

LYSIS OF FUNGAL MYCELIUM BY SOIL

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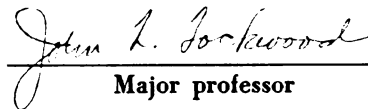
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ABSTRACT

LYSIS OF FUNGAL MYCELIUM BY SOIL

by Alan Bryce Lloyd

Soil mycolysis--the ability of soil to destroy live fungal mycelium--was assayed by direct recovery of hyphae from soil with plastic films. On Conover loam soil, mycelia of 7 fungal species lysed in 4-8 days, but some mycelia of Rhizoctonia spp. remained after 8 days. Fungi grew in sterilized soil, whereas reinfesting it with most soil streptomycetes and some soil bacteria, restored the lytic property.

Addition of glucose or peptone (0.2% of the soil's weight) temporarily annulled soil mycolysis, and fungal mycelia coexisted with large bacterial and actinomycete populations. Chitin amendment enhanced mycolysis. Live fungal mycelium, when added to natural soil (1% of the soil's weight), increased numbers of bacteria by 130-fold in 5 days and actinomycetes by 10-fold in 9 days.

Extracellular enzymes from soil microorganisms do not appear to cause soil mycolysis. Chitinase alone or in combination with other hydrolysing enzymes did not lyse live mycelia of Glomerella cingulata or Fusarium solani f. pisi, although chitinase partially degraded dead mycelium of the latter. Live mycelia of G. cingulata and Helminthosporium

victoriae autolysed completely when separated from soil by an inert membrane filter which prevented passage of enzymes. Mycelium of F. solani f. phaseoli partially autolysed. Heat-killed mycelia were consistently more resistant to soil mycolysis than live mycelia. These results are consistent with an autolytic hypothesis for soil mycolysis.

When fungal mycelia were kept in a state of continual starvation on a dialysis tube inflated with circulating mineral salt solution, and any of several antifungal antibiotics added to the mycelia, G. cingulata and H. victoriae frequently lysed completely, and F. solani f. phaseoli partly lysed. Antifungal antibiotics or starvation conditions, separately, induced only a slight degree of autolysis. Some of the assumptions made with this autolytic model were then tested in natural soil.

Evidence for starvation conditions is the low level of organic nutrients present in soil, and annulment of soil mycolysis with organic nutrients. Evidence for induction of autolysis by antibiotics in soil was the following: 1) A significant correlation ($r = 0.61$) between sizes of inhibition zones and mycolytic zones produced by the same streptomycetes on agar containing, respectively, conidia or mycelium of G. cingulata. 2) Of 8 selected bacterial isolates tested, only those 2 which inhibited spore germination also restored the lytic property to sterilized soil. 3) An antibiotic substance was extracted with ethanol from soil supplemented with live fungal mycelium (1% of the soil's weight).

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The following explanation is proposed for soil mycolysis. Soil fungistasis prevents spore germination. In the presence of organic nutrients, the spore germinates and coexists as mycelium with a large number of other soil microorganisms. On depletion of nutrients, antibiotics already formed by antagonistic microorganisms or formed with fungus itself as a substrate, induce autolysis. With some fungi, autolysis results in complete destruction of the mycelia, whereas with others, the mycelia is killed before complete autolysis. Extracellular enzymes degrade the remaining parts of the cell wall.

LYSIS OF FUNGAL MYCELIUM BY SOIL

by

Alan Bryce Lloyd

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	v
LIST OF FIGURES	vii
INTRODUCTION	1
LITERATURE REVIEW	3
METHODS AND MATERIALS	10
Preparation and assay of chitinase	10
Purification of chitinase	12
Incubation of fungal mycelium in chitinase and other enzymes	16
Mycolysis and inhibition of fungal spore germination by streptomycetes	18
Reinfesting sterilized soil with microorganisms ..	19
Direct assay for soil mycolysis	20
Supplementing soil with live fungal mycelium	21
RESULTS	23
Lysis of different fungi by soil	23
Restoring the mycolytic property to sterilized soil with streptomycetes and bacteria	31
Organic nutrients reverse soil mycolysis	33
Soil microorganisms benefit nutritionally from lysing fungal mycelium in soil	40

	Page
Mycolytic activity of chitinase and other enzymes	40
Lysis by streptomycetes	45
Lysis of fungal mycelium separated from soil by a filter	47
Lysis of dead mycelium by soil	56
Induced partial autolysis of fungal mycelium by antibiotics and fungicides	57
Induced partial autolysis of fungal mycelium by starvation conditions	60
Complete autolysis in the absence of micro- organisms and soil	65
Application of the autolysis model to natural soil	70
DISCUSSION	76
LITERATURE CITED	88

LIST OF TABLES

TABLE	Page
1. Comparison of 3 methods for partial purification of chitinase from a culture filtrate of <u>Streptomyces</u> sp., isolate 8	17
2. Lysis of the mycelia of several fungi by natural soil and by sterilized soil infested with 2 mycolytic isolates of soil streptomycetes	25
3. Lysis of young and old mycelia of several different fungi by natural soil	34
4. Lysis of the mycelia of <u>Glomerella cingulata</u> and <u>Fusarium solani</u> f. <u>phaseoli</u> by 4 different soils	35
5. Restoration of the mycolytic property to sterilized soil by infesting it with different isolates of soil streptomycetes and soil bacteria	36
6. Effect of organic supplements on lysis of the mycelia of <u>Glomerella cingulata</u> and <u>Fusarium solani</u> f. <u>phaseoli</u> by natural soil and streptomycete-infested soil	38
7. Changes in the numbers of bacteria and actinomycetes in soil supplemented with live fungal mycelium of <u>Glomerella cingulata</u>	41

8.	Relations between lysis of live mycelium and killed mycelium of <u>Glomerella cingulata</u> , hydrolysis of chitin, and inhibition of fungal spore germination by 92 actinomycetes....	48
9.	Lysis of live fungal mycelia on the upper surface of membrane filters, the lower surface of which was in close contact with natural soil	51
10.	Lysis of live and heat-killed mycelia of different fungi by natural soil	58
11.	Effect of killing mycelia of <u>Glomerella cingulata</u> and <u>Fusarium solani</u> f. <u>phaseoli</u> by exposure to heat, propylene oxide gas or ionizing radiation, on rate of lysis by natural soil	59
12.	Induced partial autolysis of <u>Glomerella cingulata</u> mycelium in agar by antibiotics, metabolic inhibitors and fungicides	61
13.	Induced partial autolysis of <u>Fusarium solani</u> f. <u>phaseoli</u> mycelium by 2 antibiotics as measured by changes in turbidity of a mycelial suspension in an antibiotic solution	63
14.	Ability of bacterial isolates from soil to both inhibit the germination of <u>Glomerella cingulata</u> conidia and restore the mycolytic property to sterilized soil	73

LIST OF FIGURES

FIGURE	Page
1. Effect of chitinase concentration on the weight of chitin hydrolysed per unit volume (ml) of the standard chitinase preparation	13
2. Relationship between chitinase activity (mU) and weight of chitin (mg) hydrolysed in 20 min., or change in initial optical density (0.40)	14
3. Progressive stages in the lysis of <u>Fusarium solani</u> f. <u>phaseoli</u> hyphae on natural soil	26
4. Segmentation of the protoplasm in hyphae of <u>Verticillium albo-atrum</u> after 4 days on natural soil	29
5. Formation of chlamydo spores by <u>Mucor ramannianus</u> on natural soil	30
6. Formation of "chlamydo spores" from germinated conidia after 4 days on soil	32
7. Effect of an organic supplement on the rate of lysis of <u>Fusarium solani</u> f. <u>phaseoli</u> mycelium on natural soil	39
8. Effect of several hydrolysing enzymes on live and dead mycelium of <u>Fusarium solani</u> f. <u>pisi</u> incubated at 28°C; O.D. measured at 400 mμ	44

9. Mycolytic zones produced by 4 streptomycete isolates grown on the surface of 0.5% peptone agar containing 4-day-old culture of Glomerella cinquilata 46
10. Lysis of live fungal mycelia when separated from natural soil by a membrane filter with 5 μ pores 52
11. The dialysis system in operation 64
12. Partial autolysis of fungal mycelia on membrane filters which rested on dialysis tubing inflated with circulating mineral salt solution 66
13. Lysis of fungal mycelia on membrane filters which rested on dialysis tubing inflated with circulating mineral salt solution 68
14. Relationship between lysis of live mycelium and inhibition of fungal spore germination by 92 streptomycetes 72
15. Inhibition zones surrounding filter paper discs containing an antibiotic substance, extracted with ethanol from soil supplemented with live fungal mycelium 75

INTRODUCTION

Most natural soils are both fungistatic and mycolytic. Fungistasis prevents germination of fungal spores in soil under conditions considered to be favorable for germination. And mycolysis destroys vegetative hyphae if germination does occur, or if germinated spores are artificially introduced into soil. Both properties are widespread in soils and non-specific, affecting most of the fungal species so far tested.

Fungal parasites which attack the roots of plants must survive, in the absence of a host, in soil where its activity is restricted by both the fungistatic and mycolytic properties of the soil. In parts of soil free from available organic nutrients, most root parasites and soil saprophytes survive as resistant propagules or spores. In the presence of nutrients, soil fungistasis and mycolysis are temporarily overcome, the spore germinates and the fungus coexists as vegetative mycelium with other soil microorganisms. After depletion of nutrients, the mycelium lyses and only the fungal propagules remain, inhibited by soil fungistasis. For it is as resistant propagules and spores that most fungi survive in parts of soil relatively free from such organic nutrients as those in rhizospheres or those released from decomposing plant and animal residues.

Sterilized soils are not fungistatic or mycolytic, but when infested with certain soil actinomycetes or soil bacteria, these properties are restored. Also, these properties can be reproduced with certain soil microorganisms in agar plates.

Much of this background information is from work on soil fungistasis and mycolysis already done at this University (26, 27, 28, 29, 30, 31). But it was with the recent description of a direct method for assay of soil fungistasis by making a plastic peeling of the soil (26) that a technique became available for the first time to adequately observe lysing fungal hyphae in soil.

The study described here utilizes this direct method for observing soil mycolysis. Some experiments already described for restoring the lytic property to sterilized soil were repeated with this direct method.

Finally, an effort was made to understand how certain soil microorganisms induce lysis of live mycelium in soil, and whether this was an autolytic or heterolytic mechanism. A model was developed whereby mycolysis could be reproduced in the absence of microorganisms and soil. Some of the assumptions made with this model were then tested in natural soil.

LITERATURE REVIEW

Fungal mycelia or germinated spores, when added to natural soil are rapidly lysed (29, 34, 49, 60, 61). The same is frequently true of germ tubes or hyphae from spores stimulated to germinate naturally in soil by addition of nutrients (9, 10, 19, 43, 52, 54, 62). Although few soils have been tested, the lytic property of soil--soil mycolysis--is probably a characteristic of all soils with an active microbial population. And all vegetative fungal mycelia is probably capable of being lysed by natural soil, although fungi do differ in their relative susceptibility to soil mycolysis.

The mycolytic property of soil can be demonstrated by several different methods. Germ tubes and hyphae of Fusarium oxysporum f. cubense (54), F. oxysporum f. niveum (19), Helminthosporium sativum (10) and 7 other fungi (9) frequently lysed when spores embedded in agar on a glass slide were stimulated to germinate in soil by addition of organic nutrients and plant materials. Mycelia of 20 different plant pathogenic fungi in agar media partially or completely lysed after an agricultural loam soil was spread over the agar surface (29). Also germ tubes of nematode-trapping fungi in agar discs were frequently lysed on the surface of a number of different field soils (34). And mycelium of

F. oxysporum on glass threads began to lyse after burial in a sandy soil for 7-10 days (40, 46), although glass (25) or nylon threads do sometimes temporarily protect mycelium from soil mycolysis.

Soil smears spread on glass slides and examined with a microscope, have also been used for observing lysis in soil previously supplemented with fungal conidia. Chopped plant materials (52) or extracts from plant residues (62), stimulated the germination of F. solani f. phaseoli chlamydospores, and this was frequently followed by lysis of the developing germ tubes and hyphae. A similar fate occurred with chlamydospores germinating in the vicinity of decomposing plant residues (62), and germinating seeds or rhizospheres of many nonsusceptible plants (52). Park (43) directly observed lysis of several fungi in soil supplemented with glucose.

The rate of lysis depends on the test fungus and the type of soil. Germinated spores embedded in agar media on glass slides were buried in soil (9, 49, 60, 61). With the garden soil, Fusarium culmorum lysed in 2-4 days, and Trichoderma lignorum and Phoma betae in 4-7 and 7-14 days, respectively. Mycolysis was most rapid in plant-mould soil, and consecutively less in garden soil, clay-limestone soil, heath and peat (49). Hyphae of several different fungi also lysed in a loam soil (9), and of F. vasinfectum in both a sandy compost and black cotton soil (60, 61). Fungal conidia are also lysed in soil, although at a slower rate than germ tubes and hyphae (45, 46, 49).

More indirect evidence that fungal hyphae do not survive for long in soil is the inability to detect much in natural soil. Jones and Mollison (24) counted the hyphal fragments in soil suspended in agar. Only in soil supplemented with farmyard manure was there more than 1 mm total length of hyphae per 20 low power objective fields of a microscope. There were a few mycelial fragments in natural soil, but most appeared empty. In a later publication (55), the same authors reported incredible yields of hyphal fragments from similar soils: 1 million hyphal fragments per g dry weight of soil, equivalent to 25-60 meters of hypha. The authors did not comment on these yields. Conn (12), after examining soil smears on glass slides, concluded that there were few hyphal fragments in most soils. Sometimes in the 5 mg subsample of soil, as few as 4 or 5 hyphal fragments were observed. Only when the soil contained large amounts of undecomposed organic matter, were hyphal fragments plentiful. Jensen (21) used the soil smear method to examine 30 Danish soils, and confirmed Conn's belief that most agricultural soils contained few hyphal fragments. However, there were abundant mycelia in acid-humus soils, especially those of peaty character, but many of these mycelia were dead and lacked protoplasm.

Recently Daniels (14) picked out hyphae from washed and sieved agricultural soil and grew them on an agar medium. Hyphae occurred only rarely in the soil: viable hyphal

fragments of Corticium solani were isolated, suggesting that this fungus has at least sparse growth in soil. Parkinson and Williams (48) washed a garden soil through a series of graded sieves to remove most fungal spores, and then plated the washed soil on a nutrient agar medium. Few heavily sporing species were isolated from the washed soil, suggesting that these species are present in soil mainly as spores. The authors assume that fungi isolated from the washed soil are derived from hyphal fragments, but they could equally well originate from spores embedded in plant residue fragments or even from mineral grains coated with organic matter.

Warcup (64) has successfully picked out individual hyphal fragments from soil and grown them on agar media. With this technique, he showed that most fungi occurring as hyphae in a wheat-field soil were either basidiomycetes or sterile isolates (65). Fungi isolated by the dilution plate or soil plate method were seldom seen as hyphae; these fungi apparently exist in soil mainly as spores. Few hyphal fragments were seen in soil during the dry season, and of these, only about 3-15% were viable. With the arrival of seasonal rains and following incorporation of plant residues in the soil, many fungi were active as vegetative mycelia. From these results, it appears that most soil fungi have restricted periods of active mycelial growth in soil. For the remaining periods, they survive as spores or resistant propagules. A few fungi, such as some basidiomycetes and sterile isolates may have sustained vegetative growth in soil,

and may even survive adverse conditions as resistant hyphae. For example, sterile hyphal fragments were found in dry soil under conditions unlikely for active growth (65).

The paucity of fungal hyphae in soil free of decomposing organic nutrients, and the rapid lysis of introduced fungal hyphae by natural soil, does not mean that mycelia are absent from soil. It does mean, however, that mycelia tend to be restricted to such microhabitats as microsites rich in plant residues (46, 52, 59, 62), rhizospheres (52, 53, 59), on or within roots of symptomless plants (14, 53, 59) and in the roots of host plants. Fungal mycelia may also be active in localized regions after release and redistribution of soil nutrients by such physical forces as those caused by freezing and thawing, wetting and drying (33). In the bulk of the soil, which tends to be deficient in available organic nutrients for long periods during the year, fungi survive as spores or other resistant propagules, prevented from germinating by the fungistatic property of soil (15, 30).

Most fungal pathogens of plant roots appear to survive in soil as resistant spores or sclerotia (29, 59). Of 18 root pathogens listed by Stover (59), only the genera Helminthosporium and Ophiobolus do not form special survival structures, and Helminthosporium may be a special case where the conidia are adapted for survival, similar to chlamydospores of many root pathogens. Conidia of most other fungal-root pathogens are slowly lysed in soil, or converted to

chlamydospores, e.g., F. oxysporum f. cubense (54), F. solani f. phaseoli (39, 52) and Thielaviopsis basicola (58).

Conidia, sporangiospores or hyphae of other fungi may also develop into resistant structures with characteristics similar to chlamydospores (45). Finally, there are a few basidiomycetes and sterile fungi which survive for sustained periods as mycelia in soil, apart from undecomposed organic matter (65). The hypha is probably adapted for survival against lysis, and therefore equivalent to the resistant structures formed by many other fungi.

Certain soils, such as highly acid podzols (5, 6) and acid peat soils, (21) have a microflora which is characteristically fungal, present mainly as vegetative mycelia. These exceptions cannot be taken as evidence for the widespread distribution of mycelia in other natural soils. The low mycolytic property is thought to be due to their acidic nature which prevents the development of a large bacterial and actinomycete population.

The ability of soil to lyse fungal mycelium is believed to be associated with soil microorganisms. Mycelia were rapidly lysed in garden soil, high in microbial numbers, whereas it lysed slowly in heath soil and peat, which were low in microbial numbers (49). Many actinomycetes, when grown on live fungal mycelium in a weak agar medium, lyse the mycelium surrounding the actinomycete colony without direct parasitism (8, 31, 67). Some soil bacteria also lyse fungal mycelium growing in culture (31, 37, 44, 45).

Individual actinomycetes from soil lysed more fungal mycelium in agar than individual bacteria. Combining several bacterial isolates increased lysis, but a combination of actinomycetes was no more effective than a single one (35). When sterilized soil was inoculated with mycolytic actinomycetes (28, 31, 57) or mycolytic bacteria (37, 44, 45) its lytic property was restored, whereas sterilized soil alone supported abundant mycelial growth.

MATERIALS AND METHODS

Preparation and assay of chitinase.--Colloidal chitin was prepared as previously described (27) except for minor modifications. Chitin (Pfanstiehl Chemical Co.) was ground to a fine powder in a Wiley mill with a 60-mesh screen. After stirring the ground chitin for 30 min. in concentrated HCl at 20°C, the dissolved portion of the chitin was precipitated in stirred cold distilled water as colloidal chitin. Approximately 80% of the original chitin was recovered at 20°C, compared with only 30% at 0°C, the temperature previously recommended (27).

Two liters of Reynolds' mineral salt solution (pH 7.0) (51) containing 0.25% colloidal chitin as the sole carbon and nitrogen source was seeded with Streptomyces sp., isolate 8, a known chitinase producer (41). Sterile air was bubbled through the culture which was incubated at 28°C. Most of the chitin was hydrolysed in 2-3 days. The culture was then filtered through cheesecloth and centrifuged in a cream separator. The culture filtrate was pervaporated in dialysis tubing to about 1/4 its original volume, and frozen for storage. Chitinase was partially purified from this pervaporated culture filtrate by 3 separate methods; ethanol precipitation, ammonium sulfate precipitation, and adsorption on chitin.

Chitinase activity was assayed as follows: the chitinase preparation and a suspension of colloidal chitin (3 mg/ml) in 0.005 M phosphate buffer (pH 6.8) were warmed separately for 5 min. in a water bath at 38°. One ml of the warmed chitin suspension was then mixed with 3 ml of the warmed chitinase preparation in a cuvette, and the initial optical density (O.D.) quickly measured with a spectrophotometer (Bausch & Lomb Spectronic 20) at 425 m μ (O.D. approximately 0.40). The decrease in O.D. was measured after 20 min. incubation at 38°. A single batch of colloidal chitin containing 0.01% thimerosal was kept for all assay work. One unit (U) of chitinase was defined as the amount of enzyme which would hydrolyse in 1 min. the weight of chitin equal to 1 microequivalent of N,N-diacetylchitobiose, the repeating unit of the chitin polymer, under the defined assay conditions. For convenience, activity was expressed as milli-units (mU).

The weight of chitin hydrolysed per unit amount of a standard chitinase solution, prepared by ethanol-precipitation of a pervaporated culture filtrate, decreased with increasing enzyme concentration (Fig. 1). Therefore the concentration giving the highest efficiency (0.1125 mg chitin hydrolysed per ml of the standard chitinase per min.) was chosen to calculate the activity of the standard chitinase preparation. This value divided by the microequivalent weight of the N,N-diacetylchitobiose repeating unit (0.406 mg), gave an activity of 280 mU of chitinase per ml. The logarithm of

chitinase activity is a linear function of the logarithm of amount of chitin hydrolysed (Fig. 2). The slope and position of the dosage-response curve for the standard chitinase solution remained unchanged for a period of 2 years during which the enzyme was stored at -18°C .

The Lowry's procedure (phenol reagent) was used for protein determination (32). Specific activity was expressed as units of chitinase activity per mg protein.

Partial purification of chitinase.--Pervaporated culture filtrate from Streptomyces sp., isolate 8, was centrifuged to remove a precipitate formed during cold storage. Chitinase was then partially purified by the following 3 methods:

- 1) Cold (-18°) ethanol was slowly added to chilled pervaporated culture filtrate previously adjusted to pH 3.5, to give a final concentration of 70% ethanol. The precipitate was immediately collected by centrifugation and redissolved in a minimum volume of 0.005 M phosphate buffer, pH 6.8. Insoluble residue remaining in the phosphate buffer was removed by centrifugation, and the supernatant fluid containing the chitinase was dialysed for 4 hrs. against a large volume of cold buffer.

- 2) Solid ammonium sulfate was added to chilled pervaporated culture filtrate (pH 7.0) to give a final 70% saturated solution. The precipitate was collected by centrifugation, dissolved in phosphate buffer, recentrifuged, and dialysed as described above for ethanol-precipitated

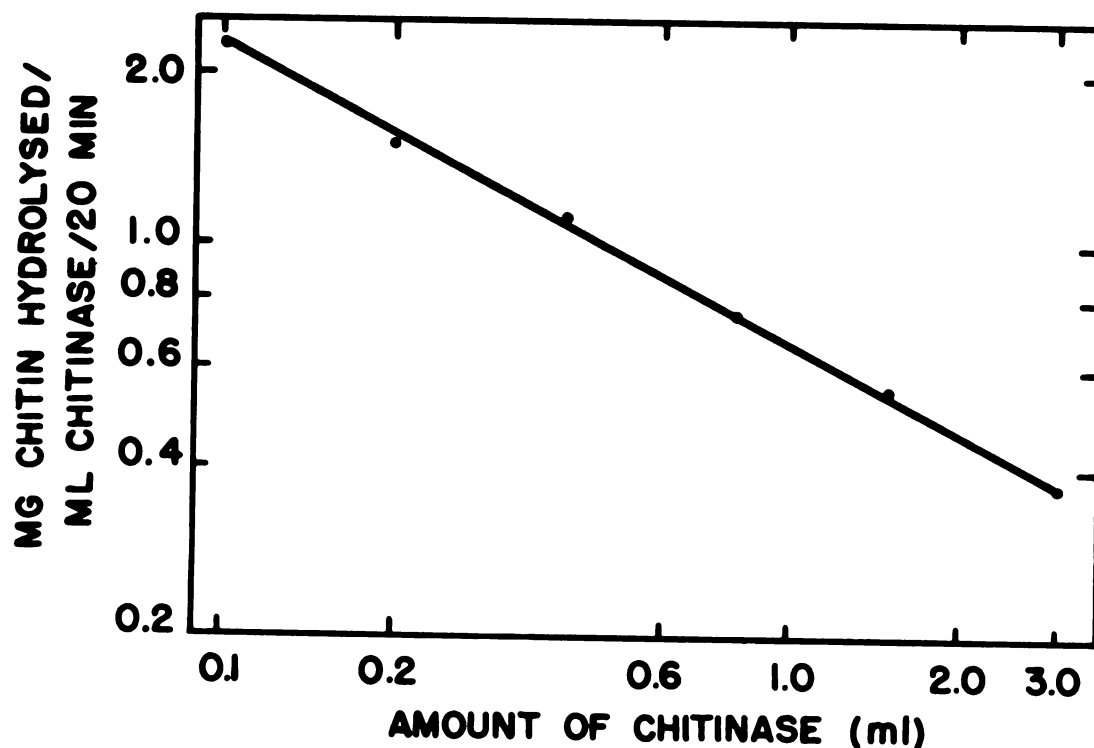


Fig. 1. Effect of chitinase concentration on the weight of chitin hydrolysed per unit volume (ml) of the standard chitinase preparation. One ml of standard colloidal chitin suspension and 3 ml of various concentrations of the enzyme preparation were incubated together at 38°C for 20 min., and changes in O.D. measured at 425 mμ.

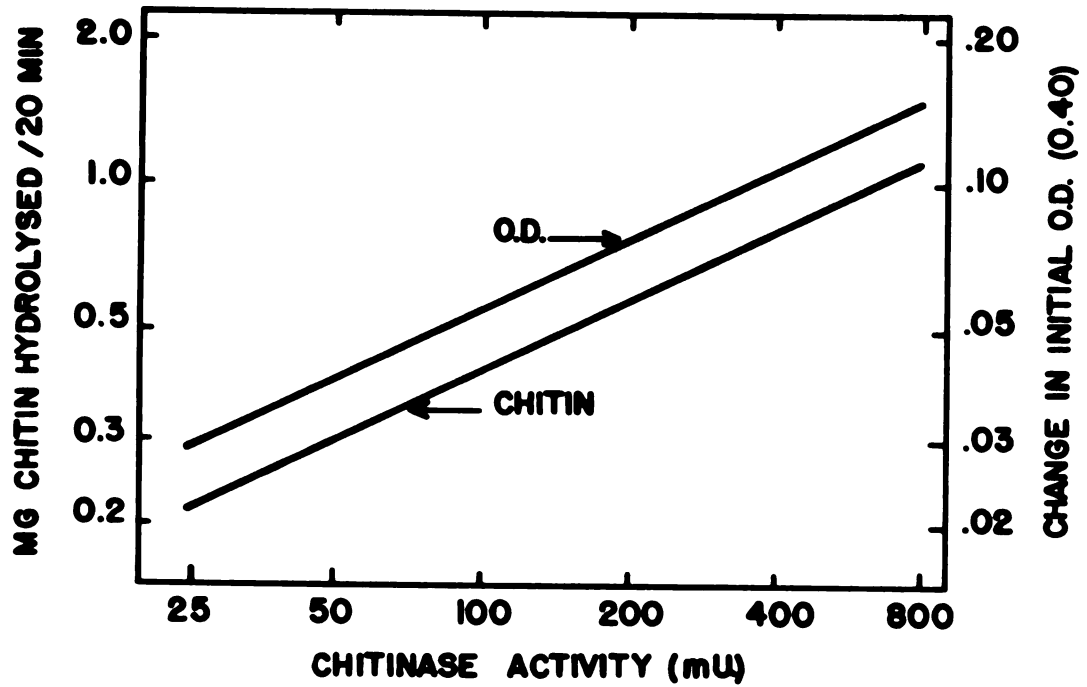


Fig. 2. Relationship between chitinase activity (mU) and weight of chitin (mg) hydrolysed in 20 min., or change in initial optical density (0.40).

chitinase. A similar procedure has already been described (2, 22).

3) Sufficient colloidal chitin was added to per-vaporated culture filtrate (pH 6.8), so that approximately half the chitin would be hydrolysed in 20 min. at 38°. After 10 min. at 2-3°C, the colloidal chitin containing the adsorbed chitinase was collected by centrifugation, washed with distilled water, recentrifuged, and resuspended to 1/10 the original volume in 0.005 M phosphate buffer containing 0.01% thimerosal and incubated at 38° until 3/4 of the chitin was hydrolysed. The remaining chitin was then removed by centrifugation and the supernatant fluid dialysed against phosphate buffer. This is a modification of a procedure described by Jeuniaux (22).

All purification methods were repeated 3 times using 3 separate culture filtrates. Similar results were obtained in all 3 replications except for chitin-adsorbed chitinase, which was recovered in only 1 of the 3 tests. Table 1 shows typical results for each method.

The mean yields for ammonium sulfate-precipitated chitinase and ethanol-precipitated chitinase were 54% and 66%, and specific activities were increased 5- and 3.5-fold, respectively. Ammonium sulfate precipitation removed more foreign protein than ethanol precipitation, but was less effective in removing nonproteinaceous matter. Yield for chitin-adsorbed chitinase, was 42%, with a specific activity

of 1573, the highest activity for the 3 purification methods. However, as ethanol-precipitated chitinase was relatively simple and reliable to prepare it was used as the enzyme preparation for most tests.

Incubation of fungal mycelium in chitinase and other enzymes.--Conidia of Glomerella cingulata (Ston.) Spauld. & Schrenk and Fusarium solani f. pisii (F.R. Jones) Snyder & Hans. were germinated in a medium containing 0.5% peptone and 0.5% glucose until the germ tubes were approximately twice the length of the conidia, and then washed twice by centrifugation, using a dilute mineral salt solution. Portions of the washed germinated spores were killed either by exposure to propylene oxide gas for 8 hrs. or heat at 80°C for 4 min. in a water bath. The enzymes used were as follows: β -D-1,3 glucanase (72 U activity/mg dry weight) (50), protease ("Pronase", 45,000 PU activity/mg dry weight), and snail digestive juice. The enzymes were obtained respectively, from E.T. Reese, Quartermaster Research and Engineering Center, Natick, Mass.; Calbiochem., Los Angeles, Calif.; and from L'Industrie Biologique Francaise, 35 à 49, Quai du Moulin de Cage, Gennevilliers (Seine), France. The final enzyme concentrations used were as follows: pervaporated culture filtrates from 4 different streptomycetes previously grown in chitin medium, 55-58 mU/ml; partially purified chitinase, 118-616 mU/ml; β -D-1,3 glucanase, 0.1 mg/ml; protease, 0.1 mg/ml; snail digestive juice, diluted 1/10. Chitinase, glucanase and snail digestive juice were diluted

Table 1. Comparison of 3 methods for partial purification of chitinase from a culture filtrate of Streptomyces sp., isolate 8

Treatment ^a	Activity/ mg dry wt (mU/mg)	Specific activity ^b (mU/mg)	Activity/ ml (mU/ml)	Total activity (mU)	Yield (%)
Culture filtrate	17	208	25	44,500	100
Pervaporated					
culture filtrate	15	231	81	43,740	98
Ethanol-precipi-					
tated chitinase	478	766	574	30,995	70
(NH ₄) ₂ SO ₄ -precipi-					
tated chitinase	309	1200	432	23,330	52
Chitin-adsorbed					
chitinase		1573	236	18,530	42

^aFor purposes of comparison, 150 ml of the same batch of pervaporated culture filtrate was used for each of the 3 purification methods. The final volume of partially purified chitinase was 15 ml. Values are from a typical test.

^bSpecific activity is the activity/mg protein.

in 0.05 M phosphate buffer, pH 6.0, and protease in phosphate buffer at pH 7.0. All solutions were sterilized by passage through a membrane filter of 0.20 μ pore size.

Mycolysis and inhibition of fungal spore germination by streptomycetes.--Streptomycetes were isolated from Conover loam soil by spraying diluted soil suspensions on the surface of chitin agar (27), or on the surface of 4-day-old cultures of G. cingulata growing in 0.5% peptone agar (8). Ninety-two randomly selected colonies were transferred to yeast-extract-dextrose-maltose agar slants (31). Bacteria were isolated by spraying a diluted soil suspension on the surface of nutrient agar. All bacterial isolates were tested for ability to inhibit G. cingulata conidia by the agar streak method.

Mycolytic activity was assayed by growing each streptomycete on both live and propylene oxide-killed mycelium in agar (7). Conidia of G. cingulata were mixed with melted agar (0.5% peptone, 2% Bacto (Difco) agar, 0.05 M phosphate buffer, pH 7.0), and 16 ml of the mixture (50,000 spores/ml) poured in each petri dish. Following 4 days of mycelial growth at 24^o, the agar surface was streaked with each of the streptomycetes, and the plates incubated for 12 days at 28^o. Measurements were then made of the width of the lysed mycelial zone surrounding each streptomycete colony (Fig. 9). Similar measurements were made of the lysed zones surrounding streptomycete colonies grown on 4-day-old mycelium of G. cingulata in agar killed with propylene gas.

Chitinase activity was assayed by measuring the zone of hydrolysed chitin surrounding each streptomycete grown on the surface of chitin agar (0.25% colloidal chitin, 2% Bacto (Difco) agar, 0.05 M phosphate buffer, pH 7.0) (27).

Ability to inhibit fungal spore germination was assayed by streaking each streptomycete on the surface of 0.5% peptone agar, incubating for 2 days, and then spraying conidia of G. cingulata on the surface of the same agar (ca. 250,000 conidia/plate). Following 1 and 2 days further incubation at 28°, the inhibition zone surrounding each streptomycete colony was measured. Similar inhibition zones on nutrient agar were measured for bacterial isolates from soil.

Reinfesting sterilized soil with microorganisms.--

Soil was heat-sterilized in 250 ml Erlenmeyer flasks by autoclaving for 40 min. on 3 consecutive days. When sterilizing soil with gas, the flasks were kept in a closed container with propylene oxide gas overnight and then stored for several days to remove the residual gas. To sterilize with gamma radiation, soil in small glass jars were exposed to a dose of 4 megarads from a Co⁶⁰ source. This work was kindly supervised by Mr. H.G. Olson, Head, Phoenix Memorial Laboratory, Ann Arbor.

Sterilized soil was infested with a mixed conidial and mycelial suspension of soil streptomycetes, or cell suspensions of a soil bacteria, and incubated for 6-8 days at 24°.

Direct assay for soil mycolysis.--Lysis of fungal mycelium by soil was assayed by a direct method, similar to the one previously described for assay of soil fungistasis (26). Conover loam soil, or sterilized soil infested with soil streptomycetes, was adjusted to about 25% moisture content, firmly compacted into small petri dishes, and then the surface of the soil smoothed. Conidia of the test fungus were germinated in a medium containing 0.5% peptone and 0.5% glucose until the germ tubes were 2-3 times the length of the conidia. After washing 3 times in 1% Reynolds' salt solution (51) by slow centrifugation, the germinated conidia were added uniformly over the soil surface. In some cases the fungus was grown in the nutrient solution, the young mycelium harvested and gently fragmented in a Waring blender, washed, and then the pieces of hyphae added to the soil. Chlamydospores of Fusarium solani (Mart.) Appel & Wr. f. phaseoli (Burk.) Snyd. & Hans. were obtained by keeping a high concentration of washed conidia in glass distilled water for about 5 weeks, when cells of the macroconidia rounded and became chlamydospores. These were germinated in the nutrient solution, washed, and put directly on the soil in the petri dishes. After keeping the soil plates in a high humidity at 24° for different time intervals, the fungal hyphae were stained on the soil surface and recovered with plastic films (26). Amount of soil mycolysis was estimated on a 0-4 scale: 0, no lysis; 1, 1-10% of mycelium lysed; 2, 10-50% of mycelium lysed; 3, 50-90% of mycelium lysed; and

4, 90-100% of the mycelium lysed (31). Pregerminated spores recovered from soil in plastic-soil peelings immediately after deposition, acted as a control.

Supplementing soil with live fungal mycelium.--

Germinated conidia of G. cingulata were added to soil in an amount equivalent in dry weight of mycelium to 1% of the dry weight of the soil. The supplemented soil was kept in a polyethylene carton and occasionally the soil surface sprayed with a fine mist of water to prevent drying. At different days, estimates of the numbers of soil microorganisms were made by the standard soil-dilution-plate method. A sample from the soil was suspended in 0.5% carboxy-methylcellulose, diluted, and then added to the melted agar in petri dishes. For bacterial counts, the final soil dilution was 1×10^{-8} in soil-extract agar (4), and for actinomycete counts, 1×10^{-6} dilution in chitin agar (27). Bacterial-dilution plates were incubated for 5-7 days at 25° and actinomycete plates for 6-8 days at 28° . The number of colonies in each plate was then counted, and the mean for the 6 replicates expressed as number of organisms per g dry weight of soil. Concurrently, the rate of soil mycolysis was estimated by 2 separate methods: 1) Germinated spores were added to the smooth surface of natural soil and recovered each day with plastic films. 2) A small portion of the supplemented soil was mixed with phenolic rose bengal, diluted with water, and a smear on a glass slide examined with a microscope. Both methods gave similar lytic ratings.

Definition of mycolysis.--For the purpose of clarity, I have defined mycolysis as loss of protoplasm and enzymatic dissolution of fungal cell walls. Hyphae containing segmented protoplasm and empty but intact hyphae formed by leakage or self-digestion of protoplasm are incomplete lysis, although these may be stages in complete lysis of live mycelium.

RESULTS

Lysis of different fungi by soil.--Washed germinated spores of several fungi, and fragmented mycelia of Rhizoctonia spp. were lysed on the surface of natural soil (Table 2). The sequence shown in Fig. 3 demonstrates the progressive lysis of the hyphae of F. solani by natural soil. At 2 days the hyphae were lightly stained and the protoplasm frequently segmented (Fig. 3B). By 4 days much of the hyphae was lysed, especially the young portions farthest from the empty conidia (Fig. 3C). At 8 days all the hyphae were lysed, and abundant bacteria and actinomycetes appeared in the region previously occupied by the hyphae (Fig. 3, D&E). There was some limited growth of F. solani on soil prior to lysis, the extent depending on the number of times the germ tubes were washed. Only a small amount of contaminating nutrient permitted limited growth and even sporulation on soil before final lysis of the hyphae. Occasionally a cell of an ungerminated macroconidium rounded and became a chlamydospore, but hyphae from germinated spores rarely produced chlamydospores. The sequence for lysis with the other fungi tested was essentially similar to F. solani: limited or usually no growth, segmentation of protoplasm (Fig. 4), uneven staining, disintegration of hyphae, followed by accumulation of microorganisms, especially bacteria and actinomycetes, and also protozoa. Mucor ramannianus Möller occasionally formed

terminal and intercalary chlamydospores (Fig. 5), and Penicillium frequentans Westling and V. albo-atrum both formed rounded "chlamydospores" about the diameter of the hypha, which persisted in soil after lysis of the vegetative hyphae (Fig. 6). The dark-pigmented conidiophores and old mycelium of Helminthosporium victoriae Meehan & Murphy resisted lysis, whereas young hyphae lysed rapidly. Mycelia of Rhizoctonia spp. grew on soil. The thin, deeply stained hyphae, which were sparsely branched, spread over the soil in a linear pattern. This was the only fungus tested whose mycelium sustained vegetative growth on natural soil. Eventually, after 8 days on soil, much of this mycelium was lysed.

The rate of mycolysis depends on the fungus (Table 2). Mycelium of G. cingulata was completely destroyed in 4 days, mycelia of most other fungi in 8 days, whereas some mycelia of Rhizoctonia spp. remained after 8 days. This difference in resistance to lysis was not obviously associated with root-parasitic fungi or soil saprophytes. Nor was resistance of mycelium to lysis associated with the type of spore which the fungus produced. Conidia of H. victoriae and chlamydospores of Fusarium solani f. phaseoli are the resistant propagules by which these 2 fungi survive in soil, yet hyphae from these resistant propagules lysed as rapidly as hyphae from conidia of F. solani f. phaseoli and other fungi. The light, thin hyphae of V. albo-atrum were consistently more resistant to mycolysis than hyphae of most of the

Table 2. Lysis of the mycelia of several fungi by natural soil and by sterilized soil infested with 2 mycolytic isolates of soil streptomycetes

Fungus	Lysis rating after 2,4 and 8 days ^a					
	Natural soil			Streptomycete soil ^b		
	2	4	8	2	4	8
<i>Glomerella cingulata</i>	2	4	4	0	3	4
<i>Mucor ramannianus</i>	2	3	4	0	3	4
<i>Helminthosporium victoriae</i>	2	3	4	0	3	4
<i>Penicillium frequentans</i>	2	3	4	0	2	3
<i>Fusarium solani</i> f. <i>pisii</i>	0	3	4	0	2	3
<i>Fusarium solani</i> f. <i>phaseoli</i>	0	3	4	0	2	3
<i>Fusarium solani</i> f. <i>phaseoli</i> ^c	0	3	4			
<i>Verticillium albo-atrum</i>	0	2	4	0	2	3
<i>Rhizoctonia solani</i>	0	2	3	0	1	2
<i>Rhizoctonia solani</i>	0	1	3	0	1	2
<i>Rhizoctonia praticola</i>	0	1	3	0	1	2

^aLysis was rated 0-4: 0=no lysis, 1= 1-10% of mycelium lysed, 2= 10-50%, 3= 50-90%, 4= 90-100%. Values are from 1 of 3 experiments.

^bAutoclaved soil was infested with 2 mycolytic streptomycetes and incubated 6-8 days before placing fungal mycelium directly on the soil surface.

^cGerminated chlamydospores ^{of} *F. solani* f. *phaseoli*.

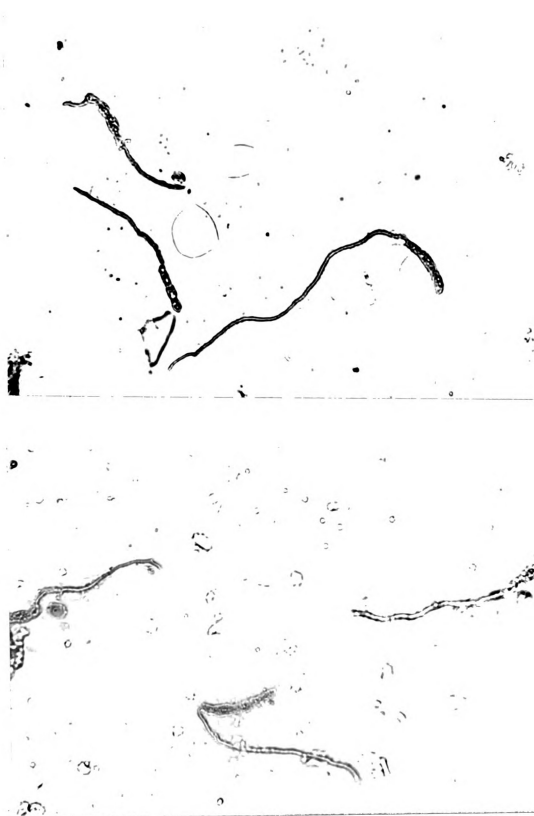


Fig. 3. Progressive stages in the lysis of Fusarium solani f. phaseoli hyphae on natural soil. A) 0 days. Germinated conidia were placed on the soil surface and recovered immediately. X 450. B) 2 days. The hyphae are lightly stained. X 450.

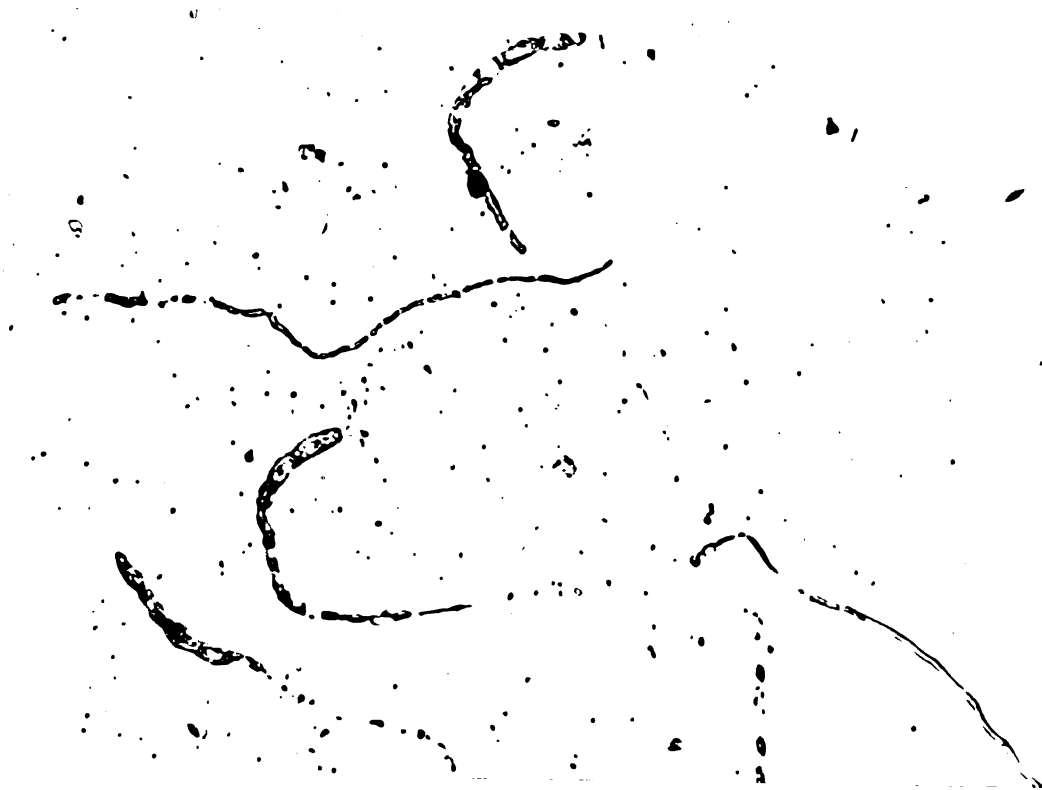


Fig. 3C. 4 days. Hyphae are partially disintegrated.

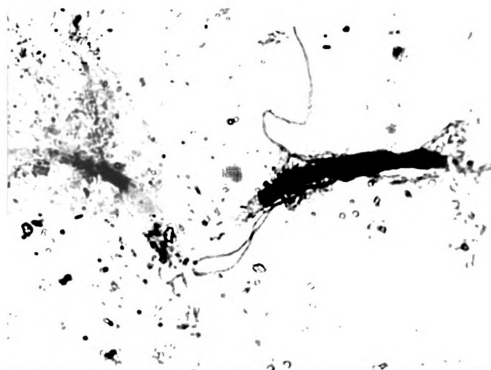


Fig.3. D) X 450. 8 days. Hyphae have completely disappeared, but remains of the macroconidia are still present. Abundant bacteria and actinomycetes appear in the region previously occupied by the hyphae. X 450.
E) An enlargement of a portion of Fig. 3D. The empty macroconidium at the right is surrounded by the thin mycelium of an actinomycete. X 900.

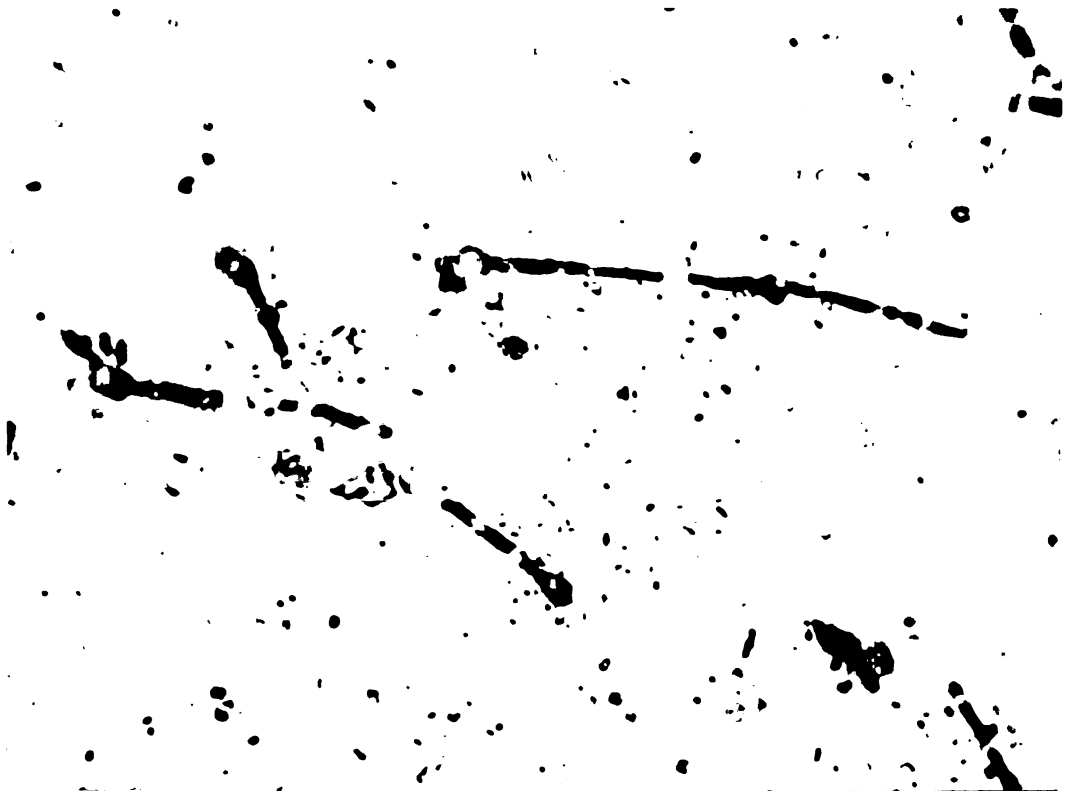


Figure 4. Segmentation of the protoplasm in hyphae of Verticillium albo-atrum after 4 days on natural soil. From each germinated conidium there is a long germ tube with segmented protoplasm.
X 450.

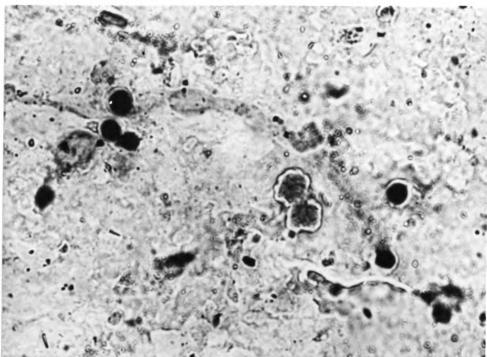
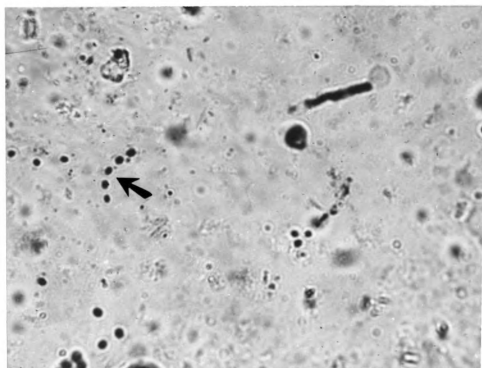


Figure 5. Formation of chlamydospores by Mucor ramannianus on natural soil. A) Four chlamydospores are attached to the hypha arising from the germinated conidium after 2 days on soil. B) The hyphae have lysed and only the chlamydospores remain after 4

Figure 6. Formation of "chlamydospores" from germinated conidia after 4 days on soil. A) A segmented and partially lysed hypha from a germinated conidium of Verticillium albo-atrum terminates in 4 "chlamydospores", about the same diameter as the hypha. B) Isolated "chlamydospores", sometimes in a row, trace the previous existence of Penicillium frequentans hyphae. One large empty conidium can be seen in the center of the photograph. X 450.



other fungi, including the coarse, deeply stained mycelium of H. victoriae. The relative resistance of V. albo-atrum hyphae was previously noted (29).

On natural soil, germ tubes of M. ramannianus lysed at a slightly faster rate than hyphal fragments from 6-day-old mycelium of the same fungus (Table 3). Germ tubes and mycelia of G. cingulata, F. solani f. phaseoli and V. albo-atrum lysed at about the same rate, indicating that older hyphae are not appreciably more resistant to lysis than very young hyphae.

The rate of soil mycolysis differs with the type of soil. Mycelia of G. cingulata and F. solani f. phaseoli were lysed more rapidly by both Conover loam soil and a greenhouse compost soil than by a loam soil from Colorado (18), or by sterilized soil reinfested with 2 mycolytic streptomycetes, originally isolated from soil (Table 4).

Restoring the mycolytic property to sterilized soil with streptomycetes and bacteria.--Whereas natural soil rapidly lysed live fungal mycelium, soil sterilized by autoclaving, radiation or propylene oxide gas supported abundant mycelial growth of all the fungi tested (Table 5). When this sterilized soil was reinfested with certain soil streptomycetes or soil bacteria, its mycolytic property was restored (Table 5). Of 10 selected streptomycetes, all but 1 restored the lytic property to sterilized soil, and this isolate also did not lyse fungal mycelium in agar. Combinations of mycolytic streptomycetes were no more effective than many of

the single isolates. Of the 8 soil bacteria tested, 2 restored the lytic property to sterilized soil; the same 2 bacterial isolates also inhibited fungal conidia in agar tests.

The mycelia of 9 different fungal spp. were lysed by streptomycete-infested soil (Table 2). When the fungi were ranked in order of increasing resistance to soil mycolysis, the order was the same for both natural soil and streptomycete-infested soil, although with each fungus, the rate of lysis was always faster on natural soil. Mycelia of G. cingulata, M. ramannianus and H. victoriae were completely lysed on streptomycete-infested soil in 8 days, whereas at least 50% of the mycelia of Rhizoctonia spp. remained after 8 days. All fungi grew for a limited period and sporulated before lysing. The sequence for lysis was similar to that for natural soil: segmentation of protoplasm, uneven light staining, disintegration of the hyphae and accumulation of streptomycete mycelium in the region previously occupied by the hyphae.

Organic nutrients reverse soil mycolysis.--One of the conditions for lysis of live fungal mycelium in agar by streptomycetes is a nutritionally deficient medium (8). With a medium high in organic nutrients, there is nolysis. As soil has a low level of available organic nutrients, addition of these nutrients should prevent soil mycolysis.

Glucose or peptone, equivalent to 0.2% of the dry weight of the soil, was added with live germinated spores of

Table 3. Lysis of young and old mycelia of several different fungi by natural soil

Fungus	Lysis rating after indicated days ^a											
	Young mycelium ^b						Old mycelium ^c					
	2	3	4	5	6	8	2	3	4	5	6	8
<i>G. cingulata</i>	3	4	4	4	4	4	3	4	4	4	4	4
<i>M. ramannianus</i>	2	3	4	4	4	4	1	2	3	3	4	4
<i>F. solani</i> f. <i>phaseoli</i>	0	1	3	3	3	4	0	1	2	3	3	4
<i>V. albo-atrum</i>	0	1	2	3	4	4	0	1	2	3	3	4
Autoclaved soil	0	0	0	0	0	0	0	0	0	0	0	0

^aLysis was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from 1 of 2 experiments.

^bYoung mycelium is germ tubes.

^cOld mycelium is hyphal fragments from 6-day-old mycelium.

Table 4. Lysis of the mycelia of Glomerella cingulata and Fusarium solani f. phaseoli by 4 different soils

Soil	Lysis rating after 2,4 and 8 days ^a					
	<u>G. cingulata</u>			<u>F. solani</u>		
	2	4	8	2	4	8
Conover loam	2	4	4	0	3	4
Compost soil	1	4	4	0	3	4
Colorado loam ^b	1	3	4	0	2	4
Streptomycete soil ^c	0	3	4	0	2	3
Autoclaved soil	0	0	0	0	0	0

^aLysis was rated 0-4: 0= no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from 1 experiment.

^bSoil collected in the vicinity of Fort Collins, Colorado (18).

^cStreptomycete soil is sterilized soil infested with 2 mycolytic streptomycetes originally isolated from soil.

Table 5. Restoration of the mycolytic property to sterilized soil by infesting it with different isolates of soil streptomycetes or soil bacteria

Infested soil ^b	Lysis rating after 2,4 and 8 days ^a					
	<u>G. cingulata</u>			<u>F. solani</u>		
	2	4	8	2	4	8
Streptomycete						
Isolate No.						
27	0	0	2	0	0	0
55				0	1	2
116	0	3	4	0	1	2
65	0	3	4	0	2	2
14	0	3	4	0	1	3
3	0	3	4	0	2	3
61				0	2	3
64				0	2	4
84				0	2	4
155	0	3	4	0	3	4
62 + 64	0	3	3	0	2	3
28 + 61	0	2	4	0	1	3
10 + 61 + 64	0	3	4	0	2	3
27+55+61+64+84				0	2	3
28 + 54 + 65 ^c	1	3	4	0	2	3
Bacterium						
Isolate No.						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
17	0	0	0	0	0	0
14	0	0	0	0	0	0
4	0	0	1	0	0	0
5	0	2	3	0	0	0
11	0	3	4	0	0	2
Natural soil	2	4	4	0	2	4
Autoclaved soil	0	0	0	0	0	0
Radiation sterilized soil ^c	0	0	0	0	0	0

^aLysis was rated 0-4: 0= no lysis, 1= 1-10% of mycelium lysed, 2= 10-50%, 3= 50-90%, 4= 90-100%. Values are from a single experiment.

^bAutoclaved soil infested with a streptomycete or bacterium and incubated 6-8 days before placing fungal mycelium directly on the infested soil surface.

^cSoil sterilized by gamma radiation (4 megarads) from a Co⁶⁰ source.

the test fungus to the smooth surface of natural soil or streptomycete-infested soil in a small petri dish. Chitin was mixed with the soil at the same rate 4 days before addition of the fungus to the soil surface. The soil plates were incubated at 24°, and the lytic rate estimated by recovery of the residual mycelium with plastic films.

Glucose or peptone greatly decreased the rate of lysis on both natural soil and streptomycete-infested soil by stimulating temporary fungus growth, whereas chitin increased the lytic rate on both soils (Table 6 & Fig. 7). Mycelium of *F. solani* f. *phaseoli* was completely lysed in 12 days by glucose- or peptone-supplemented soil, in 8 days by natural soil, and in 4 days by soil supplemented with chitin. Both glucose and peptone stimulated the development of large bacterial and streptomycete populations as well as abundant vegetative mycelia of the test fungi, *F. solani* f. *phaseoli* and *G. cingulata*. The nutrients also stimulated germination of other fungal spores in the soil: coenocytic hyphae of phycomycetes and the sporangia of mucoraceous fungi were frequently seen. In streptomycete-infested soil, glucose or peptone stimulated active mycelial growth of both the fungus and the streptomycete, and they continued to coexist while nutrients were available. When organic nutrients became depleted, the mycelium lysed.

Supplementing natural soil or streptomycete-infested soil with chitin greatly increased the rate of lysis relative to unsupplemented soil. Chitin is unavailable as an organic nutrient to the fungus, but is hydrolysed by chitinolytic

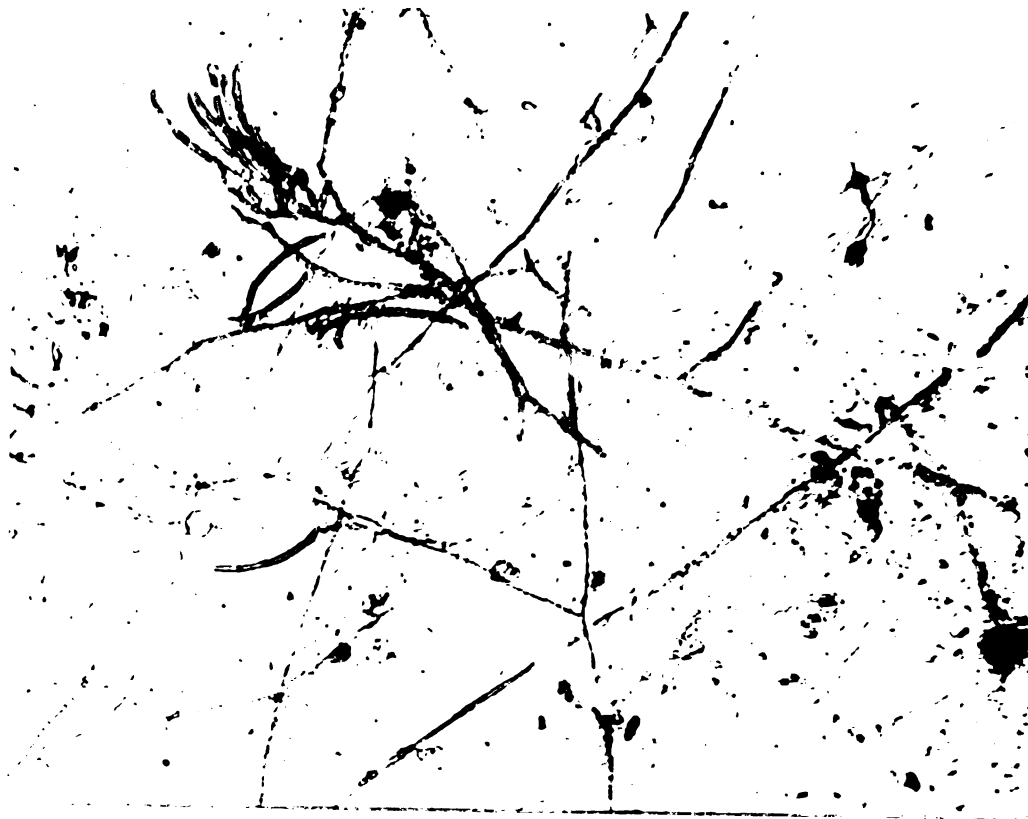
Table 6. Effect of organic supplements on lysis of the mycelia of Glomerella cingulata and Fusarium solani f. phaseoli by natural soil and streptomycete-infested soil

Supplement ^b	Lysis rating after 2,4,8 and 12 days ^a							
	<u>G. cingulata</u>				<u>F. solani</u>			
	2	4	8	12	2	4	8	12
Natural soil								
water	2	4	4	4	1	2	4	4
glucose	0	2	4	4	0	0	3	4
peptone	0	2	4	4	0	0	3	4
chitin	4	4	4	4	3	4	4	4
Streptomycete soil								
water	0	3	4	4	0	1	3	4
glucose	0	2	4	4	0	0	2	3
peptone	0	2	4	4	0	0	2	4
chitin	3	4	4	4	2	4	4	4
Autoclave soil	0	0	0	0	0	0	0	0

^aLysis was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from 1 of 2 experiments.

^bGlucose or peptone, equivalent to 0.2% of the dry weight of the soil, was added with the fungal mycelium. Chitin was mixed with the soil 4 days before addition of the fungus to the soil. Streptomycete soil is autoclaved soil infested with 2 mycolytic streptomycetes.

Figure 7. Effect of an organic supplement on the rate of lysis of Fusarium solani f. phaseoli mycelium on natural soil. A) Abundant fungal hyphae and conidial formation occurs after 4 days on soil supplemented with glucose at a rate equivalent to 0.2% of the dry weight of the soil. B) With unsupplemented soil, part of the hyphae are lysed after 4 days. X 450.



microorganisms, many of which are mycolytic. Therefore addition of chitin selectively favors those microorganisms most probably responsible for causing mycolysis.

Soil microorganisms benefit nutritionally from lysing fungal mycelium in soil.---When natural soil was supplemented with live fungal mycelium of G. cingulata, the bacterial population increased from 1×10^8 to a maximum of 138×10^8 in 7 days and the actinomycete population from 6×10^6 to a maximum of 60×10^6 in 9 days (Table 7). Fungal mycelium began to lyse on the second day and had completely disappeared by the fourth day. Concurrently, bacterial numbers began increasing on the third day and continued to increase until the fifth day when the population remained constant for several days. This suggests a close relationship between lysis of live fungal mycelium and increase in bacterial activity. Increase in propagule numbers of actinomycetes did not occur until the fourth day at which time the fungal mycelium had already disappeared. However, this lag in actinomycete numbers probably reflects a period of mycelial growth prior to sporulation. The prevalence of actinomycetes was supported by the visual appearance of abundant actinomycete mycelia on the surface of the soil on the third day and the characteristically strong earthy odor associated with these microorganisms. A large number of chitinolytic bacteria also appeared on the chitin agar plates.

Mycolytic activity of chitinase and other enzymes.---

Some actinomycetes and bacteria restore the lytic property to

Table 7. Changes in the numbers of bacteria and actinomycetes in soil supplemented with live fungal mycelium of Glomerella cingulata

Days	Colonies/g oven-dry soil ^a				Lysis rating in supple- mented soil ^b
	Bacteria x 10 ⁸		Actinomycetes x 10 ⁶		
	Natural soil	Supplemented soil	Natural soil	Supplemented soil	
0	1a	1a	6v	6v	0
1		1a		5v	0
2	2a	13b	6v	6v	2
3		28c		7v	3
4	2a	94d	5v	13w	4
5		126e		29x	4
7	2a	138e	5v	46y	4
9			6v	60z	

^aEach value is an average of 6 replicates from 1 of 2 experiments. Soil was supplemented with live mycelium, in an amount equivalent in dry weight of mycelium to 1% of the dry weight of the soil. Numbers followed by the same letter are not significantly different from each other at the 1% level of probability.

^bLysis of fungal mycelium was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%.

sterilized soil, but it is not certain how these microorganisms induce mycolysis. The hyphae could lyse either by self-digestion from their own intracellular enzymes, or by extracellular enzymes from adjacent soil microorganisms. If extracellular enzymes alone lyse hyphae in soil, then those enzymes which hydrolyse chitin and laminarin, major cell-wall constituents of filamentous fungi, should cause heterolysis of mycelium in vitro.

Suspensions of washed live germinated spores of F. solani f. pisi or G. cingulata were incubated with each of the following enzyme preparations: pervaporated streptomycete culture filtrates, partially purified chitinase obtained by ammonium sulfate precipitation, ethanol precipitation or chitin adsorption, protease, β -D-1,3 glucanase, and snail digestive juice. Combinations of enzymes were also used, in which case the germinated spores were first incubated in protease for 3 hr. and then washed prior to addition of the other hydrolysing enzymes. Germinated spores in an enzyme preparation were incubated in a water bath at 28^o, and changes in turbidity at 400 m μ measured at time intervals. The final suspension contained approximately 0.5 mg dry weight of germinated spores per ml.

Live germ tubes of either test fungus were not lysed by culture filtrates or other enzyme preparations; on the contrary, the germ tubes continued to grow, resulting in an increase in the turbidity of the mixture. In most cases, growth in enzyme preparations exceeded that in buffer alone.

Typical results for F. solani f. pisi are shown in Fig. 8.

Washed, live germinated spores of G. cingulata, F. solani f. pisi or H. victoriae, incubated at 28° in per-vaporated culture filtrates or partially purified chitinase preparations, were observed microscopically. In all cases the germ tubes continued to elongate, and after 22 hr. incubation there was a substantial increase in amount of hyphae. No abnormal growth or lysis was seen during the incubation period.

No lysis was observed when chitinase solutions were applied to an agar medium containing hyphae of the 3 test fungi. From all of these results, it is apparent that chitinase alone does not lyse live germ tubes of the test fungi.

Heat-killed germ tubes of F. solani f. pisi were not lysed after 20 hr. incubation in solutions of protease, glucanase or buffer. But chitinase, snail digestive juice (which contains chitinase), or a combination of all enzymes, decreased the O.D. by approximately 25% (Fig. 8). Killed germ tubes of G. cingulata were not lysed in any of the enzyme preparations.

Heat-killed germ tubes of F. solani f. pisi lysed more rapidly than did gas-killed germ tubes during the first 2-3 hr. incubation in chitinase (Fig. 8A). This difference in initial rates of lysis may be a reflection of the manner in which the germ tubes were previously killed. Heat treatment caused no change in turbidity of a germinated spore

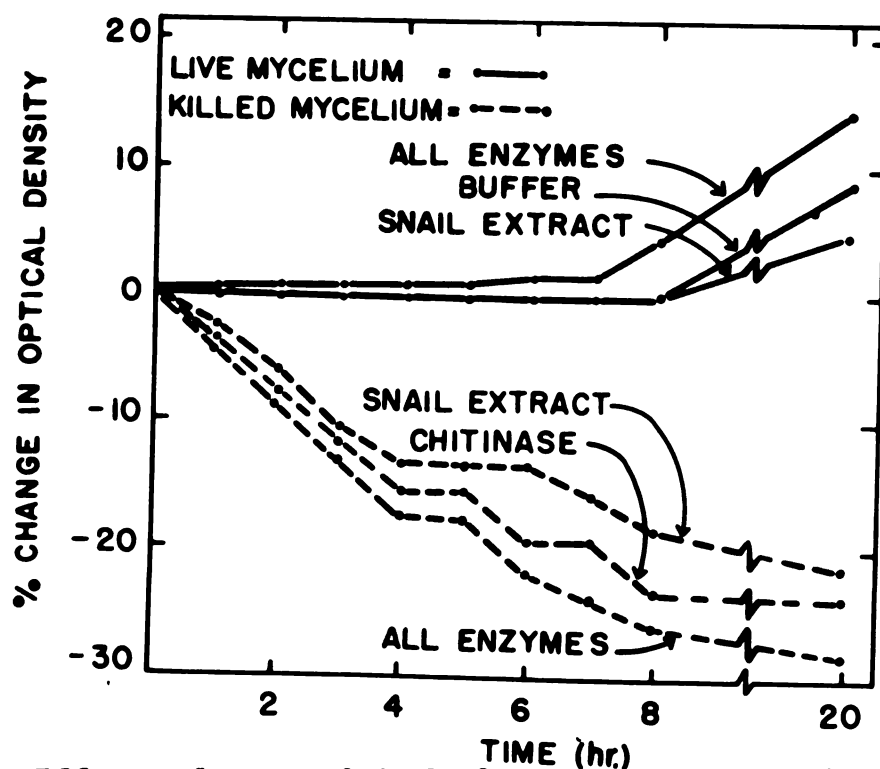
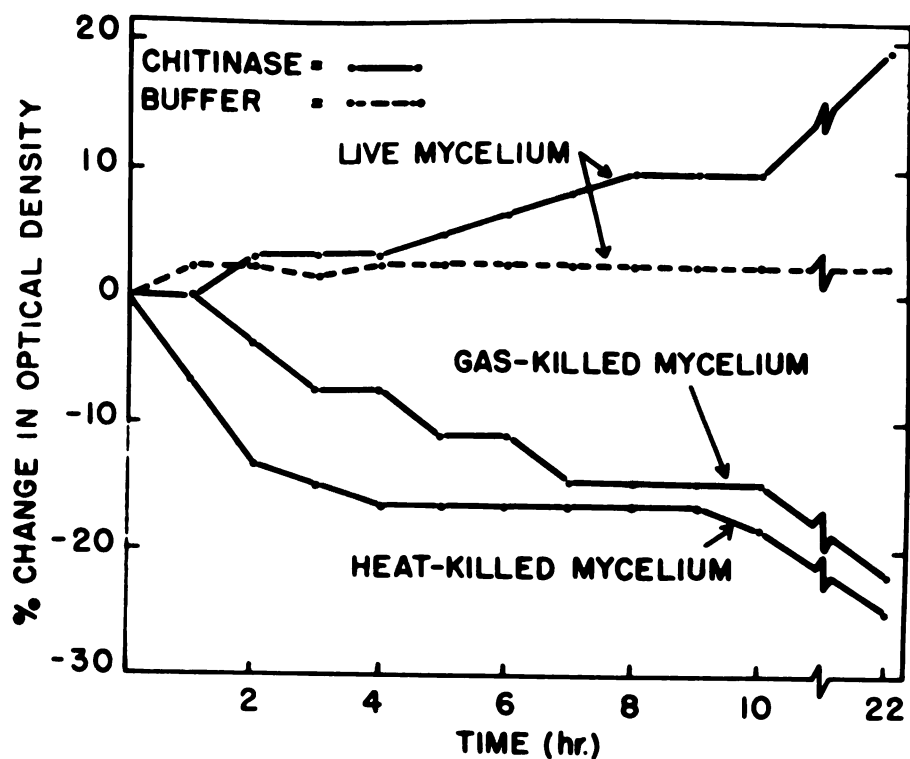


Figure 8. Effect of several hydrolysing enzymes on live and dead mycelium of *Fusarium solani* f. *pisi* incubated at 28°C; O.D. measured at 400 mμ. A) In ethanol-precipitated chitinase. B) In phosphate buffer, snail digestive juice or a combination of protease, chitinase, β-D-1,3 glucanase and snail digestive juice.

suspension, whereas gas treatment decreased it by 16%, presumably as a result of autolysis.

Reduction in turbidity of dead germinated spores of F. solani f. pisi in chitinase seems to result from partial hydrolysis of cell-wall chitin rather than from leakage of protoplasm, as the turbidity of dead germinated spores in phosphate buffer did not change during the same incubation period. Also, the walls of the germ tubes incubated in chitinase appeared thin and almost invisible when seen with a microscope.

Lysis by streptomycetes.--Many streptomycetes both lyse fungal mycelium and hydrolyse chitin. Therefore the importance of extracellular chitinase was further evaluated by comparing the amount of fungal mycelium lysed and amount of chitin hydrolysed in agar by the same streptomycetes.

Of 92 streptomycete isolates, 77 completely or partially lysed live fungal mycelium, all 92 isolates partially lysed killed mycelium and completely hydrolysed colloidal chitin and 65 isolates inhibited fungal spore germination. The mycolytic zones produced by these streptomycetes on live mycelium (Fig. 9) were mainly free of fungal hyphae and streptomycete growth; mycolysis occurred without direct parasitism of the fungal mycelium.

Sizes of mycolytic zones surrounding the same streptomycetes grown on live mycelium and killed mycelium were significantly correlated (1% level) (Table 8), but the value is low ($r = 0.37$) and is of doubtful biological

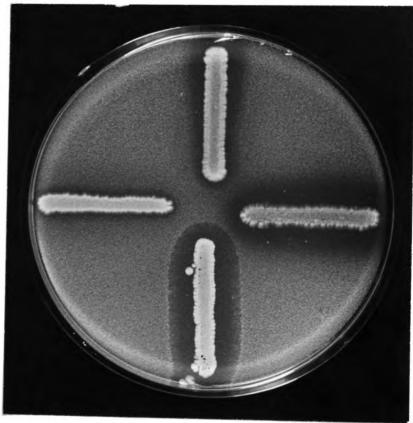


Figure 9. Mycolytic zones produced by 4 streptomycete isolates grown on the surface of 0.5% peptone agar containing a 4-day-old culture of Glomerella cingulata.

Table 8. Relations between lysis of live mycelium and killed mycelium of G. cingulata, hydrolysis of chitin, and inhibition of fungal spore germination by 92 actinomycetes

Association tested	Correlation coefficient (r)
Killed mycelium X Chitin	0.07
Live mycelium X Chitin	-0.20
Live mycelium X Killed mycelium	0.37**
Live mycelium X Inhibition	0.61**

**Significant at 1% level.

significance. The sizes of cleared zones produced by streptomycetes grown on chitin agar were not significantly correlated with those produced on live or killed mycelium ($r = 0.20$ and 0.07). Thus, the quantity of extracellular chitinase formed by streptomycetes was not associated with the amount of live or killed mycelium lysed by the same streptomycetes.

Lysis of fungal mycelium separated from soil by a filter.--If live fungal mycelium were lysed by extracellular enzymes alone, then separating soil from fungal mycelium by a filter which prevents passage of enzymes, should also prevent mycolysis. If lysis did occur free from extracellular enzymes, this would suggest autolysis rather than heterolysis as the lytic mechanism.

Natural soil was placed in a petri dish, the surface smoothed, moistened, and then covered with a washed membrane filter. Three types of filters were used: 5 and 10 μ filters, about 130 μ thick and previously sterilized by ionizing radiation (Millipore Corporation, Bedford, Mass.); 4 μ filters (Gelman Company, Ann Arbor, Mich.), 220 μ thick and sterilized by propylene oxide gas. The 5 μ filters were donated by the Millipore Corporation. All 3 filters were about 50 mm diameter, composed of cellulose esters and biologically inert. Filters were preincubated on the soil surface for 1 day, and then washed germinated spores of the test fungus put directly on the upper surface of the filter, i.e., the filter separated the germinated spores from the soil.

As a control, membranes with germinated spores were placed on the surface of autoclaved soil. Water was occasionally added to the soil surface to compensate for loss in soil moisture during the 4 or 8 days incubation at 24°. The filters were then removed from the soil surface, and the soil particles washed off. The mycelium was stained with phenolic rose bengal, and excess stain removed by placing the filter on a Büchner funnel. After drying, the filters were mounted in immersion oil where they became transparent and stained mycelium could then be seen with a microscope. Unstained filters were also examined by phase contrast microscopy.

Mycelium of G. cingulata was almost completely lysed in 8 days on all 3 types of filters; only conidia and a few hyphal remnants remained after 8 days (Table 9; Fig. 10, A&B). Scattered clumps of conidia suggested that some mycelial growth and sporulation occurred on restricted areas of the filter prior to mycolysis. When unstained preparations were observed with a phase microscope, only small fragments of hypha could be seen. Mycelium of H. victoriae was completely lysed in 8 days on the upper surface of membranes with 5 and 10 μ spores, and 50-90% lysed on 220 μ thick membranes with 4 μ pores (Table 9). Only conidia, conidiophores and old pigmented hyphae remained on the surface of the 10 and 5 μ membranes (Fig. 10, C&D). Mycelium of F. solani f. phaseoli grew on the membrane surface, especially the 220 μ thick membrane, sporulated, and then was partially

destroyed. After 8 days, 50-90% of the mycelium was destroyed on the thin membranes, leaving pieces of hypha, conidia and scattered chlamydospores joined to discontinuous hyphal remnants (Fig. 10, E&F). With phase contrast, pieces of unstained hyphae were frequently seen. The mycelia of all 3 fungi grew abundantly over the surfaces of filters on autoclaved soil.

Soil was also placed on the upper surface of a membrane filter, the lower surface being placed in close contact with 3-day-old G. cingulata mycelium in 0.5% peptone agar. The soil (about 9 g dry weight) was supplemented with water, peptone, glucose or chitin, equivalent to 0.2% of the dry weight of the soil. Colloidal chitin was added 4 days before placing the soil on a filter. As controls, natural soil and supplemented soils without filters, or membranes alone were placed directly on the agar surface. After 8 days incubation at 28°, the amount of lysis occurring beneath each filter was estimated by comparison with a G. cingulata culture killed by steam, and then stored in a refrigerator.

Partial lysis occurred under the 4, 5 and 10 μ filters which contained soil supplemented with water or colloidal chitin; there was little or no lysis beneath filters containing soil supplemented with peptone or glucose. Without filters the results were similar although more pronounced.

In contrast to these results, Mitchell (36) reported that when a dialysis membrane of regenerated cellulose was

Table 9. Lysis of live fungal mycelia on the upper surface of membrane filters, the lower surface of which was in close contact with natural soil

Filter ^b	Lysis rating after 4 and 8 days ^a					
	<u>Glomerella</u>		<u>Helminthosporium</u>		<u>Fusarium</u>	
	<u>cingulata</u>		<u>victoriae</u>		<u>solani</u>	
	4	8	4	8	4	8
10 μ pore size						
filter	4	4	1	4	2	3
5 μ pore size						
filter	3	4	1	4	2	3
4 μ pore size						
filter	3	4	2	3	2	3
Autoclaved soil	0	0	0	0	0	0

^aLysis was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from 1 of 2 experiments.

^bMembrane filters were biologically inert and composed of cellulose esters. The 10 and 5 μ filters were 130 μ thick and sterilized by ionizing radiation; the 4 μ filters were 220 μ thick and sterilized by propylene oxide gas.

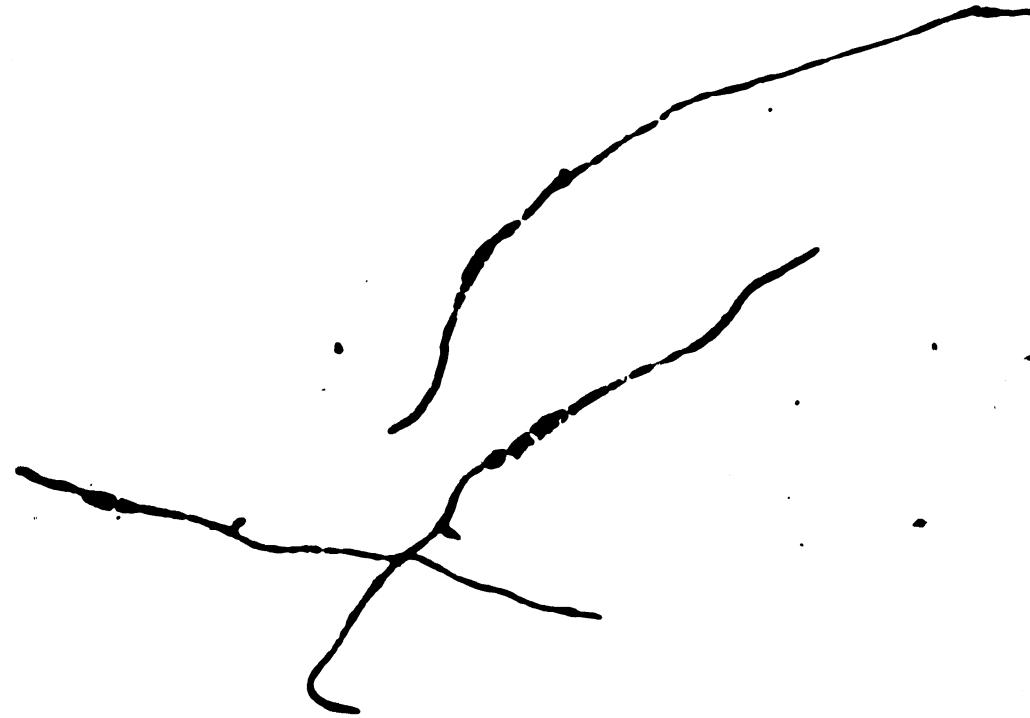


Figure 10. Lysis of live fungal mycelia when separated from natural soil by a membrane filter with 5 μ m pores. A) germinated spores of Glomerella cingulata on the filter surface before placing the filter on soil. X 450. B) After 8 days the mycelium is almost completely lysed, X 200.

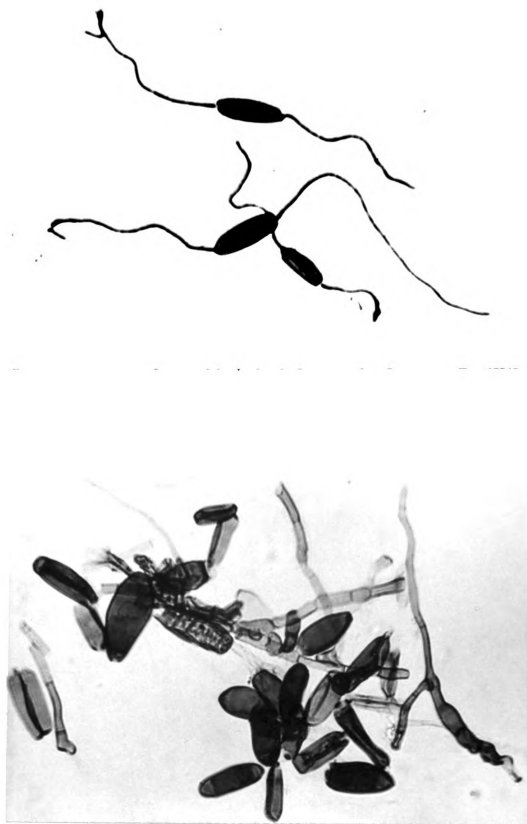


Figure 10. C) Germinated spores of Helminthosporium victoriae on the filter surface before placing the filter on soil. X 450. D) After 8 days the mycelium is almost completely lysed, leaving only conidia, conidiophores and old pigmented hyphae. X450.

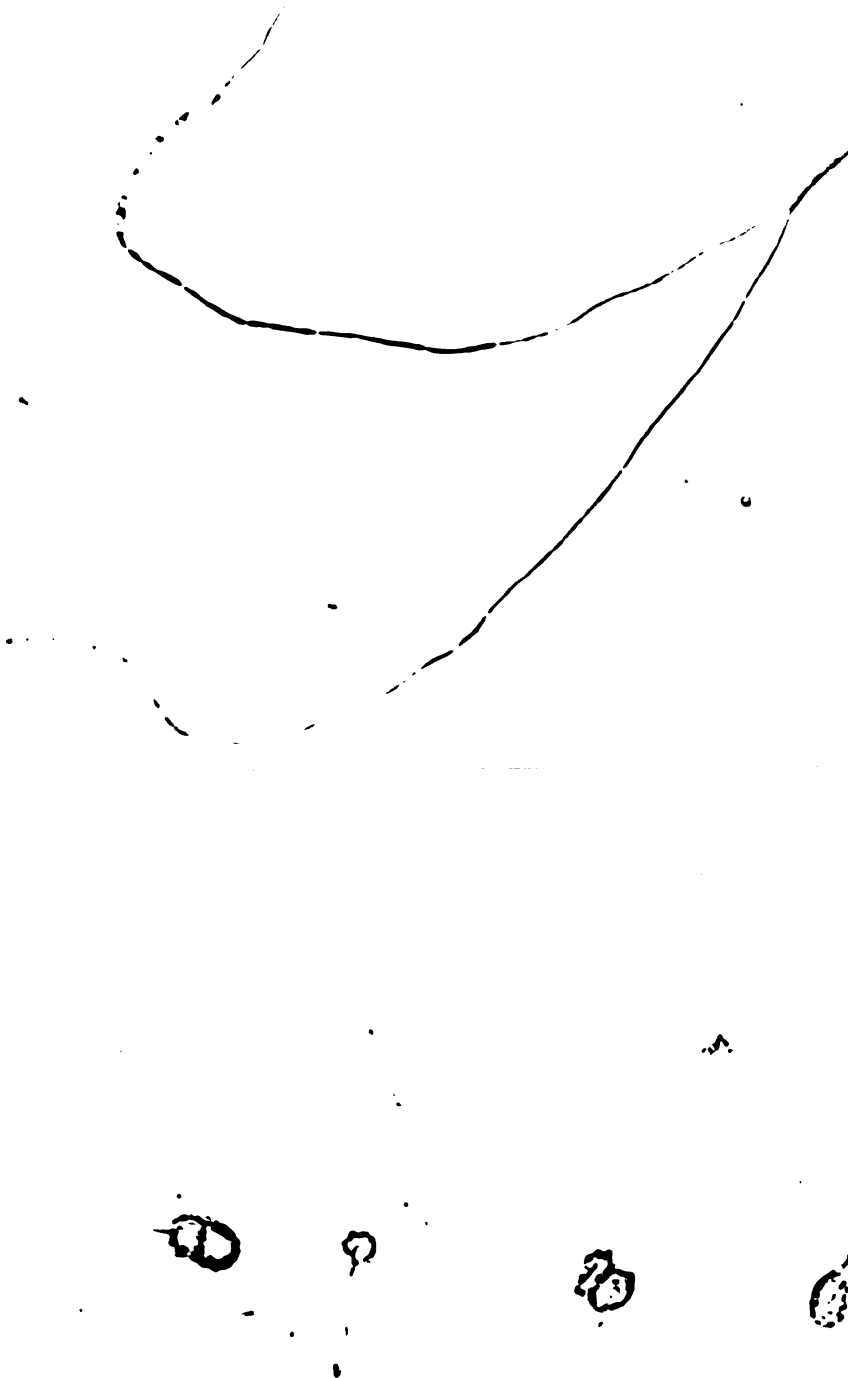


Figure 10. E) Germinated spores of Fusarium solani f. phaseoli on the filter surface before placing the filter on soil. X 450. F) After 8 days the mycelium is largely destroyed, leaving conidia, pieces of hyphae and scattered chlamydo-spores. X 450.

inserted between a 0.22 μ membrane filter and fungal mycelium in agar, soil on the filter surface did not cause lysis of mycelium in the agar. This was cited as evidence that extracellular enzymes are essential for soil mycolysis. Yet, when I attempted to repeat this experiment, with dialysis tubing or cellophane, these materials were partially degraded in contact with soil. Both G. cingulata mycelium in the agar and cellulolytic microorganisms from the soil can hydrolyse the cellulose, releasing hydrolytic products into the agar which would then be available as an organic nutrient source for the fungus. Since nutrients temporarily annul mycolysis, the inability to demonstrate lysis beneath the dialysis tubing is probably due to the presence of hydrolytic products from the dialysis membrane and not to preventing the passage of extracellular enzymes from the soil. For this reason, an inert membrane filter with a 5 μ pore size is superior to a dialysis membrane.

There are several reasons why the results with membrane filters were not conclusive evidence that fungal mycelium autolyses on the filter surface, Soil microorganisms could colonize the sterile surface from the outer edge towards the fungal mycelium in the center. Although no special precautions were taken to prevent such contamination, bacteria or actinomycetes were not seen in the area of the lysed fungal mycelium. If present, they should have stained and been observed. Occasionally when an air pocket was

trapped between a filter and the soil, fungal mycelium did not lyse on the filter surface above the airpocket, suggesting that if some bacterial contaminants were present, they were ineffective in causing mycolysis. Also, when soil was placed on the upper surface of the filters, no bacterial or actinomycete colonies appeared on agar media beneath the filters, even after prolonged incubation. Washings from the surface of these small-pore filters, when spread over the surface of nutrient agar, did not reveal contaminating microorganisms. However, when 130 μ thick filters were put on the surface of nutrient agar, streaked with streptomycetes, incubated 2 days and the filters then transferred to the surface of chitin agar or live fungal mycelium in agar and incubated further at 28^o, after 6 days the streptomycete mycelia penetrated the filters and lysed small areas of chitin or fungal mycelium below the filters. Apparently in time, streptomycete mycelia from a large inoculum can penetrate 130 μ thick filters, but not the 220 μ ones. With soil there was no evidence that actinomycete mycelium ever penetrated the 130 μ filters. Furthermore, lysis did occur beneath the 4 μ pore size filters, which are impregnable to streptomycete mycelium.

Lysis of dead mycelium by soil.--Live fungal mycelium should lyse in soil at the same rate as dead mycelium if mycolysis is caused by extracellular enzymes alone. However, if soil mycolysis is an autolytic mechanism, then dead mycelium should be more resistant. When live and heat-killed

fungus mycelia of 9 different fungi were placed directly on natural soil, the live mycelium was always lysed faster than the dead mycelium (Tables 10 & 11). Live mycelium of G. cingulata, the most sensitive test fungus, was completely lysed in 4 days, whereas dead mycelium was not completely lysed until 8 days. With the other fungi, except Rhizoctonia spp., 50-90% of the live mycelia, but only 1-10% of the dead mycelia were lysed on natural soil in 4 days (Table 10).

The faster rate of lysis with live mycelium suggests an autolytic process. However, when mycelia of G. cingulata and F. solani f. phaseoli were killed by either propylene oxide gas or gamma radiation, they lysed on soil at the same rate as live mycelium (Table 11). During the long treatment required for gas or radiation killing, the mycelium partially autolyzes. This may account for its faster rate of lysis on soil as compared with heat-killed mycelium, the enzymes of which are inactivated before autolysis can occur.

Induced partial autolysis of fungal mycelium by antibiotics and fungicides.--From the results with hydrolytic enzymes, membrane filters, and dead mycelium, it appears that extracellular enzymes alone do not lyse fungal mycelium. Rather, the evidence suggests that live mycelium autolyzes in soil. If this is the mechanism for destruction of live fungal mycelium, what induces autolysis? Antibiotics and fungicides have been reported to induce partial autolysis

Table 10. Lysis of live and heat-killed mycelia of different fungi by natural soil

Fungus	Lysis rating at 2,4,8 and 12 days ^a							
	Live			Dead ^b				
	2	4	8	2	4	8	12	
<i>Glomerella cingulata</i>	2	4	4	0	2	4	4	
<i>Mucor ramannianus</i>	2	3	4	0	1	3	4	
<i>Helminthosporium victoriae</i>	2	3	4	0	2	4	4	
<i>Penicillium frequentans</i>	2	3	4	0	1	4	4	
<i>Fusarium solani</i> f. <i>pisi</i>	0	3	4	0	1	4	4	
<i>Fusarium solani</i> f. <i>phaseoli</i>	0	3	4	0	1	4	4	
<i>Verticillium albo-atrum</i>	0	2	4	0	1	4	4	
<i>Rhizoctonia praticola</i>	0	2	3	0	0	2	4	
<i>Rhizoctonia solani</i>	0	2	3	0	0	3	3	
<i>Rhizoctonia solani</i>	0	1	3	0	0	2	4	

^aLysis was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from a single experiment.

^bFungal mycelia were killed by exposure to 80°C for 4 min. in a water bath.

Table 11. Effect of killing mycelia of Glomerella cingulata and Fusarium solani f. phaseoli by exposure to heat, propylene oxide gas or ionizing radiation, on rate of lysis by natural soil

Soil ^b	Mycelial treatment ^c	Lysis rating on 2,4,6 and 8 days ^a							
		<u>G. cingulata</u>				<u>F. solani</u>			
		2	4	6	8	2	4	6	8
Natural soil	heat-killed	0	3	4	4	0	1	2	3
Natural soil	gas-killed	3	4	4	4	1	3	4	4
Natural soil	radiation-killed 1.2 megarads	3	4	4	4	1	3	4	4
Natural soil	radiation-killed 3.0 megarads	3	4	4	4	1	4	4	4
Natural soil	live mycelium	3	4	4	4	1	3	4	4
Radiation soil	live mycelium	0	0	0	0	0	0	0	0
Autoclaved soil	live mycelium	0	0	0	0	0	0	0	0

^aLysis was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from 1 of 2 experiments.

^bRadiation-sterilized soil received 4 megarads from a Co⁶⁰ source.

^cMycelium was placed in a water bath at 80°C for 4 min., exposed to propylene oxide gas for 8 hr. or to gamma radiation for the indicated dosage.

of live fungal mycelium (8), and this has now been confirmed (Table 12). Of 7 antibiotics tested, all but griseofulvin induced partial autolysis of 3-day-old mycelium of G. cingulata in 0.5% peptone agar, when added to the surface of the agar in small filter discs. Three of 5 fungicides, but neither of the 2 metabolic inhibitors, were also effective.

Live germinated spores of F. solani f. phaseoli, when added to a solution of endomycin or cycloheximide, autolysed slightly during 22 hr. incubation (Table 13). With 100 μ g per ml endomycin and cycloheximide, the optical density decreased by 13% and 3%, respectively. Results were similar when washed germinated spores were kept in 10% Reynolds' mineral salt solution for 1 day before adding the antibiotics.

Induced partial autolysis of fungal mycelium by starvation conditions.--As an alternative to antibiotics or toxic substances, autolysis may be induced by lack of available nutrients, similar to the mechanism proposed as an explanation for soil fungistasis (30). Addition of organic nutrients to soil temporarily nullified mycolysis. Streptomycetes induce lysis in a nutritionally deficient medium (7), and partial autolysis follows depletion of an organic nutrient in a liquid culture (11).

Conidia were spread over the surface of sterilized membrane filters (0.45 or 0.65 μ pore sizes) which rested on a dilute nutrient agar medium. After about 24 hr. incubation, the filters were washed several times in 10% Reynolds'

Table 12. Induced partial autolysis of Glomerella cingulata mycelium in agar by antibiotics, metabolic inhibitors and fungicides

Inhibitors ^a	Concentration ($\mu\text{g/ml}$)	Lysis rating ^b
Cycloheximide	100	++
Amphotericin B	100	++
Endomycin	100	++
Filipin	100	++
Griseofulvin	100	+
Nystatin	100	++
Pimaricin	100	++
2-4-dinitrophenol	10 mM ^c	+
Sodium azide	10 mM ^c	+
Captan	1,000	+
Phenyl mercuric acetate	100	++
Thiram	1,000	+
Copper sulfate	100	++
Mercuric chloride	100	++
Heat-killed mycelium		0

^aCaptan is N-Trichloromethylmercapto-4-cyclohexene-1, 2-dicarboximide. Thiram is bis(dimethylthiocarbamyl) disulfide.

^bLysis ratings: 0 = no lysis, + = Inhibition of mycelial growth or weak lysis; ++ = definite but incomplete lysis.

^cMilli-moles.

Table 13. Induced partial autolysis of Fusarium solani f. phaseoli mycelium by 2 antibiotics as measured by changes in turbidity of a mycelial suspension in an antibiotic solution

Time	% decrease in optical density ^a						Buffer
	Endomycin			Cycloheximide			
	1,000	100	10	1,000	100	10	
(hr)	(µg/ml)			(µg/ml)			
0	0	0	0	0	0	0	0
2	7	5	4	2	2	0	0
4	10	10	8	4	2	0	0
6	12	13	10	4	2	0	+2
8	14	13	10	6	3	0	+2
10	15	13	12	6	3	+1	+2
22	15	13	12	8	3	+2	+6

^aOptical density was measured at 425 mµ. All values are decreases in O.D. except for those marked with a +. Values are from 1 of 2 experiments. Endomycin and cycloheximide solutions contained 1% ethanol which was also added to the phosphate buffer control.

mineral salt solution, and then put directly on an inflated dialysis tubing (Fig. 11). The dialysis system functioned as follows: a centrifugal pump with a nylon head slowly circulated 10% Reynolds' solution through vinyl tubing (200 ml/min.) into inflated dialysis tubing, about 30 cm long and 3 cm inflated diameter, which rested on a sheet of plexiglass. Closely adhering to the outer surface of the dialysis tubing were membrane filters with fungal mycelia on their outer surface. A plexiglass cover surrounded the entire dialysis tubing to maintain a high relative humidity. After passing through the dialysis tubing the circulating solution entered a 3 liter reservoir which was also continually drained from an outlet connected to the centrifugal pump. The circulating solution was kept at 25°, and sometimes streptomycin sulfate added (40 µg/ml) to inhibit bacteria. Membrane filters and dialysis tubing were sterilized, and aseptic precautions used where practical.

The object of the dialysis system was to create a diffusion gradient away from the fungus so that the mycelium would be in a state of continual starvation. If mycelium leaked organic nutrients, they would pass through the dialysis tubing and be diluted by the large volume of circulating solution. On the second day the membrane filters were removed, stained with phenolic rose bengal, washed, dried, mounted in immersion oil, and examined with a microscope.

Mycelia of the 3 test fungi grew for a limited period on the membrane filters. The protoplasm of G.cingulata

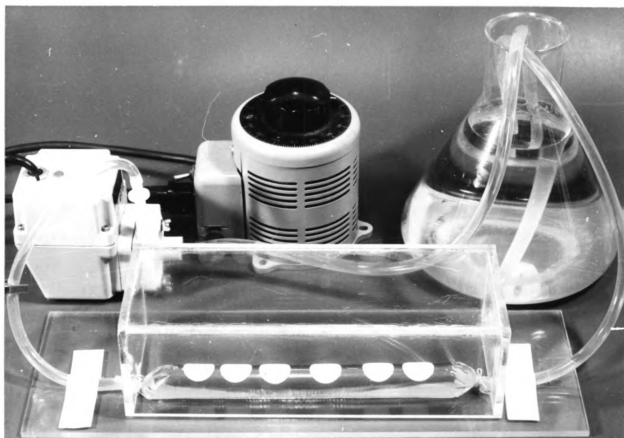


Figure 11. The dialysis system in operation. Fungal mycelium is on the outer surface of the 6 round membrane filters resting on the inflated dialysis tubing. The water pump at the left circulates a mineral salt solution through the dialysis tubing and back via the reservoir at the right.

and H. victoriae segmented, but with these 2 fungi and F. solani f. phaseoli the hyphae otherwise remained intact without obvious loss of protoplasm or cell wall breakdown (Fig. 12).

Complete autolysis in the absence of microorganisms and soil.--Continual starvation alone failed to induce complete autolysis, as did antibiotics added to the fungal mycelium in agar or in buffer. The 2 treatments were therefore combined on the dialysis system. Membrane filters containing live fungal mycelia were placed on top of the dialysis tubing. After 3-4 hr., about 0.2 ml of an antibiotic was added to the surface of each filter, and the application repeated twice at about 3 hr. intervals and twice again after 1 day. Antibiotics were dissolved in ethanol, the final concentration being 100 μ g per ml in 1% ethanol.

With addition of antibiotics, complete autolysis frequently occurred on the dialysis system (Fig. 13), although in some tests the mycelium failed to autolyse. Such variables as humidity within the plexiglass cover, temperature of the circulating solution and type of membrane, when changed within reasonable limits, did not noticeably affect autolysis. But in spite of occasional failure, the dialysis system clearly demonstrated that in a starved condition, the addition of an antibiotic will induce complete autolysis with some fungi and partial autolysis with others.

Addition of the antifungal antibiotic filipin or endomycin (supplied by Upjohn Co., Kalamazoo, Mich.) to fungal



Figure 12. Partial autolysis of fungal mycelia on membrane filters which rested on dialysis tubing inflated with circulating mineral salt solution. A) Mycelium of Glomerella cingulata on the filter surface before placing the filter on dialysis tubing. X300. B) After 2 days the protoplasm is segmented but the hyphae remain intact. X300.

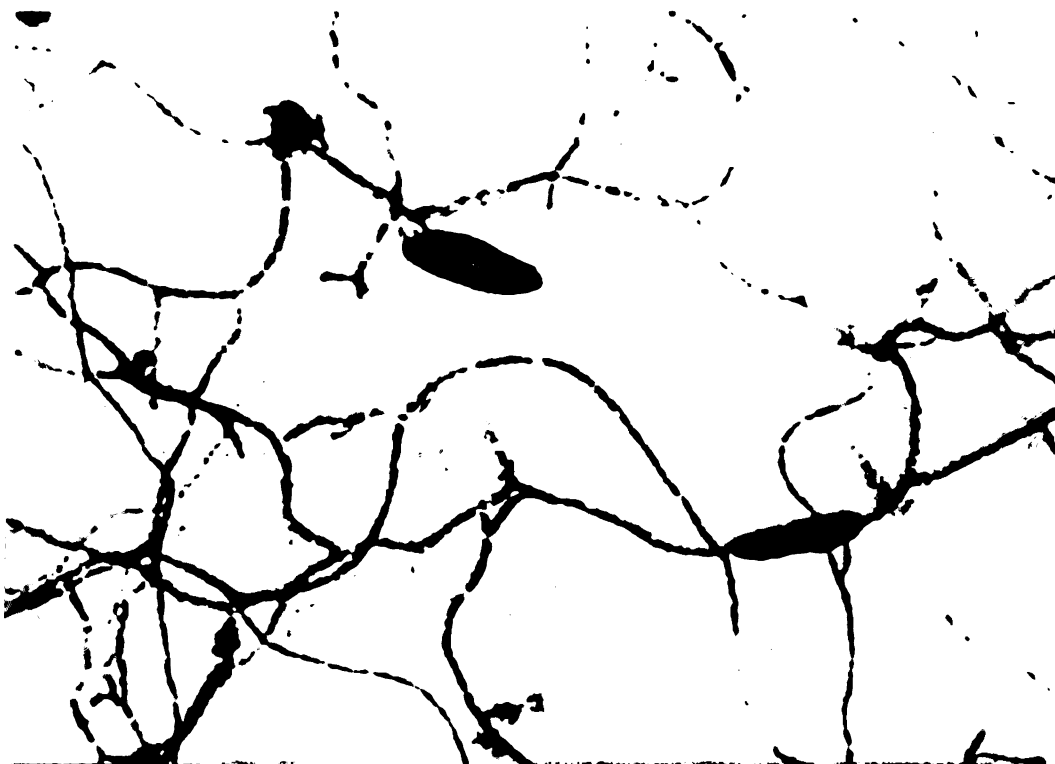


Figure 12. C) Mycelium of Helminthosporium victoriae on the filter surface before placing the filter on the dialysis tubing. X300. D) After 2 days, some of the protoplasm is segmented, but the hyphae remains intact. X300.

Figure 13. Lysis of fungal mycelia on membrane filters which rested on dialysis tubing inflated with circulating mineral salt solution. The antibiotic filipin (100 $\mu\text{g/ml}$) was added to the surface of the filters. A) Mycelium of Glomerella cingulata on the filter surface before placing the filter on the dialysis tubing. X300. B) After 2 days with antibiotic added, the mycelium is almost completely autolysed, leaving conidia and small hyphal fragments. X300.



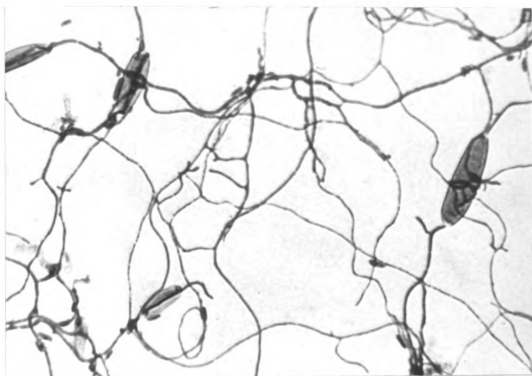


Figure 13. C) Mycelium of Helminthosporium victoriae on the filter surface before placing the filter on dialysis tubing. X300. D) After 2 days with antibiotic added, the mycelium is almost completely lysed, leaving conidia and small hyphal fragments. X300.

mycelia of G. cingulata and H. victoriae on the dialysis system induced much autolysis after 1 day. The remaining mycelia were lightly stained and frequently segmented. On the second day the mycelia of both fungi were almost completely autolysed: conidia, small hyphal fragments and some old pigmented hyphae of H. victoriae remained (Fig. 13). In unstained preparations, few hyphae could be seen with phase contrast. Occasional hyphal tips of H. victoriae did not lyse, presumably because these tips were aerial and somehow escaped lysis. With F. solani f. phaseoli, only a small amount of autolysis occurred, the majority of the mycelium being lightly stained but apparently intact.

Nystatin (supplied by The Squibb Inst., New Brunswick, N.J.) and cycloheximide also induced complete autolysis of the mycelia of G. cingulata and H. victoriae. Pimaricin was less effective.

Using the dialysis system, it is inevitable that some bacterial contamination will occur on the filter surface. These contaminants are believed unimportant for several reasons: 1) few stained bacteria were seen on the membrane filters, 2) the use of streptomycin sulfate in the circulating solution did not interfere with lysis, 3) mycelium on the control filters without antifungal antibiotics remained intact.

Application of the autolysis model to natural soil.--

How good is the dialysis system as a model for soil mycolysis? In areas of soil free from plant residues, there is indeed a

low level of available organic nutrients. And there is some evidence that those soil microorganisms which produce antibiotics also induce lysis of live hyphae.

There was a significant correlation ($r = 0.61$; 1% level) between sizes of inhibition zones and sizes of mycolytic zones produced by the same 92 streptomycetes when grown respectively on agar seeded with G. cingulata conidia and live fungal mycelium in agar (Table 8). Figure 14 shows a scatter diagram with the zone measurements for each streptomycete, and the estimated regression line. With 1 exception, these streptomycetes which produced well-defined mycolytic zones greater than 1 mm in width also produced inhibition zones (Fig. 14). A few streptomycetes inhibited conidial germination but did not lyse live mycelium, or formed very small lytic zones. In summary, those streptomycetes which distinctly lysed live fungal mycelium also inhibited fungal spore germination, and degree of mycolysis seems to be associated with degree of fungal spore inhibition. With the 8 bacterial isolates from soil, only the 2 which clearly inhibited germination of G. cingulata conidia were able to restore mycolysis to sterilized soil (Table 14). Bacterial isolates which were not antagonistic were also ineffective in sterilized soil. None of the bacterial isolates produced chitinase.

If antibiotic producing microorganisms do induce autolysis of fungal mycelium in soil, then antibiotics must be formed in the vicinity of lysing fungal hyphae.

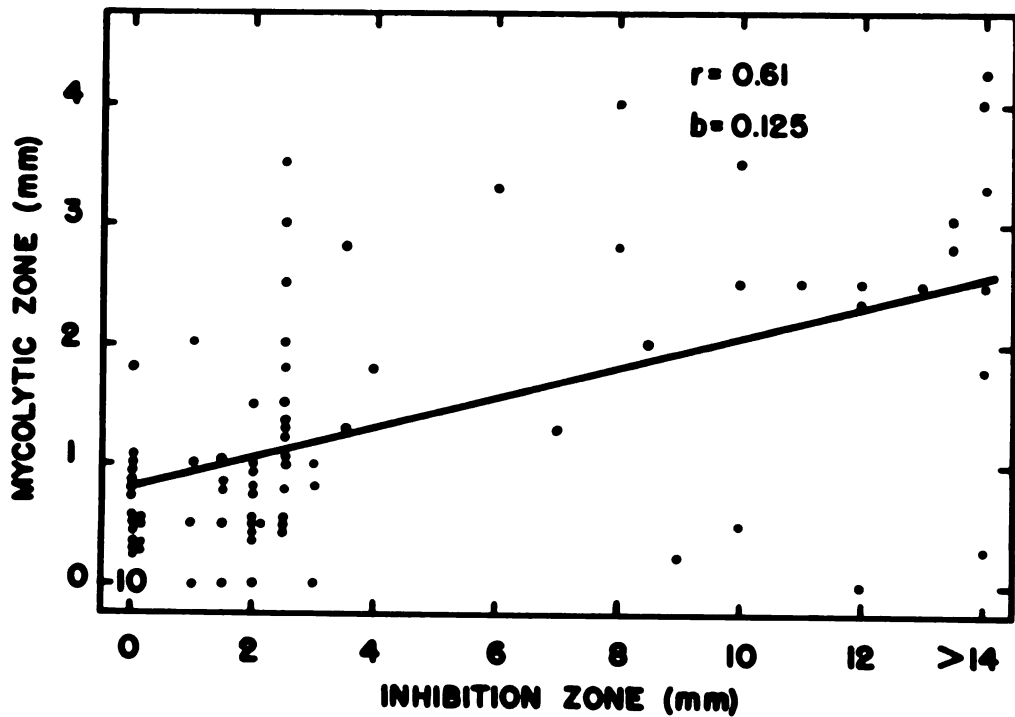


Figure 14. Relationship between lysis of live mycelium and inhibition of fungal spore germination by 92 streptomycetes. Each point indicates the zone widths produced by the same streptomycete when grown on live mycelium of Glomerella cingulata in agar (mycolytic zone), and on the surface of agar seeded with G. cingulata conidia (inhibition zone). r = correlation coefficient; b = regression coefficient; 10 = ten streptomycetes which neither inhibited nor lysed.

Table 14. Ability of bacterial isolates from soil to both inhibit the germination of Glomerella cingulata conidia and restore the mycolytic property to sterilized soil

Bacterial isolate ^a	Inhibition zone (mm)	Lysis rating at 2, 4 and 8 days ^b		
		2	4	8
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
14	0	0	0	0
17	1	0	0	0
4	1	0	0	1
5	8	0	2	2
11	11	0	3	4
Autoclaved soil		0	0	0

^aAutoclaved soil was infested with a bacterial isolate and incubated 6 days before placing the mycelium directly on the soil surface.

^bLysis was rated 0-4: 0 = no lysis, 1 = 1-10% of mycelium lysed, 2 = 10-50%, 3 = 50-90%, 4 = 90-100%. Values are from a single experiment.

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Live fungal mycelium of G. cingulata was added to natural soil in an amount equivalent in dry weight of the mycelium, to 1% dry weight of the soil. After 2-4 days incubation, the antibiotic substance was extracted by mixing 40 g of supplemented soil with 40 ml ethanol, centrifuging, and then evaporating the supernatant fluid to dryness with vacuum. The precipitate was redissolved in a minimum amount of ethanol, sterilized by passage through a sintered-glass filter (1.0-1.5 μ pore size), added to a small filter paper disc, air-dried, and then placed on the surface of 0.5% peptone agar medium. After 2-3 hr. storage at 4°, the surface of the agar was sprayed with a conidial suspension of either G. cingulata or F. solani f. phaseoli and the plates incubated at 24° for 1 and 2 days.

The antibiotic substance in the filter disc inhibited germination of the conidia of both G. cingulata and F. solani f. phaseoli (Fig. 15). Of 5 soils which were supplemented with live mycelium and kept for either 2 or 4 days, extracts from 3 of these soils formed large and well-defined inhibition zones; with the other 2, the zones were doubtful. In one case the radius of the inhibition zone measured 14 mm, exclusive of the disc. No inhibition occurred with discs containing ethanol extractions from natural soil. Apparently in natural soil, free of decomposing organic matter, sufficient nutrients leak from lysing fungal hyphae to promote antibiotic production by adjacent antagonistic microorganisms.

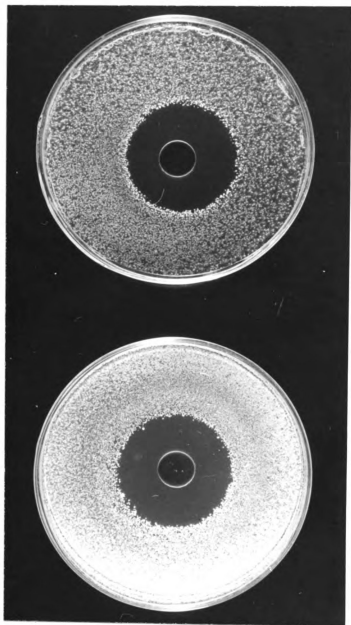


Figure 15. Inhibition zones surrounding filter paper discs containing an antibiotic substance, extracted with ethanol from soil supplemented with live fungal mycelium. Top, Glomerella cinquilata. Bottom, Fusarium solani f. phaseoli.

DISCUSSION

Most fungal hyphae, when added to natural soil, are rapidly lysed, although fungi differ in their rates of lysis. Mycelia of G. cingulata, a parasite of apples and not found in soil, was completely lysed in 4 days, mycelia of 6 other species in 8 days, whereas some mycelia of Rhizoctonia spp. remained after 8 days.

Soil mycolysis appears to be a universal property of all soils with a high microbial population. A garden soil, high in microbial numbers, lysed live fungal mycelia at a faster rate than heath or peat soil, which were low in microbial numbers (49). Moreover, soils which do contain a large amount of fungal mycelia are frequently acidic and unsuitable for growth of soil bacteria and soil actinomycetes (5, 21), the organisms most likely involved in mycolysis. The microflora of acid podzols (5) and acid peat soils (21) is characteristically fungal, containing much mycelia.

The ability of soil to destroy live fungal mycelia is associated with soil microorganisms. Many soil streptomycetes lysed live fungal mycelium of G. cingulata growing in an agar medium. And these same streptomycetes restored the lytic property to sterilized soil, a relationship already noted (31). Two of the 8 selected soil bacteria also restored the lytic property to sterilized soil.

Soil mycolysis does not appear to result from direct parasitism of the live hyphae. Live mycelium of G. cingulata

in agar lysed at some distance from the lytic streptomycete colonies. Further, bacteria and actinomycetes do not accumulate round lysing fungal hyphae, but appeared after complete decomposition has occurred. In contrast, there are several reports that on a glass slide buried in soil, bacteria do accumulate round an intact hypha before decomposition (12, 13, 56). Perhaps the presence of the inert glass surface permits coexistence of both the hypha and bacteria for a limited time before lysis of the hypha. A similar situation exists with soil fungistasis: the presence of a glass surface temporarily annuls the fungistatic property of soil, permitting fungal spores to germinate on the glass surface in soil (15).

Soil mycolysis tends to restrict mycelia to microsites where organic nutrients are present. With addition of glucose or peptone to natural soil, both fungal mycelia and large numbers of actively growing bacteria and actinomycetes coexisted until depletion of the nutrients, when the fungal mycelia lysed. Supplementing soil with chitin, a substrate unavailable to the fungal mycelium, but hydrolysed by chitinolytic microorganisms in soil, enhanced lysis. Hence, organic nutrients which are available to both the fungus and other soil microorganisms temporarily annul soil mycolysis, whereas organic nutrients available to some soil microorganisms but not to the fungus mycelium, enhance lysis. As the soil mass free from decomposing organic substrates has a low level of organic nutrients, most fungi exist in these

parts as resistant propagules and not as mycelia (29, 59).

Most of the root-inhabiting fungi possess resistant spores or other resistant propagules (29, 59), but many of the soil-inhabiting fungi do not appear to have obvious, large resistant structures. In some of these cases, survival might depend on small resistant "chlamydospores", similar to those which persisted in soil after decomposition of the vegetative hyphae of P. frequentans and V. albo-atrum. And a few basidiomycetes and sterile fungi do survive as mycelia for sustained periods in the soil mass free from decomposing organic matter (65).

Soil microorganisms which cause lysis of live fungal mycelium in soil seem also to benefit nutritionally from lysing mycelium, as undoubtedly non-lytic microorganisms do. In natural soil supplemented with live mycelium of G. cingulata, bacterial numbers increased about 100-fold in 7 days and the soil actinomycetes about 10-fold in 9 days. A similar increase has also been reported for soil supplemented with live mycelium of F. oxysporum f. cubense (37). Other soil biota must also benefit indirectly from lysing fungal mycelia and a characteristic succession may develop. Protozoa for instance, frequently appeared in the vicinity of the lysed mycelium.

If mycolysis is caused by soil microorganisms, the mechanism must be either heterolytic or autolytic. For convenience, heterolysis is defined as lysis of live mycelium by extracellular enzymes liberated by adjacent microorganisms.

Conversely, autolysis is lysis of live mycelium by intracellular enzymes, resulting in self-digestion of the fungal mycelium. The 2 mechanisms differ in the origin of the lytic enzymes, but a clear distinction between these two possible alternatives seems fundamental to an understanding of the mechanisms of soil mycolysis.

Extracellular enzymes alone do not appear to lyse live fungal hyphae. When soil was separated from fungal mycelia by an inert membrane filter having pore sizes small enough to prevent passage of enzymes, mycelia of G. cingulata and H. victoriae lysed completely; mycelium of F. solani f. phaseoli partially lysed. Complete lysis also occurred ~~in~~ when the same filters separated mycelium of G. cingulata in agar from soil. Assuming that there ~~was~~ no microbial contamination or movement of enzymes through the filters, then the live hyphae must be autolysed. Heat-killed mycelia of 9 different fungi were consistently more resistant to soil mycolysis than live mycelia, which also suggests that live hyphae autolyse in soil.

However, there are some reports that enzymes alone lyse live mycelium. An enzyme preparation, precipitated from the culture filtrate of a Streptomyces sp., produced protoplasts from several different fungi, apparently by dissolution or weakening of some cell-wall component in a restricted area, causing extrusion of protoplasm (1). This is a different phenomenon from soil mycolysis. Empty hyphae or those with bulges were not seen when hyphal remains were recovered from soil by plastic peelings.

If extracellular enzymes do lyse live mycelium, one of these enzymes would have to be chitinase, as chitin is a major constituent of most fungal cell walls (11). Further, many mycolytic actinomycetes produce chitinase (41), and the few mycolytic bacteria examined produce chitinase and also laminarinase (37), which hydrolyses laminarin, another major constituent of the cell wall of filamentous fungi. Streptomyces chitinases are reported as partially lysing live fungal mycelia of G. cinquilata (41) and Aspergillus oryzae (20) at 37°.

Chitinase was not, however, a lysin for live fungal mycelia in the present work at the incubation temperature of 28°. This enzyme, either separately, or in combination with other hydrolysing enzymes, including laminarinase, did not lyse live mycelia of G. cinquilata and F. solani f. pisi, although it did partially lyse dead mycelium of the latter. A chitinase preparation from table mushrooms also did not lyse live mycelium of F. oxysporum f. cubense (37). The 2 reports on lysis of live fungal mycelia by chitinase (20,41) were from tests done at 37°, which is approaching a lethal temperature for hyphae of these fungi. It is likely then, that the fungi were in a moribund state, and reacted to chitinase more like dead than live mycelia. This is especially supported in the case with A. oryzae where the optical density of the mixture decreased during incubation at 37° by approximately 20%, a value similar to my results

for partial lysis of dead mycelium of F. solani f. lisi in chitinase.

The quantity of extracellular chitinase produced by streptomycetes is also not a critical factor determining their mycolytic ability. There was no correlation between the amount of chitin hydrolysed and amount of live or killed mycelium lysed in agar by the same streptomycetes. Moreover, 2 bacterial isolates which did not produce extracellular chitinase, both restored the lytic property to sterilized soil.

Although chitinase alone is not a lysin for live fungal mycelia, the facts remain that most mycolytic actinomycetes and mycolytic bacteria produce chitinase. Further, supplementing soil with live fungal mycelium results in a large increase in chitinolytic bacteria which are otherwise uncommon in soil (63). This evidence suggests a functional role for extracellular chitinase. One possibility would be that mycolytic microorganisms both induce autolysis of fungal hyphae and produce chitinase which would hydrolyse the chitin remnants after incomplete cell wall breakdown from autolysis. The parallel evolution of ability to induce autolysis and to produce extracellular chitinase in bacteria and actinomycetes would then be of selective advantage to these organisms. Among the antagonists which induce autolysis, selection would tend towards those which could also compete for cell-wall remnants by formation of such extracellular enzymes as chitinase.

Much of the experimental evidence reviewed so far suggests that soil mycolysis is an autolytic mechanism: 1) lysis of live mycelia separated from soil by a membrane filter, 2) the relative resistance of heat-killed mycelia to soil mycolysis, and 3) ineffectiveness of chitinase and other hydrolysing enzymes as lysins of live mycelia. Other evidence also supports this view. Controlled local autolysis involving restricted cell-wall breakdown during hyphal anastomosis and gametangial union is common among the fungi. And Park (47) has recently described autolysis of hyphae in staled cultures of F. oxysporum.

If soil mycolysis is an autolytic process, what induces lysis? Accumulation of toxic substances are reported to induce autolysis in an aging pure culture (47). Partial autolysis of hyphae in agar was also induced by antibiotics and fungicides, as were germ tubes in a solution of an antibiotic.

Starvation conditions may also induce partial autolysis. Exhaustion of nutrients, especially organic nutrients, results in reduced mycelial weight in aging pure fungus cultures (11). And lysis of live mycelium by antibiotics occurs in a nutrient-deficient medium but not in one high in organic nutrients (7). When fungal mycelia were kept in a state of continual starvation on a dialysis tube inflated with circulating mineral salt solution, the protoplasm within the hyphae segmented, but autolysis did not proceed further.

Thus neither starvation alone nor antibiotics alone induced complete autolysis. However, when any of several antifungal antibiotics were added to live mycelia of G. cingulata and H. victoriae, kept continually starved on the dialysis system, complete autolysis frequently occurred. With F. solani f. phaseoli, the protoplasm segmented, but the hyphae remained intact. Therefore the dialysis system clearly demonstrated that in a starved condition, antibiotics will induce complete autolysis with some fungi and partial lysis with others. To my knowledge, this is the first report that mycolysis, similar to that occurring in soil, could be reproduced in the absence of microorganisms and soil.

More attention has been given to the mechanism for lysis of bacterial cells, and in some cases this is somewhat similar to that shown on the dialysis system for mycolysis. Complete destruction of some bacterial cells results from a combination of an antibiotic and extracellular enzymes, produced by the lytic microorganism. Some actinomycetes, or sterile culture filtrates from these organisms, lysed dead bacterial cells and live, gram-positive bacteria, but not live gram-negative ones (23, 38, 66). An antibiotic, isolated from the culture filtrate, killed gram-positive bacteria and so made them susceptible to destruction by extracellular enzymes in the filtrate, which alone were ineffective against live bacteria. A similar mechanism apparently operates with lysis of bacterial cells by culture filtrates from a

Myxococcus sp. (42). These examples demonstrate that the requirement of an antibiotic for lysis, as used on the dialysis system, is not unique. The main difference is that with some fungi the antibiotic can induce complete autolysis, whereas the bacterial cells were apparently killed before much autolysis occurred.

Mycolysis of live fungal mycelia on the dialysis system was similar to that occurring in soil. However, similarity is insufficient reason to believe that the dialysis system is a model for soil mycolysis. For it is necessary to show that in soil, fungal hyphae in a state of starvation are induced to autolyse by antibiotics from adjacent antagonistic microorganisms. There is at least some evidence that this may be true.

A significant correlation ($r = 0.61$) existed between sizes of inhibition zones produced by the same streptomycetes, when grown on agar containing fungal conidia and live mycelium of G. cinquilata. Those streptomycetes which lysed live mycelium also secreted antibiotics, a relationship already noted with a different group of actinomycetes (31). A similar close association existed between ability of bacterial isolates to inhibit fungal spore germination and to restore mycolysis to sterilized soil.

Within the soil, lysing fungal mycelium may provide a nutrient substrate for production of antibiotics by other soil microorganisms. When natural soil was supplemented with live fungal mycelium, an antibiotic substance was

extracted from the soil. Apparently soluble nutrients, leaking from the lysing hyphae (11), form a microsubstrate for growth and production of significant amounts of antibiotics by antagonistic microorganisms. Such a situation is compatible with current ecological concepts (3, 17).

Evidence that starvation conditions are also a requirement for soil mycolysis is the known low level of available organic nutrients in the bulk of soil free from decomposing organic matter. This is further supported by recent work with soil fungistasis (30). Only those spores which have an adequate nutritional reserve can germinate in soil apart from decomposing organic substances, and it is this part of the soil where fungal mycelium is rapidly lysed. Supplying natural soil with such organic nutrients as peptone or glucose prevents mycolysis until the nutrients are depleted, when the mycelium then lyses.

Antibiotics may induce only partial autolysis in some fungi. Destruction of the remaining dead hyphae would then depend on extracellular enzymes from other microorganisms. The incomplete autolysis of F. solani f. phaseoli mycelium on the dialysis system and also on the membrane filters in contact with soil, suggests that such a mechanism operates with this fungus, and probably with some other fungi.

Finally, Payen (49) believes that in soil, hyphae are first killed by a preformed external toxin which is not of microbial origin. After death, the protoplasm leaks

from the hyphae and then extracellular enzymes destroy the empty hyphae. The results from my work, on the contrary, indicate that lysis of hyphal walls occurred concurrently with disappearance of protoplasm. Moreover, there is no clear evidence that preformed soil toxins cause death of fungi in soil.

It is from the work of other people and my experimental results described here that I suggest the following short explanation for soil mycolysis. The fungistatic property of soil prevents germination and wastage of fungal spores in the absence of potentially colonizable organic substrates. In the presence of organic nutrients, the fungal spore germinates and colonizes the new substrate, followed by bacteria and actinomycetes. Under conditions of high organic nutrients, vegetative fungal mycelium coexists with high populations of both bacteria and actinomycetes. Antibiotics are produced by antagonistic microorganisms within the nutrient substrate, but these antibiotics are ineffective until organic nutrients become depleted, or the fungus is unable to compete for the remaining nutrients. Now the mycelium is starved, and the antibiotics or toxic metabolites from antagonistic microorganisms induce autolysis. Soluble nutrients leaking from lysing hyphae may also form a micro-substrate for growth and production of antibiotics. The hyphae of some fungi are completely destroyed by induced autolysis, but with other fungi, death of the hyphae occurs

before complete autolysis. Final dissolution of the remains of the cell walls would then depend on extracellular enzymes, which in soil come from adjacent microorganisms.

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