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INDUCTION OF CHLAMYDOSPORE FORMATION IN FUNGI IN AGAR MEDIA BY STREPTOMYCETES

Thesis for the Degree of M. S.
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MARINA INN CHIANG
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ABSTRACT

INDUCTION OF CHLAMYDOSPORE FORMATION
IN FUNGI IN AGAR MEDIA
BY STREPTOMYCETES

By

Marina Inn Chiang

Each of thirty Streptomyces isolates from soil induced chlamydospore formation by Fusarium solani f. sp. phaseoli isolates 20 and 151, F. solani f. sp. pisi and Mucor ramannianus in co-culture with the fungus. All isolates also inhibited fungal growth on the co-culture plates. Sixteen isolates produced substances in solid and liquid cultures which were capable of inducing chlamydospore formation and inhibiting germination of growth of the fungus. Agar discs from the area adjacent to streptomycete colonies, when transferred to fresh medium, induced chlamydospore formation in all four fungi. Chlamydospore-inducing substances from streptomycetes were detected when paper discs impregnated with sterile filtrates of liquid cultures were placed on agar seeded with F. solani f. sp. phaseoli. The chlamydospore-inducing substances were extraced from liquid cultures of two selected isolates with amyl alcohol. The

extract from one of the cultures inhibited macroconidial germination and induced chlamydospore formation from ungerminated macroconidia. The other inhibited germling growth followed by chlamydospore formation from the germlings.

The remaining fourteen Streptomyces isolates failed to produce detectable chlamydospore-inducing substances in culture, and the possibility that these isolates induced chlamydospore production by nutrient deprivation was examined. When nutrients were rapidly exhausted from an agar medium by aqueous leaching, 100% chlamydospore formation was induced within 6 days. When four Streptomyces isolates, two of which produced chlamydospore-inducing substances, and two of which did not, were grown on agar containing only glucose and glutamic acid, 90% of both compounds were exhausted within the area of chlamydospore formation after six days. Therefore, nutrient deprivation in co-culture seemed to be the mechanism by which chlamydospore formation was induced for those Streptomyces isolates which do not produce chlamydospore-inducing substances.

INDUCTION OF CHLAMYDOSPORE FORMATION IN FUNGI IN AGAR MEDIA BY STREPTOMYCETES

By

Marina Inn Chiang

A THESIS

Submitted to
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in partial fulfillment of the requirements
for the degree of

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to my parents

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INTRODUCTION

Resting structures of a number of soil-borne fungi have been reported to develop as a result of co-culture with other microorganisms, particularly bacteria. For example, production of sporangia by Phytophthora cinnamomi (2, 30) as well as of chlamydospores of Fusarium solani were induced in co-cultures with soil bacteria in agar (9, 29) and in soil (9), whereas such structures were not produced when the fungi were grown alone. Substances produced by the soil bacteria in these co-cultures were postulated as playing a morphogenetic role (9, 30). It is also possible that nutrient competition may have been involved in the induction of resting structures. Recently (22), it was reported that sudden withdrawal of exogenous nutrients from several form species of F. solani in liquid culture caused rapid and abundant chlamydospore formation. Chlamydospore were also produced when the environment was deficient in energy but contained appropriate mineral salts (14).

Previous work in our laboratory showed that some antagonistic streptomycetes inhibited the development of fungi in agar media by production of antibiotics which could be detected in solid and liquid cultures, whereas others which failed to produce antibiotics caused

inhibition by means of nutrient deprivation (13). Therefore, my research attempted to determine whether chlamydospore production in Fusarium spp. could be induced by co-culture with streptomycetes, and to elucidate the mechanism of such induction.

LITERATURE REVIEW

Chlamydospores provide the principal means of survival of Fusarium spp. during unfavorable periods in soil. They are produced in response to a variety of stimuli, among which are staling product accumulation (25, 26, 27, 29), low C/N ratio of the culture medium (33), low concentration of a carbon source (5), specific chlamydospore-inducing substances (9, 31), starvation (12, 20, 21) or withdrawal of a carbon source (22), weak salt solutions in the absence of an energy source (14), and co-culture with other organisms (9, 30).

Staling substances accumulated in the culture medium not only had a general inhibitive effect on F. oxysporum, but also had a positive morphogenetic effect with respect to chlamydospore formation (25, 27). Following a period of rapid growth, normal mycelial elongation was inhibited and the hyphal apices abstricted conidia. As staling increased, swelling of the apices resulted in chlamydospore formation, and macroconidium germination was inhibited. Later, there was complete cessation of growth followed by mycelial autolysis. At this point, production of staling substances ceased and their concentration began to decrease the medium. It is not known whether the

separate morphological effects were all caused by a single substance or were due to several substances each with its own specificity.

Vankata Ram (30) found that certain bacteria stimulated chlamydospore formation by Fusarium solani on agar media. He suggested, without other evidence, that the phenomenon was due to the production of antibiotics by the bacteria.

Three, and possibly four, substances obtained in aqueous extracts from one Salinas Valley soil induced chlamydospore formation by one or more clones of Fusarium solani f. sp. phaseoli (8). Clones of the fungus responded differentially to each of the chlamydospore-inducing fractions. Extracts made from soil samples collected at different times of the year varied in their capacity to induce three clones to form chlamydospores. Since non-sterilized extracts of soil induced the fungus to produce more chalmydospores than did sterilized extracts, soil bacteria were thought to be involved in the production of chlamydospores. In further work (9), isolates of three genera of soil bacteria (Protaminobacter, Arthrobacter and Bacillus) induced chlamydospore formation in co-culture with the fungus. Substances isolated from culture filtrates of some of the bacteria showed specificity in inducing chlamydospore formation by different clones of the F. solani f. sp. phaseoli under axenic conditions. Therefore, specific bacteria were

thought to be responsible for producing chlamydospore-inducing substances in soil.

Ford, et al. (10) also showed that a limited amount of energy source allowed chlamydospore production, whereas excess nutrients decreased the effect of chlamydospore-inducing substance. They interpreted their work as indicating that the potential for chlamydospore formation was regulated primarily by the concentration of the chlamydospore-inducing substances and to a lesser extent by the amount of energy source.

Other evidence indicates that carbon starvation may be a primary cause of chlamydospore formation. Low levels of carbon in the culture medium favored chlamydospore formation by F. oxysporum f. sp. cubense (33). Carlile reported that a low C/N ratio favored chlamydospore formation in F. oxysporum (5), but it was not determined whether the determining factor was the proportion of carbon to nitrogen or the actual carbon and nitrogen levels. Lockwood (21) proposed that as exogenous nutrients become exhausted, hyphal lysis and resting spore formation occur. For several species of Fusarium, the sudden withdrawal of exogenous carbon source from a liquid culture medium (22), or incubation in the presence of suitable salts without a carbon source (14) favored chlamydospore formation. According to Lockwood microbial activity at the immediate surface of spores or other resting structures will keep the fungal propagales

in a more or less continual state of nutrient deprivation (20).

Deprivation of energy substrate per se does not explain all aspects of chlamydospore formation in F. solani f. sp. phaseoli. For example, it doesn't explain why sterilized extracts of soil support greater chlamydospore formation in F. solani than sterilized water alone (8). In a recent study (24), chlamydospores of F. solani were formed in the same amount when washed nongerminated macroconidia incubated on Nuclepore filters were floated on dilute Na_2SO_4 solutions as on soil. Incubation in water or in phosphate buffer at pH 7.0 did not result in chlamydospore formation. Germlings of five Fusarium form species borne on membrane filters on Na_2SO_4 solution or water also formed chlamydospore rapidly. Chlamydospores also were formed from germlings incubated on acid-washed sterilized sand continually leached with water or phosphate buffer (pH 6.9), but fewer chlamydospores were formed without leaching. Therefore, chlamydospore production may require an environment deficient in energy but containing appropriate mineral salts.

METHODS AND MATERIALS

Preparation of Streptomycetes and Fungi

Thirty unidentified *Streptomyces* isolates were isolated from Conover loam soil from the Michigan State University Farm (17) by means of soil dilution plates, using chitin agar (19) as a selective medium. The isolated selected differed in morphological characteristics and colony color but none were identified to species. The isolates were maintained on yeast extract-maltose-glucose agar slants (per liter: yeast-extract 4g, maltose 10g, dextrose 4g, agar 20g). The fungi used were *Fusarium solani* (Mart.) Appel & Wr. f. sp. *phaseoli* (Burk.) Snyd. & Hans. isolates 20 and 151, *Mucor ramannianus* Möller and *Fusarium solani* f. sp. *pisi* (F. R. Jones) Snyd. & Hans.

Tests for chlamydospore induction were made in petri dishes on peptone-glucose agar (per liter: peptone 5g, glucose 5g, agar 20g) with and without 0.1 M phosphate buffer (pH 7.0). *Streptomyces* isolates were streaked on the medium and incubated at 24C for 7 days. Then spore suspensions of the test fungi were sprayed onto the agar surface with a medicinal atomizer attached to a compressed air line

mounted on a ring stand with the nozzle 25 cm from the surface of the agar. Approximately 2.5×10^5 spored were sprayed onto each plate. After 1, 2 and 3 days the plates were examined for chlamydospore formation. Agar discs adjacent to the Streptomyces colonies were removed aseptically with a 3 mm diameter cork borer, and stained with phenolic rose bengal and observed with a microscope. Chlamydospore production was expressed as the percentage of macroconidia or germlings forming one or more chlamydospores.

Detection of Chlamydospore-Inducing Substances

To detect chlamydospore-inducing substances found in agar media, agar discs 5 mm in diameter were cut with a sterile cork borer from glucose-peptone agar adjacent to Streptomyces colonies and transferred to fresh peptone agar (per liter: peptone 5g, agar 20g) plates. After 24 hours conidial suspensions of the four test fungi were sprayed onto the agar surface. One, 2, 3, 4, 5 and 6 days later the induction of chlamydospore formation was observed in the manner previously described.

For the production of chlamydospore-inducing substances in liquid media, streptomycetes were cultured in duplicate, shaken 125 ml Erlenmyer flasks containing 25 ml glucose-peptone broth (per liter: peptone 5g, glucose 5g) for 7 days at 24C. Filter paper antibiotic assay discs

(13 mm diameter) were impregnated with sterile filtrates by applying 0.1 ml at a time, then alternately air-drying the discs. The discs were then placed on the surface of peptone agar plates and allowed to stand for 12 hours, when conidial suspensions of the four test fungi were sprayed onto the plates. The induction of chlamydospores was observed microscopically 1, 2, 3, 4, 5 and 6 days after application of the fungal spores. Uninoculated liquid medium treated in the same way as the culture served as the control.

Liquid cultures of three selected Streptomyces isolates were passed through Millipore filters (0.22μ) and the filtrates were extracted with an equal volume of amyl alcohol. The amyl alcohol extracts were first dried, then redissolved in 5 ml sterilized distilled water. The extracts were placed into small glass petri-dishes, and Nuclepore-membrane filters (10 mm diam., 0.5 μ pore size) bearing washed macroconidia of F. solani f. sp. phaseoli isolate 151 were floated on the distilled water or placed on Conver loam soil. After incubation for 0.25, 0.5, 1, 3, 4, 5, 6 and 7 days, one filter was removed from each of the three substrates. To observe chlamydospore formation, the fungi were stained with phenolic rose bengal, and the membranes made transparent by mounting them in clove oil on glass slides (14).

Utilization of Nutrients in Agar by Streptomycetes

An agar medium was prepared in 0.1 M phosphate buffer (pH 7.0) and contained, per liter: glucose 2g, glutamic acid 2g, agar 20g or 30g. Two layers of agar were prepared: a bottom layer (5 ml of 30g agar/liter) was poured in the petri-dishes. After hardening, the top layer (10 ml of 20g/liter) was poured onto the bottom layer. Streptomyces isolates 10, 11, 15 and 29 were streaked on the agar surface. This medium supported good growth of F. solani f. sp. phaseoli isolate 151 and the streptomycetes. On the 1st, 2nd, 4th, 6th and 8th day, 6 agar discs 3 mm in diameter were removed from the top layer of agar adjacent to the Streptomyces colonies and melted in 3 ml distilled water. Glucose in the agar was measured colorimetrically at 420 mu. Glucose at concentrations of 5, 10, 15, 20 ug/ml was used as a standard. Glutamic acid was assayed colorimetrically at 570 mu using the ninhydrin reagent (23). Glutamic acid at concentrations of 2, 3 and 4 ug/ml was used as a standard.

Induction of Chlamydospore Formation on Leached Sand

Discs 3 mm in diameter were cut from glucose-glutamic acid agar (per liter: glucose 2g, glutamic acid 2g, agar 20g) and inoculated with macroconidia of F. solani f. sp. phaseoli isolate 151. The seeded discs were immediately transferred

to the surface of a sand bed in a leaching apparatus (15). This was composed of a separatory funnel equipped with a sealed-in dripping tip to maintain a constant flow rate. The funnel stem was connected by plastic tubing to a needle valve, then to a glass petri-dish fitted with an inlet in the lid and an outlet at the bottom on the opposite side. The dish contained a 5 mm layer of acid-washed silica sand. The separatory funnel was filled with distilled water. The entire apparatus was sterilized by autoclaving before use. Control discs were incubated on glucose-peptone agar. After leaching 1, 2, 3, 4, 5 and 6 days, two discs were removed from the sand bed, stained with phenolic rose bengal, and observed microscopically. Chlamydospore production was determined in 100 macroconidia on each disc.

RESULTS

The Characteristics of Chlamydospore Induction by Streptomycetes

All of the thirty streptomycete isolates induced chlamydospore formation when co-cultured with F. solani f. sp. phaseoli isolates 20 and 151, F. solani f. sp. pisi and M. ramannianus (Tables 1, 2). Twenty-three of the isolates also inhibited macroconidial germination resulting in clear zones surrounding the streptomycete colonies. Chlamydospores were formed within 3-5 days from mycelia around the edges of the inhibition zones as well as from ungerminated macroconidia near the periphery of the inhibition zones. The other seven streptomycete isolates did not inhibit macroconidial germination but did inhibit germling growth, producing a partially clear zone. Chlamydospores were formed throughout the area of growth inhibition as well as at the edge of the zone. Apparently mature chlamydospores were observed as early as one day after macroconidia were applied to the agar.

Since the pH of the non-buffered agar medium around the Streptomyces colonies increased from 6.9 to 7.6-8.3, Chlamydospore induction of F. solani f. sp. phaseoli

Table 1. Chlamydospore-inducing and inhibitory characteristics of streptomycetes toward *Fusarium solani* f. sp. *phaseoli* isolate 151 and *Mucor ramannianus* in nonbuffered agar and liquid media.

Group	Isolate	*Streptomyces*+*Fusarium* or *Mucor* co-culture		*Streptomyces* culture
		Germination inhibition	Chlamydospore induction	Chlamydospore-inducing factors in agar and liquid cultures
Control		-[a]	-	-
I	1	+	+	-
	2	+	+	-
	3	+	+	-
	4	+	+	-
	5	+	+	-
	6	+	+	-
	7	+	+	-
	8	+	+	-
	9	+	+	-
	10	+	+	-
	11	+	+	-
	12	+	+	-
	13	+	+	-
	14	+	+	-

	15	+	+	+
	16	+	+	+
	17	+	+	+
	18	+	+	+
II	19	+	+	+
	20	+	+	+
	21	+	+	+
	22	+	+	+
	23	+	+	+
	24	+[b]	+	+
	25	+[b]	+	+
	26	+[b]	+	+
III	27	+[b]	+	+
	28	+[b]	+	+
	29	+[b]	+	+
	30	+[b]	+	+

[a] + = Germination inhibition or chlamydospore induction; - = no inhibition or chlamydospore induction.

[b] Inhibition of germ tube growth.

Table 2. Chlamydospore-inducing and inhibitory characteristics of selected streptomycetes towards Fusarium solani f. sp. phaseoli isolate 20 and F. solani f. sp. pisi.

Group	Isolate	Streptomyces+fungus co-culture		Streptomyces culture
		Germination inhibition	Chlamydospore induction	Chlamydospore-inducing factors in agar and liquid cultures
Control		-[a]	-	-
I	9	+	+	-
	10	+	+	-
II	15	+	+	+
III	29	+[b]	+	+

[a] + = Germination inhibition or chlamydospore induction; - = no inhibition or chlamydospore induction.

[b] Inhibition of germ tube growth.

isolate 151 was retested using buffered agar (pH 7.0) to determine if chlamydospores were pH-induced. Chlamydospores were formed on the buffered plates at the same rate as on non-buffered plates (Table 3); on the buffered agar, the pH remained at 7.0. The size of inhibition zones produced by the same isolates was similar in both buffered and non-buffered agar with the exception of isolates 10, 11 and 23 which produced smaller zones in buffered agar. The aberrant result with these three isolates appeared to be due to poor growth of the streptomycetes in the presence of phosphate rather than to any effect of pH.

In another experiment F. solani f. sp. phaseoli isolates 20 and 151, F. solani f. sp. pisi and M. ramannianus failed to form chlamydospores in agar buffered at pH 7.0, 7.3, 7.7 and 8.3 with 0.1 M phosphate buffer, even though growth at all pHs was substantial. Therefore, pH of the agar seemed to have no effect on chlamydospore formation.

Production of Chlamydospore-Inducing Substances in Solid Culture

To determine whether the Streptomyces isolates produced substances which induced formation of chlamydospores in the agar medium, discs cut from the area adjacent to the Streptomyces isolates were transferred to fresh peptone agar plates seeded with conidia of F. solani f. sp. phaseoli isolate 151 or Mucor ramannianus. Agar discs from the

Table 3. Chlamydospore-inducing characteristics of streptomycetes toward *Fusarium solani* f. sp. *phaseoli* in buffered agar (pH 7.0).

Group	Isolate	*Streptomyces*+*Fusarium* co-culture (buffered agar)		pH of *Streptomyces*+ co-culture agar (unbuffered)
		Germination inhibition	Chlamydospore induction	
Control		-[a]	-	6.9
I	1	+	+	7.8
	2	+	+	7.7
	3	+	+	7.6
	4	+	+	7.8
	5	+	+	7.8
	6	+	+	7.7
	7	+	+	7.9
	8	+	+	7.9
	9	+	+	8.0
	10	+[b]	+	7.8
	11	+[b]	+	7.8
	12	+	+	7.8
	13	+	+	7.8
	14	+	+	7.8

		+	+	7.8
II	15	+	+	7.8
	16	+	+	8.3
	17	+	+	8.0
	18	+	+	8.2
	19	+	+	8.0
	20	+	+	7.8
	21	+	+	7.8
	22	+	+	7.8
	23	+[b]	+	7.8
	24	+[c]	+	8.3
	25	+[c]	+	8.0
	26	+[c]	+	8.0
	27	+[c]	+	8.2
	28	+[c]	+	8.0
	29	+[c]	+	7.9
	30	+[c]	+	7.8

[a] + = Germination inhibition or chlamydospore induction; - = no inhibition or chlamydospore induction.

[b] Smaller inhibition zone than that on non-buffered medium.

[c] Inhibition of germ tube growth.

medium adjacent to isolates 15, 16, 17, 18, 19, 20, 21, 22 and 23 caused new inhibition zones surrounding them, and chlamydospores were formed in the mycelium and germ tubes at the edges of such zones (Table 1). Discs from near isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 neither caused chlamydospore formation nor inhibited germination or growth. Agar removed from the vicinity of a third group of isolates, 24, 25, 26, 27, 28, 29 and 30, inhibited germ tube growth which was followed by chlamydospore formation both from germ tubes and conidia.

Production of Chlamydospore-Inducing Substances in Liquid Media

To further test whether chlamydospore-inducing substances were produced by the *Streptomyces* isolates, all 30 isolates were grown in a liquid medium. Sterile culture filtrates of isolates 1-14, which failed to produce chlamydospore-inducing substances on agar media, also failed to induce chlamydospore formation in *F. solani* f. sp. *phaseoli* isolate 151 and *M. ramannianus* (Table 1). The selected isolates 10 and 11 also had no effect on *F. solani* f. sp. *phaseoli* isolate 20 and *F. solani* f. sp. *pisi*. The other 16 isolates, which produced chlamydospore-inducing substances on agar media, also induced chlamydospore formation of *F. solani* f. sp. *phaseoli* isolate 151 and *M. rammanianus*. Filtrates from 9 of these isolates (15-23) also inhibited

macroconidial germination surrounding the paper discs, whereas filtrates of 7 other isolates (24-30) had no effect on germination but inhibited germling growth (Table 2). Culture filtrates from isolates 15 and 29 also caused the same effects on Fusarium solani f. sp. phaseoli isolate 20 and F. solani f. sp. pisi (Table 2). Inhibition zones for M. ramannianus usually were larger than those of other fungi.

Culture filtrates of three selected Streptomyces isolates were extracted with amyl alcohol. One (isolate 15) produced chlamydospore-inducing substances and completely inhibited macroconidial germination, another (isolate 29) produced chlamydospore-inducing substances but inhibited only germ tube growth, and the third (isolate 11) did not produce chlamydospore-inducing substances. The extracts were tested for induction of chlamydospore formation by F. solani f. sp. phaseoli isolate 151 borne on Nuclepore filters. The extract from isolate 15 induced chlamydospore formation within 12 hours, and 100% of the macroconidia had formed chlamydospores within 48 hours (Figure 1). Chlamydospore formation in the extract of isolate 29 was first observed in 6 hours, and 100% of the macroconidia had formed them within 24 hours. Chlamydospore formation in the extract from isolate 11 was first observed in 48 hours, and at the 7th day there was 70% chlamydospore formation. This slower rate of chlamydospore formation was similar to that in distilled water or on soil.

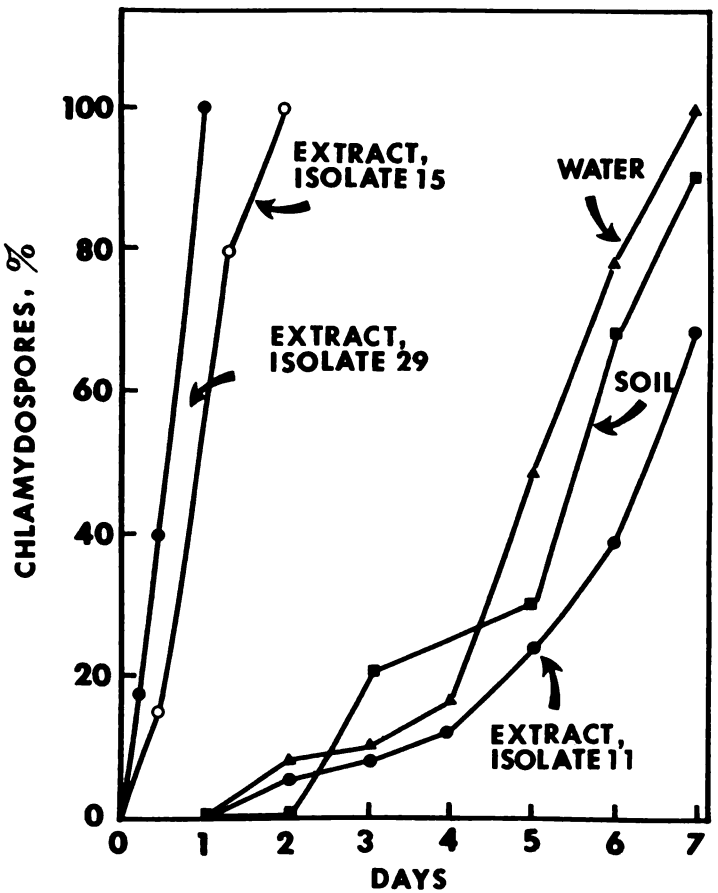

Figure 1. Rate of chlamydospore formation in macroconidia of Fusarium solani f. sp. phaseoli isolate 151 incubated on Nuclespore membranes floated (a) on amyl alcohol extracts of cultures of three Streptomyces isolates, (b) on distilled water, or (c) incubated on natural soil.

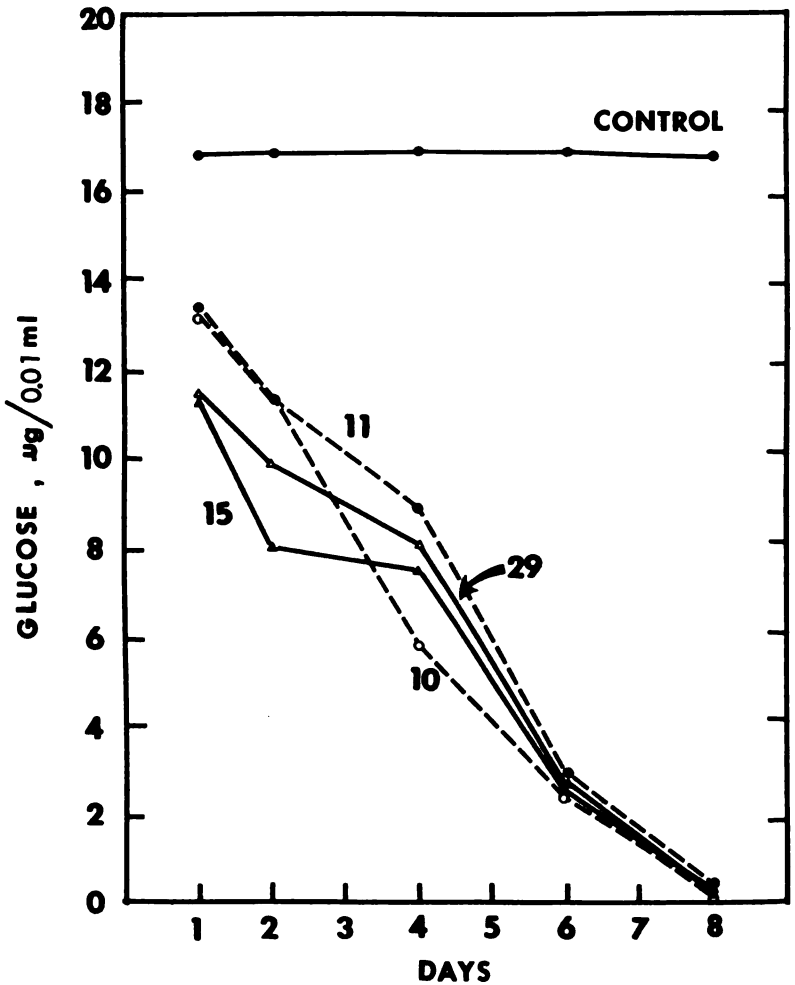

Figure 2. Loss of glucose from 3 mm diameter agar discs immediately adjacent to Streptomyces colonies growing on agar containing 0.2% glucose and 0.2% glutamic acid. Isolates 15 and 29 produced chlamydospore-inducing substances; isolates 10 and 11 did not. In the control, no streptomycetes were streaked on the agar.

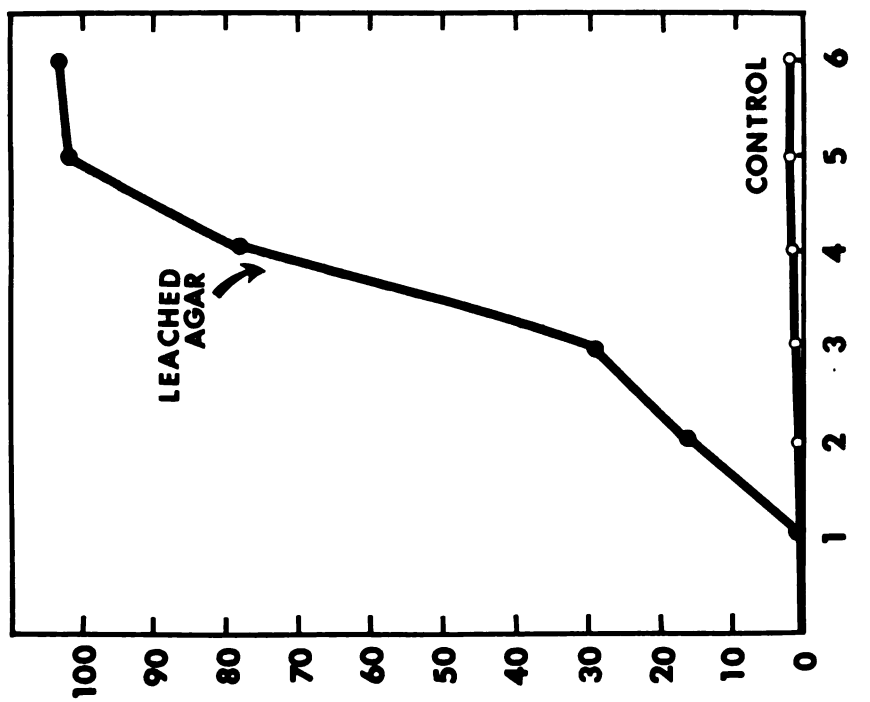

Figure 4. Chlamydospore formation by Fusarium solani f. sp. phaseoli isolate 151, when agar discs containing 0.2% glucose and 0.2% glutamic acid and seeded with macroconidia were placed in sand leached aseptically with distilled water, at the rate 1-1.5 ml/min. Control was macroconidia seeded on agar discs incubated on peptone-glucose agar.

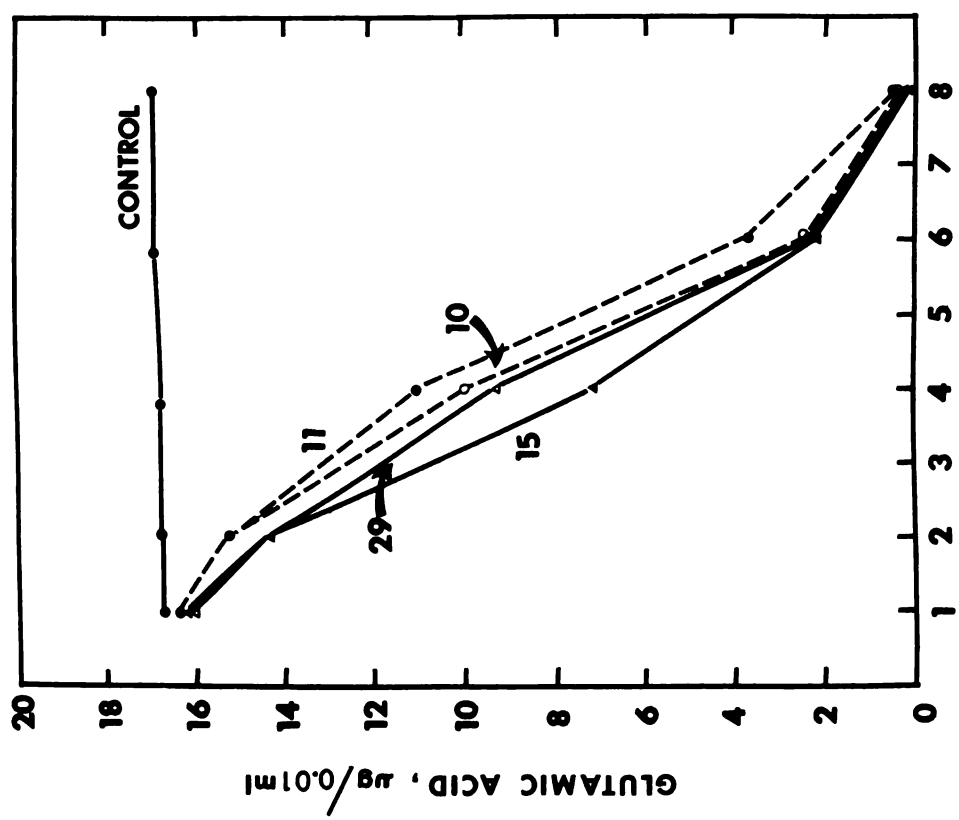

Figure 3. Loss of glutamic acid from 3 mm diameter agar discs immediately adjacent to Streptomyces colonies growing on agar containing 0.2% glutamic acid and 0.2% glucose. Isolates 15 and 29 produced chlamydospore-inducing substances; isolates 10 and 11 did not. In the control, no streptomycetes were streaked on the agar.

To determine whether the extracts would retain activity in the presence of nutrients, 1 ml glucose-peptone broth was added to 5 ml undiluted amyl alcohol extract of isolate 15. In such amended extracts, germination of F. solani f. sp. phaseoli isolate 151 was inhibited and chlamydospores were formed. However, when nutrient-amended extracts from isolate 15 were diluted with water, the macroconidia occasionally germinated with chlamydospore formation following at the tip of the germ tube and within the macroconidia. Following chlamydospore formation, the mycelia continued growing. The extract of isolate 29, in the presence of nutrients, inhibited germling growth and induced chlamydospore formation at the same rate as without nutrients.

Nutrient Status of Agar Adjacent to Streptomyces Colonies

Several lines of evidence indicated that about half of the streptomycetes induced the formation of chlamydospores by some means other than production of morphogenetic substances, possibly by nutrient deprivation. Therefore, the nutrient status of agar near the Streptomyces colonies was assayed. Four selected Streptomyces isolates, two of which did not produce chlamydospore-inducing substances (isolates 10 and 11), and two of which produced chlamydospore-inducing substances (isolates 15 and 19). Isolate 15 completely inhibited macroconidial germination, whereas isolate 29 inhibited

only germ tube growth. When each was streaked on glucose-glutamic acid agar, both substances were rapidly lost from the agar within 3 mm of the Streptomyces colonies; 30-50% was lost by the 4th day, and 80-90% was lost by the 6th day (Figures 2 and 3).

Induction of Chlamydospore Formation under Leaching Conditions

To determine directly whether exhaustion of nutrients can induce chlamydospore formation on agar, discs of glucose-glutamic acid agar bearing macroconidia of F. solani f. sp. phaseoli isolate 151 were incubated on the sand bed which was leached with distilled water. Most macroconidia germinated on the agar discs during the first day, but germlings failed to grow further. Chlamydospores began to form either from the germlings or the germinating macroconidia on the 2nd day (Figure 4). By the 4th day, about 80% of the germlings had formed chlamydospores. By day 6, there was 100% chlamydospore formation. Loss of glucose and glutamic acid in the agar discs was nearly complete within 24 hours. These results further indicated that the induction of chlamydospore formation in agar by some streptomycetes such as isolates 10 and 11 might be due to the rapid loss of nutrients from the agar.

DISCUSSION

Previous to this research, only bacteria have been implicated in the induction of chlamydospore formation by Fusarium spp. in vitro (9, 30). However, the present results show that streptomycetes also can induce chlamydospore formation in co-culture with Fusarium and Mucor. Some but not all of the streptomycetes produced chlamydospore-inducing substances. Since the increase in pH of the medium due to the growth of streptomycetes was not responsible for chlamydospore formation, the chlamydospore-inducing isolates could be classified into three groups:

Group I (isolates 1-14) which induced chlamydospore formation and produced inhibition zones by means of competition for nutrients in co-culture. These isolates failed to produce detectable chlamydospore-inducing or inhibitory substances in solid as well as liquid cultures. The rate of chlamydospore formation in an amyl alcohol extract of a culture of isolate 11 (representing Group I) was similar to that in distilled water indicating that the extract contained no chlamydospore-inducing substances.

Group II (isolates 15-23) which produced chlamydospore-inducing substances in solid as well as liquid cultures. The amyl alcohol extract of isolate 15 stimulated

rapid chlamydospore formation from the macroconidia.

Group III (isolates 24-30) which also produced chlamydospore-inducing substances in solid and liquid cultures. However, germ tube growth, rather than macroconidial germination, was inhibited by these Streptomyces colonies and by substances produced in liquid cultures. The rate of chlamydospore formation in an amyl alcohol extract of a culture of isolate 29 was more rapid than for Group II isolates.

There are two possible ways in which microorganisms may induce chlamydospore formation in the soil environment. One is through competition for nutrients, and the other is by the production of chlamydospore-inducing substances. The similarity in the rate of chlamydospore formation by Fusarium in soil to that in distilled water or the presence of isolates which did not produce chalmydospore-inducing substances (Group I) suggests that chlamydospore formation in soil is induced by nutrient deprivation. Spore germination could be prevented by slow, steady leaching with water (17), somewhat comparable to the slow, steady exhaustion of nutrients by other soil microorganisms.

Isolates 15 and 29, which produced chlamydospore-inducing substances, removed nutrients as rapidly from the agar as did isolates 10 and 11 which did not produce chlamydospore-inducing substances. Therefore, chlamydospore production in co-culture with these isolates may have

occurred through the combined inductive effects of chlamydospore-inducing substances and nutrient deprivation. Hsu and Lockwood (13) also reported that streptomycete isolates which produced antibiotics removed nutrients as rapidly from the agar as did isolates which did not produce antibiotics.

The Streptomyces isolates, their culture filtrates, and amyl alcohol extracts all induced chlamydospore production nonspecifically by the four test fungi used. Moreover, the Streptomyces isolates could be classified into the same three groups based on their effects on these fungi. In an earlier study the staling substances produced by F. oxysporum which induced chlamydospore formation were also nonspecific in activity (27). These results contrast with those of Ford, et al. (8), who found clonal specificity in extracts from different soils and in different fractions of the same soil extract. The "morphogenetic" substances found in soil extracts by Ford, et al. may be similar to the weak salt solutions used by Hsu and Lockwood (14) to induce chlamydospore formation, and which showed some specificity.

The results of the co-culture experiments suggest that growth inhibition is a prerequisite for chlamydospore formation, inasmuch as all Streptomyces isolates caused growth inhibition, and this occurred before chlamydospore formation. This sequence may be obligatory for Streptomyces isolates competing for culture nutrients. However,

such a sequence may not be obligatory to the fungus. When incubated on diluted extracts of isolates 15 in the presence of nutrients, growth continued from hyphal tips following formation of chlamydospores.

The constant association of an inhibitory capability with that for chlamydospore induction in the streptomycetes also suggests that a single substance may be involved both in inhibition and chlamydospore formation. But here also, continued growth coincident with chlamydospore formation such as occurred in the extract of a Group II culture suggests that different compounds are involved. It is also possible that the growth inhibitory aspect of a single material is reversed more readily by nutrients than the chlamydospore-inducing property.

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