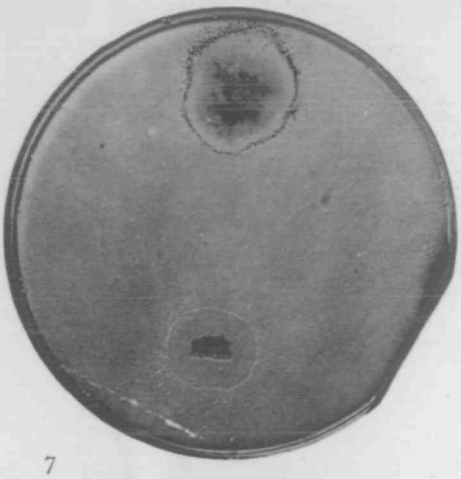

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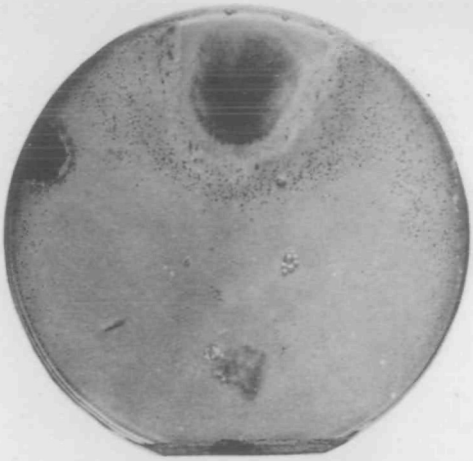

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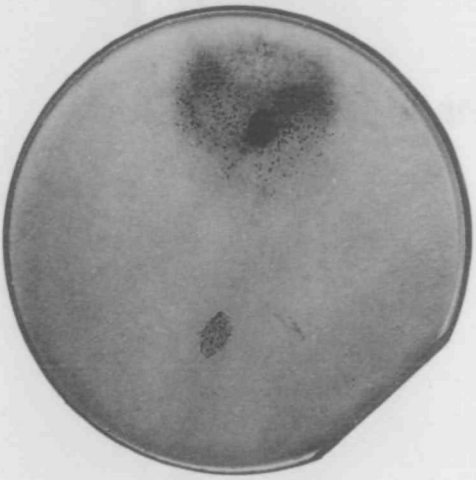

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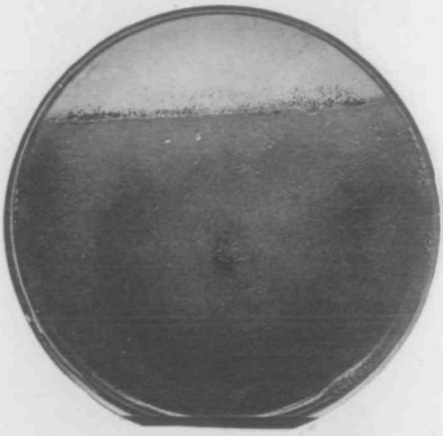

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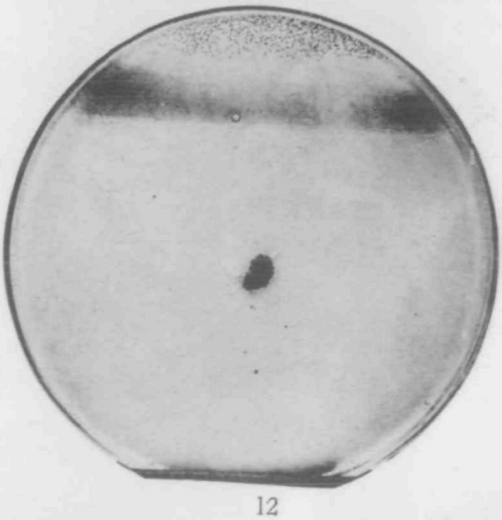

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