# Induction of Chlamydospore Formation in Fusarium solani by Soil Bacteria

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ABSTRACT

Sterile soil extracts induced three clones of *Fusarium solani* f. sp. *phaseoli* to produce fewer chlamydospores than did nonsterile extracts or extracts preincubated prior to sterilization. Isolates of three genera of soil bacteria (*Protaminobacter, Arthrobacter,* and *Bacillus*) induced chlamydospore formation by one or more clones of the fungus in mixed cultures. Substances isolated from culture filtrates of *Arthrobacter* and *Bacillus* each induced chlamydospore formation by a different clone of the fungus under axenic conditions.

Twenty-five of 50 bacterial isolates induced one clone of *F. solani* f. sp. *phaseoli* to form chlamydospores; 16 of another 50 bacterial isolates induced a second clone to form chlamydospores; and 10 of another 50 bacterial isolates induced a third clone to form chlamydospores. The hypothesis is advanced that specific bacteria are responsible for the chlamydospore-inducing substances in soil, and that changes in the numbers and types of bacteria in soil may affect the ability of specific clones of *Fusarium* to produce chlamydospores in that soil. Phytopathology 60:479-484.

Previous studies (1, 2, 4, 7) have established the presence of three and possibly more substances in soil that induce chlamydospore formation by one or more clones of *F. solani* (Mart.) Appel. & Wr. f. sp. *phaseoli* (Burk.) emend. Snyd. & Hans. These substances, as assayed by chlamydospore formation in soil extracts, apparently fluctuate in extractable concentration in the soil throughout the year (4), and may be of microbial origin (5, 16).

In addition to soil, several other sources of chlamydospore-inducing substances for *Fusarium* have been described. Venkata Ram (16) reported that a saprophytic clone of *F. solani* produced more chlamydospores when cultured in the presence of several soil bacteria or in sterile bacterial culture filtrates. Park and coworkers (10, 11, 12, 13, 14) found that "staling products" in cultures of *F. oxysporum* (Schlecht.) emend. Snyd. & Hans. and *F. roseum* (Lk.) emend. Snyd. & Hans., when added to fresh cultures of the fungi, induced chlamydospore formation. The saprophytic clones of *F. solani* and most clones of *F. roseum* and *F. oxysporum* produce appreciable numbers of chlamydospores in old potato-dextrose agar (PDA) cultures and/or in sterile, distilled water (W. C. Snyder, *personal communication*). This is in contrast to some clones of the plant pathogenic forms of *F. solani,* notably *F. solani* f. sp. *phaseoli,* which form chlamydospores only sparsely under similar conditions.

This study considers the role of bacterial products in the induction of chlamydospore formation by *F. solani* f. sp. *phaseoli.* The diversity of three bacteria involved in the induction of chlamydospore formation, and the number of bacteria potentially contributing to the pool of chlamydospore-inducing substances in soil, are also considered.

MATERIALS AND METHODS.—*Fungal and bacterial cultures.*—Three clones of *F. solani* f. sp. *phaseoli* ($S_{2a}$, $S_{2b}$, and $S_{2c}$) were used in the study. Cultures were maintained at 22 ± 2 C on PDA and exposed to room light. Single spore transfers were made at 3-week intervals.

Three isolates of soil bacteria (B, C, F) were maintained at 22 ± 2 C on yeast, dextrose, peptone agar (YDCP), or PDA, and were transferred at weekly intervals.

*Soil and extraction procedures.*—The soil was a sandy loam described previously (2, 4). It was collected from one field at irregular intervals throughout the course of the study, and stored in the laboratory in galvanized garbage cans. While in storage, the soil was maintained at approximately field capacity by periodically adding tap water.

Moist soil was extracted with one part of distilled water (w/v). The water and soil were thoroughly mixed in a large beaker. After a short period of standing, the solid particles were removed by filtration through two layers of Whatman No. 50 filter paper.

*Procedures for bioassaying chlamydospore formation.*—Standard serum vial techniques for bioassaying chlamydospore formation (4) were used in all but one experiment in which chlamydospore formation was bioassayed using small petri plates (100 mm diam.). Five ml of the test solution were dispensed aseptically in each petri plate, and 0.10 ml of an aqueous spore suspension of the test fungus was added to give approximately 2,000 macroconidia/ml. After 7 days' incubation at room temperature, the number of chlamydospores formed per ml of test solution was determined by counting the number of chlamydospores in the same number of microscope fields (×100 magnification) containing 100 germinated macroconidia. The number of chlamydospores thus obtained was multiplied by 20 to give the results in chlamydospores produced per ml of test solution based on the initial inoculum of 2,000 macroconidia/ml.

The standard control for all experiments was a sterile basal medium (BM) (4) consisting of a mineral salts solution (pH 6.6). BM was chosen as a control solution on the basis of earlier work (2) showing that chlamydospore formation is inhibited by the lack of essential minerals. Chlamydospores were rarely formed by the three clones in distilled water in 7 days; however, in BM a few chlamydospores were produced by the fungus in many experiments.

*Procedures for assaying bacterial induction of chlamydospore formation.*—Bacteria were aseptically removed from the surface of a 2- to 3-day-old culture with a wire loop and suspended in sterile distilled water to a turbidity of 25 on a Klett-Sumerson colorimeter, using a green filter; 0.10 ml of the bacterial suspension was added to 5 ml of the sterile basal medium containing 2,000 macroconidia of the test fungus/ml in each petri plate. The co-cultures were incubated at 22 ± 2 C for 7 days, then examined for chlamydospore formation.

*Fractionation of culture filtrates.*—Various liquid media in which specific bacteria had been grown were fractionated by ion exchange chromatography into cation, anion, and neutral fractions. The cation fraction was removed from the filtrate by exchange with Amberlite IR 120 ($H^+$ form); the anion fraction was removed from the filtrate by exchange with Amberlite IR 45 ($OH^-$ form); the neutral fraction consisted of the effluent culture filtrate plus the wash water. The cation fraction was removed from the IR 120 by elution with 2 N HCl, and the anion fraction was removed from the IR 45 by elution with 2 N $NH_4OH$.

All three fractions were evaporated in a rotary evaporator under vacuum at 50 C. The residue from each fraction was then redissolved in a volume of distilled water equivalent to one-twentieth the volume of the original culture filtrate. Each fraction was diluted with distilled water and the basal medium stock solution to give the desired concentration of the fraction in BM. The controls consisted of the sterile culture medium fractionated and tested in the same manner as the bacterial culture filtrates.

Each experiment consisted of three replications/treatment, and was repeated two or three times. All solutions were sterilized by autoclaving at 15 lb. pressure for 15-20 min.

RESULTS.—*Association of microbial activity with the capacity of soil extracts to induce chlamydospore formation.*—Chlamydospore formation by the three clones of *F. solani* f. sp. *phaseoli* was evaluated in sterile and nonsterile soil extracts and compared with that in sterile BM (Fig. 1). The number of chlamydospores produced by each clone in sterile soil extracts was significantly greater than the number produced in BM, with the exception of clone $S_{2a}$ in Experiment 1. Chlamydospore formation, in all cases, was increased in the nonsterile soil extracts over that in the corresponding sterile soil extracts and BM controls. Experiment 1 differs from Experiment 2 only in the time at which the soil used to prepare the soil extracts was collected. There was variation in the total number of chlamydospores produced by each clone in the sterile and nonsterile soil extracts, and in the per cent increase in number of chlamydospores formed by each clone when soil extract was not sterilized. Similar differences occurred each time a separate collection of soil was used to perform the experiment.

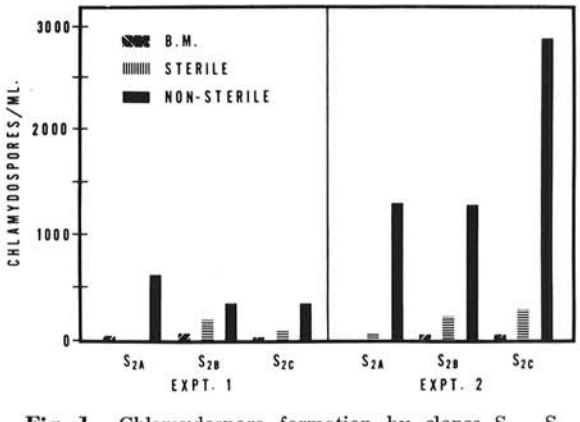


**Fig. 1.** Chlamydospore formation by clones $S_{2a}$, $S_{2b}$, and $S_{2c}$ of *Fusarium solani* f. sp. *phaseoli* in BM, sterile soil extract, and nonsterile soil extract.

Microscopic examination of the nonsterile soil extracts revealed a large increase in the number of bacteria by the end of the experiment. Thus, the increase in chlamydospore formation in nonsterile soil extracts over that in sterile soil extracts appeared to be associated with microbial activity in the extracts.

In the nonsterile soil extracts, chlamydospores were produced in the old conidial cells, intercalarily in the hyphae, and terminally in the hyphal tips (Fig. 2-A, B, C). There were proportionately fewer chlamydospores formed in the hyphal tips in the sterile soil extracts than in the nonsterile soil extracts.

Chlamydospore production was also evaluated in a series of soil extracts which were preincubated for increasing periods of time prior to sterilization and bioassay (Fig. 3). Four 100-ml portions of soil extract were dispensed into 250-ml Erlenmeyer flasks and incubated at 22 ± 2 C for 0, 1, 2, and 4 days. At each time period, the contents of one flask was filtered through a 0.22 μ Millipore filter, sterilized by autoclaving, and stored at 4 C until tested for capacity to induce chlamydospore formation.

The number of chlamydospores produced by clones $S_{2b}$ and $S_{2c}$ in sterilized soil extracts increased with the preincubation period. The logarithm of the number of chlamydospores produced approached a linear relationship with the length of the preincubation period. Apparently, the beneficial effect of microbial activity in soil extracts on chlamydospore formation was associated with a change in the chemical composition of the extracts.

*Bacterial induction of chlamydospore formation.*—Soil dilution plates ($10^{-5}$) of the Salinas Valley soil were prepared on YDCP agar. After 4-days' incubation at 22 ± 2 C, three morphologically distinct colonies of bacteria (B, C, F) were selected for study on the possible bacterial induction of chlamydospore formation.

The demonstration that certain soil-borne bacteria are capable of inducing chlamydospore formation by *F. solani* f. sp. *phaseoli* was made using mixed cultures of the fungal clones and the bacterial isolates in BM (Table 1). Bacterial isolate B induced all three fungal clones to form chlamydospores. Bacterial isolates C and F induced only clones $S_{2b}$ and $S_{2c}$ to form chlamydospores. Clone $S_{2c}$ formed more than two times the number of chlamydospores formed by clone $S_{2b}$ when

TABLE 1. Induction of chlamydospore formation in *Fusarium solani* f. sp. *phaseoli* in mixed culture with soil bacteria

| Bacterial isolate | Clone of *F. solani* f. sp. *phaseoli* | | |
|---|---|---|---|
| | $S_{2a}$ | $S_{2b}$ | $S_{2c}$ |
| | *chlamydospores/ml* | | |
| B | 620 ± 192[a] | 1,227 ± 94 | 2,227 ± 125 |
| C | 0 | 2,467 ± 24 | 5,807 ± 722 |
| F | 0 | 2,360 ± 20 | 1,240 ± 139 |
| Control | 0 | 707 ± 22 | 0 |

[a] Each value is the mean of three replications ± the standard error of the mean.

co-cultured with bacterial isolate C. In contrast, clone $S_{2b}$ formed more than 2 times the number of chlamydospores formed by clone $S_{2c}$ when co-cultured with bacterial isolate F.

The initial concentration of bacterial cells per ml of test solution were: isolate B, $1.6 \times 10^5$ cells/ml; isolate C, $1.5 \times 10^4$ cells/ml; and isolate F, $2.2 \times 10^4$ cells/ml. Because of the differences in initial concentration of the bacteria and possible differences in growth rates among the bacteria, care should be taken in interpreting differences in the numbers of chlamydospores induced in each clone by each of the bacteria.

*Chlamydospore formation in fractionated culture filtrates of soil bacteria.*—Sterile culture filtrates of each of the bacteria were tested directly and after separation into cation, anion, and neutral fractions for the capacity to induce chlamydospore formation by the three fungal clones.

The three clones did not produce chlamydospores in sterilized culture media or in the ionic fractions of media in which bacterial isolate B had been grown. The bacterium grew poorly in the three culture media tested (potato-dextrose broth, 0.2% peptone water, and nutrient broth); and possibly did not produce chlamydospore-inducing substances in amounts that were detectable by the bioassay.

One chlamydospore-inducing fraction was detected from 0.1% peptone water in which bacterial isolate C had grown for 6 days (Table 2). Chlamydospores were not produced by the three clones in the sterile, crude filtrate, or in the cation and neutral fractions of the filtrate. The anion fraction induced only clone $S_{2c}$ to form chlamydospores. Chlamydospores were not produced in the controls.

The addition of 0.5% NaCl to the 0.1% peptone water medium, as recommended by Skerman (15), increased the number of chlamydospores produced by clone $S_{2c}$ in the subsequent anion fraction (Table 2). At a concentration of 2.5 times the original culture medium volume, chlamydospore formation was greatly increased over that in other concentrations. Further concentration of the fraction led to decreased chlamydospore production. Vegetative growth increased as the concentration of the fraction was increased. Chlamydospores were not produced in the culture medium controls, but a few were produced in the BM.

One chlamydospore-inducing fraction was detected from quarter strength potato-dextrose broth in which bacterial isolate F had grown for 7 days (Table 3). Chlamydospores were not produced in the sterile, crude culture filtrate or in the cation and neutral fractions of the filtrate. The anion fraction induced chlamydospore formation in clone $S_{2b}$, and to a slight extent in clone $S_{2c}$. Chlamydospores were not formed in the culture medium controls, although a few chlamydospores were produced by clones $S_{2b}$ and $S_{2c}$ in the BM.

TABLE 2. Chlamydospore formation by *Fusarium solani* f. sp. *phaseoli* in the anion fraction of two culture media in which bacterial isolate C was cultured

| Culture medium | Relative concn. of anion fraction | Clone of *F. solani* f. sp. *phaseoli* | | |
|---|---|---|---|---|
| | | $S_{2a}$ | $S_{2b}$ | $S_{2c}$ |
| | | *chlamydospores/ml* | | |
| 0.1% Peptone water | 1.00 × | 0[a] | 0 | 96 ± 8 |
| | 0.50 × | 0 | 0 | 209 ± 6 |
| | 0.25 × | 0 | 0 | 70 ± 13 |
| Basal medium control | | 0 | 0 | 34 ± 8 |
| 0.1% Peptone water plus 0.5% NaCl | 5.0 × | | 0 | 0 |
| | 2.5 × | | 0 | 1,220 ± 29 |
| | 1.0 × | | 0 | 427 ± 58 |
| Basal medium control | | | 0 | 43 ± 10 |

[a] Each value gives the mean of three replications ± the standard error of the mean.

*Tentative identification of bacteria.*—The genera of the three soil bacteria were tentatively identified according to the keys in *Bergey's Manual of Determinative Bacteriology* (3) and Skerman (15). Isolate B most closely corresponded to the genus *Protaminobacter*. The utilization of the alkylamine, ethylamine, as a sole carbon source is definitive for this genus. Bacterial isolate C most closely corresponded to the genus *Arthrobacter*. Isolate F most closely corresponded to the genus *Bacillus*.

*Survey of the bacteria in the Salinas Valley soil capable of inducing chlamydospore formation.*—Soil dilution plates ($10^{-6}$) were prepared on PDA and incubated for 7 days at 22 ± 2 C. Fifty colonies of bacteria selected at random were tested for the ability to induce chlamydospore formation. A colony of each bacterium was placed into a 20-ml serum vial containing

TABLE 3. Chlamydospore formation by *Fusarium solani* f. sp. *phaseoli* in the anion fraction of quarter strength potato-dextrose broth in which bacterial isolate was cultured

| Treatment | Clone of *F. solani* f. sp. *phaseoli* | | |
|---|---|---|---|
| | $S_{2a}$ | $S_{2b}$ | $S_{2c}$ |
| | | *chlamydospores/ml* | |
| Basal medium control | 0[a] | 43 ± 4 | 31 ± 5 |
| Anion fraction | 0 | 769 ± 4 | 57 ± 4 |

[a] Each value gives the mean of three replications ± the standard error of the mean.

5 ml BM and $10^4$ macroconidia of the test clone. After 7 days' incubation at $22 \pm 2$ C, the cultures were evaluated for the number of chlamydospores formed and compared with the number formed in BM. The entire procedure was repeated three times, once for each fungal clone.

Clone $S_{2a}$ was induced to form chlamydospores by 10 of 50 bacterial isolates, clone $S_{2b}$ by 25 of 50 bacterial isolates, and clone $S_{2c}$ by 16 of 50 bacterial isolates. No chlamydospores were formed in the BM control solutions. Apparently, soil bacteria differ in the ability to induce chlamydospore formation in each of the three fungal clones.

DISCUSSION.—This study indicates that the origin of chlamydospore-inducing substances in soil is related to specific bacteria. Three isolates of soil bacteria induced chlamydospore formation when cultured with one or more of the *Fusarium* clones. Induction was clearly due to the release of substances by the bacteria into the BM, as BM itself does not promote appreciable chlamydospore formation (2). That the bacteria released chlamydospore-inducing substances into the medium in which they were growing was further indicated by the demonstration that the anion fraction from culture filtrates of two bacteria induced chlamydospore formation by two of the fungal clones. We were unable

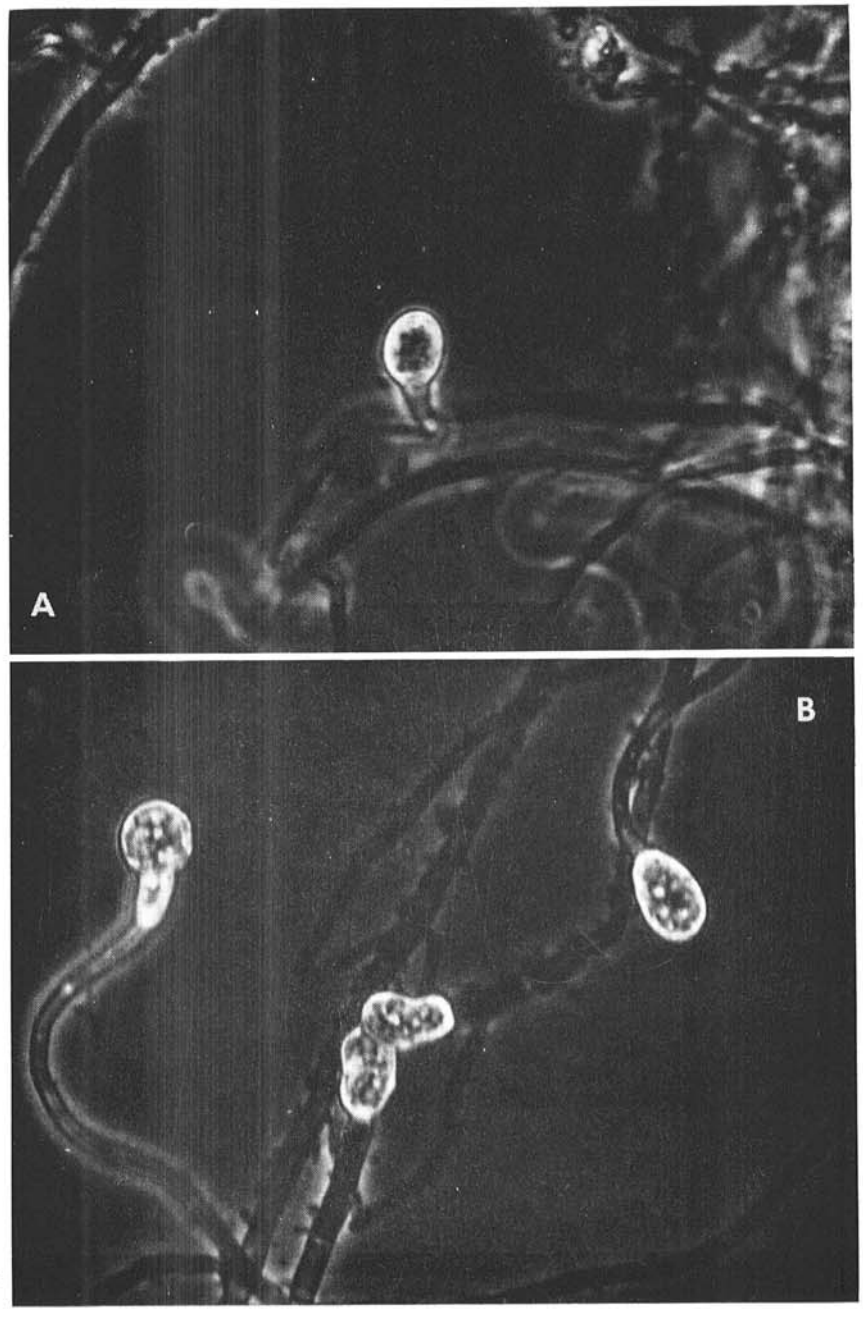


**Fig. 2.** Caption on facing page.

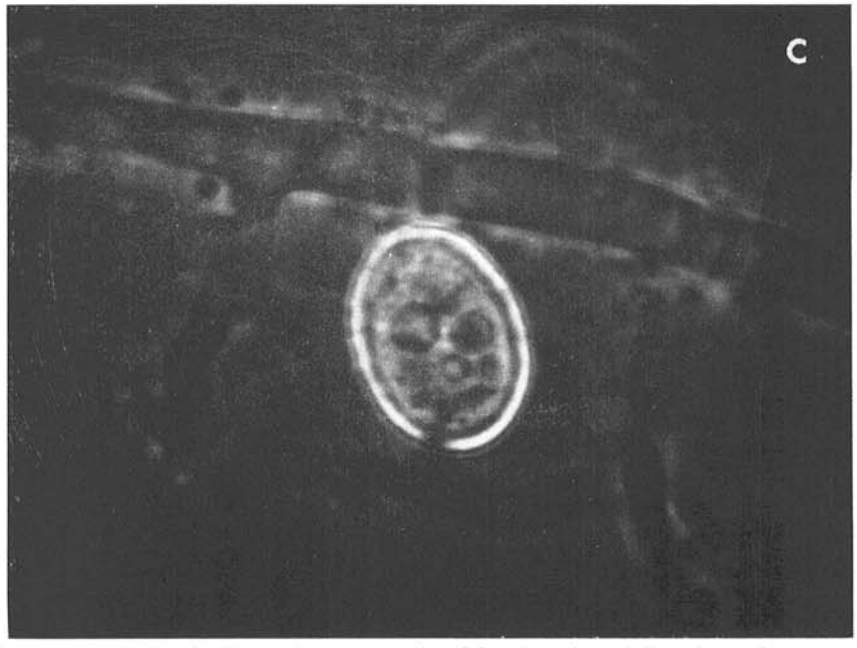


**Fig. 2.** (Facing page and above) Chlamydospores produced by clone $S_{2b}$ of *Fusarium solani* f. sp. *phaseoli* in non-sterile soil extract. **A)** Young terminal chlamydospore produced after 3 days' incubation (×2000). **B)** Terminal and intercalary chlamydospores produced after 7 days' incubation (×2000). **C)** Chlamydospore produced after 7 days' incubation (×5000).

to isolate and detect enough metabolic products from the bacteria to account completely for the induction of chlamydospore formation with some clones as expressed in the co-culture experiments. Venkata Ram (16) has also reported that bacteria produced substances which induced a saprophytic clone of *F. solani* to form chlamydospores.

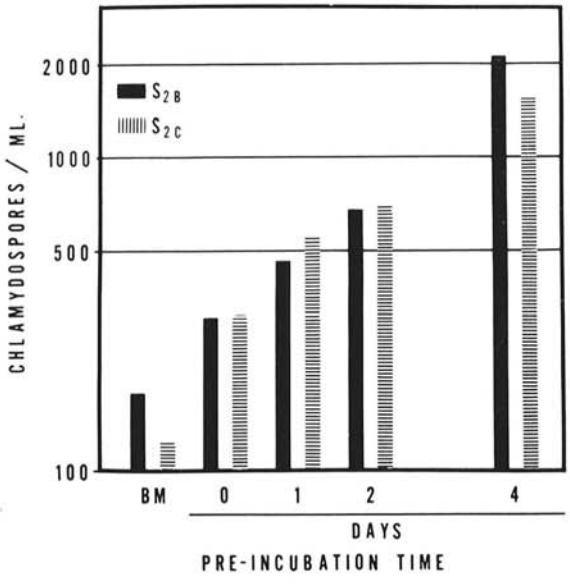


**Fig. 3.** Chlamydospore formation by clones $S_{2b}$ and $S_{2c}$ of *Fusarium solani* f. sp. *phaseoli* in a sterile basal medium, and sterile soil extracts which had been preincubated for increasing times prior to sterilization.

The induction of chlamydospore formation in *F. solani* f. sp. *phaseoli* by soil bacteria does not appear to be due to the release of a single substance affecting all clones, but rather, a response to several substances, each inducing only certain clones to form chlamydospores. This view is supported by the finding that different bacteria did not induce all three clones to form chlamydospores, nor did the fractions of culture filtrates induce all the clones to form chlamydospores. A similar specificity has been reported for chlamydospore-inducing substances extracted from soil (5).

The survival of most of the soil-borne fusaria depends on the production of thick-walled chlamydospores in soil. These spores are resistant to the lysis that rapidly destroys the thin-walled portions of the fungal thallus in soil (8, 9). If bacteria are responsible for at least certain of the chlamydospore-inducing substances in soil, the particular bacteria involved must be capable of existing and growing in most soils. *Protaminobacter, Arthrobacter,* and *Bacillus,* the three bacteria tentatively identified, are considered widespread in soils (3). The diversity of these three genera would indicate that this fungal-bacterial relationship may prevail in many soil types, and is potentially of great importance in the production of chlamydospores by *Fusarium.*

Chlamydospore formation by *Fusarium* in soil has been considered a form of soil fungistasis (4). It has been stated (1, 2, 5) that chlamydospore formation is a positive response to the presence of one or more substances, not simply a negative response to the lack of nutrition. This work indicates that these substances originate in soil from the activities of specific microorganisms.

The nature of the process by which the thin-walled, vegetative portions of the fungal thallus convert to the thickened, double-walled, resting spores is still not clear. Present knowledge indicates that the process is initiated in the thallus as a response to a group of substances. Isolation and purification of such substances should make possible a detailed experimental study of the mechanism of chlamydospore formation.

Isolates of *F. oxysporum* (10, 11, 12, 13, 14), *F. roseum* (11), and a saprophytic *F. solani* (6) have been reported to produce, as a normal sequence in vegetative growth, metabolic products that accumulate and serve to induce chlamydospore formation. Parks (11) has reported a specificity of clonal response to the metabolic products from one isolate similar to that observed for the chlamydospore-inducing substances produced by bacteria. The isolates of *Fusarium* that do not form chlamydospores in culture, but are known to do so in soil, may lack the ability to produce substances that induce chlamydospore formation.

The existence of chlamydospore-inducing substances in soil may be of great importance in enabling certain fusaria to produce their survival spores rapidly and efficiently in soil. The production of these substances by specific bacteria and the clonal specificity to each of the substances allows for many variations in the ability of different clones of *Fusarium* to produce chlamydospores in different soils, and even in one soil at different times of the year.

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