Interaction of Carbon Nutrition and Soil Substances in Chlamydospore Formation by Fusarium

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ABSTRACT

The potential for chlamydospore formation by one clone of *Fusarium solani* f. sp. *phaseoli* was regulated by the concentration of chlamydospore-inducing substances from soil. The actual number of chlamydospores produced by the fungus was determined by the amount of energy source (glucose) available. Both the chlamydospore-inducing substances and glucose showed optimal concentration for chlamydospore formation. A scheme for relating chlamydospore formation among all clones of *Fusarium* is advanced, in which chlamydospore formation occurs as a result of the interaction between a morphogen (the chlamydospore-inducing substances), other environmental factors (nutrition, etc.), and the fungus. The morphogen may be produced endogenously by the fungus itself as a by-product of mycelial growth, or be produced exogenously by other microbes in the fungus' habitat. Phytopathology 60:1732-1737.

Chlamydospores of *Fusarium* sp., which provide the principal means of survival during unfavorable periods in soil, have been reported to be produced in response to a variety of stimuli. Among them are unfavorable growing conditions (8, 17), starvation (15), C:N ratio of the culture medium (6, 7, 21, 24), and staling product accumulation (9, 14, 19, 20). All of these stimuli are general in nature, and fail to define precisely the conditions leading to chlamydospore formation; moreover, chlamydospore formation is not induced in all *Fusarium* clones by any one of these conditions. Chlamydospore formation was first placed on a solid physiological basis with the demonstration that certain soil bacteria produced specific substances which increased chlamydospore production by *F. solani* (23).

F. solani f. sp. *phaseoli* (Burk.) Snyd. & Hans. is an ideal organism for studying the physiology of chlamydospore formation, since very few chlamydospores are produced under normal laboratory cultural conditions. Recently (1, 2, 10, 13, 16), it was found that soil contains extractable substances which induce high levels of chlamydospore formation. These substances are apparently produced by specific soil bacteria (9, 11, 18).

The demonstration that one environmental factor influences an over-all physiological process does not exclude the influence of other environmental factors. Bourret et al. (3) explored the influence of CO_2 concn on chlamydospore formation in the presence of the substances extracted from soil. Other studies (2, 10) indicated that mineral salts also played a role. There were also indications that energy sources may be important in determining whether the direction of development of *Fusarium* is toward mycelial growth or chlamydospore formation.

The soil-extractable substances were effective in such low concn that it is difficult to think of them as important sources of energy. The present study was undertaken to clarify the relationship between these substances and a typical energy source. Three fractions of soil extract (neutral, anion, and anionic ethanol-insoluble fractions) were used as sources of partially purified chlamydospore-inducing substances for one clone of *F. solani* f. sp. *phaseoli* (10). Chlamydospore formation was studied as a function of both the concn of the chlamydospore-inducing fractions and the concn of glucose.

MATERIALS AND METHODS.—Many of the experimental materials and methods used here have been described more completely in an earlier publication (10).

Fungus cultures.—Cultures of *F. solani* f. sp. *phaseoli* clone S_{2b} were maintained on potato-dextrose agar (PDA) in the laboratory in ambient room light. Stock cultures were renewed by single sporing at 3- to 4-week intervals. Spores for use as experimental materials were taken from 1- to 3-week-old cultures.

Fractions of soil extract.—Neutral, anion, and anionic 90% ethanol-insoluble fractions of soil extract were used as sources of chlamydospore-inducing substances for clone S_{2b} (10). Three parts of a Salinas Valley soil (sandy overwash series) were extracted with one part of water. After filtering, the extract was passed successively through Amberlite IR 120 (H^+ form) to remove cations, and through Amberlite IR 45 (OH^- form) to remove anions. The columns were then washed with deionized distilled water.

The neutral fraction of soil extract consisted of the effluent extract plus the wash water. The solution was taken to dryness in a rotary evaporator under vacuum at 50 C. The residue remaining was then redissolved in a volume of distilled water equivalent to 1/20 the volume of the original extract volume, and the pH adjusted to 7.0 ± 0.2 with either 1 N HCl or 1 N KOH.

The anion fraction of soil extract was removed from the IR 45 resin by elution with 2 N NH_4OH. The eluant was taken to dryness in a rotary evaporator under vacuum at 50 C, and the residue redissolved in a volume of distilled water equivalent to 1/20 the original extract volume. The pH was adjusted to 7.0 ± 0.2 with 1 N HCl.

The anionic 90% ethanol-insoluble fraction was prepared by acidifying the concd anion fraction to pH 1.0 with 6 N HCl. Nine parts of absolute ethanol were added to one part of the acidified anion fraction, and the solution cooled to 4 C. The resultant precipitate was

removed from suspension by filtering through a 0.22-μ Millipore filter, and the residue on the filter was dried in a forced-air drying oven at 50 C. The residue was then redissolved in a volume of distilled water equivalent to 1/20 the volume of the original extract and neutralized with 1 N KOH to pH 7.0 ± 0.2.

All fractions were sterilized by autoclaving and treated aseptically until used.

Preparation of treatment solutions.—Treatment solutions consisted of various concn of the fractions of soil extract and the carbon source in a basal medium (BM). The BM was a mineral salts solution with a pH of 6.6 (10). The BM was prepared as a 20-fold concd stock solution, sterilized, and diluted as needed.

Glucose served as the source of carbon nutrition in these experiments. It was freshly prepared as a 1,000 ppm stock solution for each experiment.

Final concn of the chlamydospore-inducing substances, based on their concn in the original soil extract, and of the carbon sources in BM, were obtained by combining appropriate volumes of each of the stock solutions and diluting with distilled water. Treatments were dispensed as 5 ml of solution/20 ml serum vial which were plugged loosely with cotton. The vials were then sterilized by autoclaving at 15-lb. pressure for 15 min.

Procedure for assaying chlamydospore formation.—The standard serum vial techniques for assaying chlamydospore formation (2, 10) were used in all experiments. Briefly, the technique consisted of adding 1/10 ml of an aq spore suspension of the fungus to each serum vial to bring the spore concn to approximately 2,000 macroconidia/ml in the treatment solutions. In one experiment, a concn of 4,000 macroconidia/ml was used. After 7 days' incubation at 24 ± 2 C, the number of chlamydospores/ml of test solution was determined. Two drops of 0.1% phenol were added to each vial, and then the contents were sonicated to disrupt the mycelium and leave the chlamydospores in suspension. After staining with two drops of 0.85% acid fuchsin, 1 ml of the suspension was drawn through a 0.22-μ Millipore filter, leaving the chlamydospores trapped on the surface. The number of chlamydospores appearing in 10 microscope fields (×205) was determined and multiplied by the ratio (3.1) of the area of the disc counted to the area of the disc through which the suspension was drawn to give the results in chlamydospores/ml.

Each experiment consisted of three replications/treatment. The experiment was repeated 2 or 3 times to verify the results. Significance of treatments was determined at the 99% level ($P = .01$), using the Student t-test (22).

RESULTS.—*Chlamydospore formation in dilute nutrient solutions.*—Chlamydospore formation in the BM and in BM plus low concn of glucose varied in different experiments (Table 1). Several reproducible patterns appeared. When there was little or no chlamydospore formation in the BM, there was no significant increase in chlamydospore formation in any of the glucose treatments. However, when there was chlamydospore formation (~ 100/ml) in the BM, chlamydospore formation increased to a max as the glucose concn was increased. The variability in chlamydospore formation between experiments apparently was caused by variability in the culture medium, homemade PDA, since conidia from the same batch of PDA gave comparable results when used in separate experiments.

Effect of glucose on chlamydospore formation in the neutral fraction of soil extract.—The influence of carbon nutrition on chlamydospore formation was considered when three concn of the neutral fraction of soil extract were used as a source of chlamydospore-inducing substance(s) for clone S_{2b} (Fig. 1-A). Each concn of the neutral fraction (1, 2.5, and 5 times) was supplemented with 0, 5, 10, 50, 75, and 100 ppm glucose. The control system, consisting of the BM plus each of the glucose levels, did not induce chlamydospore formation, and is not depicted in Fig. 1-A.

At each of the three concn of the neutral fraction, there was an increase in the number of chlamydospores produced as the level of supplemental glucose was increased. At glucose concn above 50 ppm, the trend was reversed, and further increases in the glucose level led to decreased chlamydospore production. The opt concn of glucose for chlamydospore formation was the same at all concn of the chlamydospore-inducing substance(s).

The concn of the neutral fraction determined the number of chlamydospores produced at each glucose level. At any given glucose concn, the 2.5 times concn of the neutral fraction induced more chlamydospores to form than did comparable glucose levels in the 1 or 5 times concn of the neutral fraction. Thus, there apparently was an opt concn of the neutral fraction (chlamydospore-inducing substance[s]) for chlamydospore induction. Beyond this opt, further increases in concn inhibited the process.

Visual observations were made on the total amt of

TABLE 1. Chlamydospore formation by *Fusarium solani* f. sp. *phaseoli* in BM[a] and BM plus glucose

Exp. No.	Glucose (ppm) 0	5	10	25	50	75	100
	Chlamydospores/ml						
1	0[b]	0	0		0	0	0
2	92 ± 4			632 ± 15	1,245 ± 23	988 ± 14	774 ± 23
3[c]	105 ± 4		205 ± 13	564 ± 10	1,165 ± 28	1,525 ± 4	1,230 ± 23
4	16 ± 10	0	0		0	0	0

[a] Basal medium was a mineral salts solution, pH 6.6 (10).
[b] Each value is the mean of three replicates ± standard error of the mean.
[c] Initial inoculum was 4,000 macroconidia/ml.

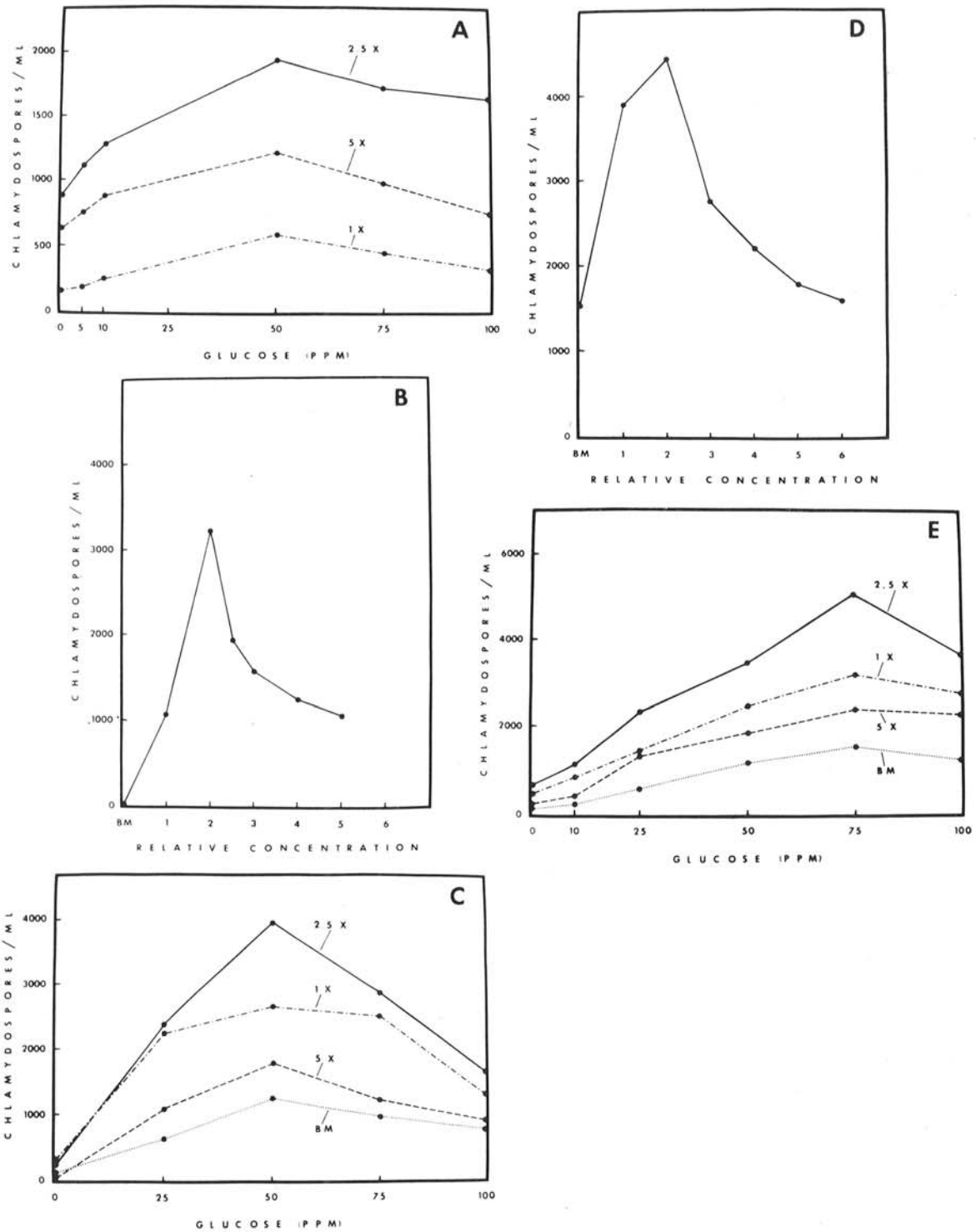

Fig. 1. Chlamydospore formation by *Fusarium solani* f. sp. *phaseoli* expressed as a function of the concn of chlamydospore-inducing substances and glucose: **A)** three concn of the neutral fraction of soil extract plus increasing levels of glucose. **B)** Increasing concn of the neutral fraction of soil extract plus 50 ppm glucose. **C)** Three concn of the anion fraction of soil extract plus increasing levels of glucose. **D)** Increasing concn of the anion fraction of soil extract plus 50 ppm glucose. **E)** Three concn of the anionic 90% ethanol-insoluble fraction of soil extract plus increasing levels of glucose.

fungal material produced in relation to the increasing glucose levels at each concn of the neutral fraction. Although precise techniques were unavailable for confirming these observations, it appeared that an increase in total fungal material occurred as the glucose concn was increased. In addition, observations were made on the type of sporulation occurring in the series. At glucose concn of 0, 5, and 10 ppm, the chlamydospore was the only spore stage produced. As the glucose concn reached 50, 75, and 100 ppm, the production of macroconidia became successively greater. At these glucose levels, chlamydospores were being produced on one portion of the thallus, while macroconidia were being produced on another.

Effect of the concn of the neutral fraction of soil extract on chlamydospore formation.—This experiment examined the production of chlamydospores as the concn of the neutral fraction supplemented with 50 ppm glucose was varied from 1 to 5 times its strength in the original soil extract (Fig. 1-B). The controls (BM, and BM plus 50 ppm glucose) did not induce chlamydospore formation.

Chlamydospore production increased to a max at concn of 2 times the neutral fraction of soil extract. At greater concn of the neutral fraction, chlamydospore production was reduced. The reduction of chlamydospore formation was not linear as the concn of the fraction increased. With each unit of increase in concn, the decrease in chlamydospore production became progressively less over the range examined.

The reduction in number of chlamydospores formed, as the concn of the neutral chlamydospore-inducing substance(s) was increased, did not appear to be due to the concn of materials present in the neutral fraction inhibitory to fungal growth. Visual observations revealed no noticeable reduction in total fungal growth as the strength of the neutral fraction was increased, nor was there any noticeable distortion of mycelium produced in the treatments.

Chlamydospore formation increased in the anion fraction of soil extract supplemented with glucose.—The influence of carbon nutrition on chlamydospore formation was also studied when three concn (1, 2.5, and 5 times) of the anion fraction of soil extract were used as sources of chlamydospore-inducing substance(s). Each concn of the anion fraction was supplemented with 0, 10, 25, 50, 75, and 100 ppm glucose (Fig. 1-C).

There was an increase in the number of chlamydospores produced in each concn of the anion fraction as glucose was increased to 50 ppm. Further increases in glucose concn led to a reduced number of chlamydospores in each anion concn. As with the neutral fraction, the 2.5 times concn of the anion fraction induced more chlamydospores to form at most glucose levels than did the 1 or 5 times concn.

In this experiment, the fungus formed some chlamydospores in the unamended BM. There was an increase in the number of chlamydospores produced as glucose was increased to 50 ppm. Further increases in glucose led to reduced chlamydospore production. The shape of the curve for the control system closely paralleled those obtained with the anion fraction, but at a much reduced level (Fig. 1-C).

Influence of the concn of the anion fraction on chlamydospore formation.—Chlamydospore formation was also studied when the concn of the anion fraction was varied from 1 to 6 times that in the original soil extract (Fig. 1-D). Each concn was supplemented with 50 ppm glucose.

The number of chlamydospores formed in the anion fraction increased very rapidly as its concn was increased to 2 times its original strength. Further increases led to decreased chlamydospore formation and eventually leveled off at that of the control. As with the neutral fraction, we could detect no inhibition of mycelial growth at the higher concn of the anion fraction.

Effect of glucose on chlamydospore formation in the anionic 90% ethanol-insoluble fraction of soil extract.—A further purification of chlamydospore-inducing substances in the anion fraction was made with 90% ethanol precipitation. Three concn (1, 2.5, and 5 times) of the anionic, 90% ethanol-insoluble fraction were supplemented with 0, 10, 25, 50, 75, and 100 ppm glucose (Fig. 1-E). In this experiment, the initial inoculum consisted of 4,000 macroconidia/ml.

The control system, consisting of BM at each of the glucose concn, supported a large increase in the number of chlamydospores produced as glucose was increased. The increase in chlamydospore production occurred at a significantly lower level than in any of the concn of the anionic 90% ethanol-insoluble fraction of soil extract. The response followed a pattern similar to that obtained in the treatments containing the chlamydospore-inducing substances.

The additions of increasing amounts of glucose to the three concn of the anionic 90% ethanol-insoluble fraction of soil extract show a similar trend to those obtained in earlier experiments using other fractions of soil extract as a source of chlamydospore-inducing substance(s). As glucose levels were increased, the number of chlamydospores produced was increased, and an opt level of glucose for chlamydospore formation was achieved. As the glucose concn was increased beyond the optimum, there was a reduction in the number of chlamydospores formed.

The effect of the concn of the fraction containing the chlamydospore-inducing substance(s) was also similar to that obtained earlier. At all levels of glucose, the concn of the fraction giving the greatest number of chlamydospores was 2.5 times. The number of chlamydospores produced/ml was reduced in the 5 times concn of the fraction. The reduction in chlamydospore formation apparently was not due to a toxic effect of concn, since inhibition of mycelial growth was not evident, as the anionic 90% ethanol-insoluble fraction was increased.

The opt glucose concn for chlamydospore production in all three concn of the anionic 90% ethanol-insoluble fraction and in the BM control system was approx 75 ppm glucose. This level of glucose is roughly twice that obtained in earlier experiments, where 2,000 macro-

conidia/ml were used rather than the 4,000 macroconidia/ml employed here.

Properties of the chlamydospore, such as the amount of storage material accumulated, the degree of wall thickening, and the amount of exuded materials on the spore surface, were observed but not evaluated. In various treatments, extremely robust chlamydospores were produced. Figure 2 presents examples of chlamydospores formed in the presence of the anionic 90% ethanol-insoluble fraction of soil extract supplemented with 50 ppm glucose. They are typical of the well-developed chlamydospores seen when the fractionated soil extracts were supplemented with glucose.

DISCUSSION.—Our findings are consistent with the concept that chlamydospore formation in *Fusarium* is initiated in response to distinct chlamydospore-inducing substances, and not merely to low nutrient or unfavorable conditions per se (9, 10, 11). The family of curves presented in Fig. 1-A, C, and E show max chlamydospore production at fixed glucose levels for each curve in the figures. It was the concn of the soil extract fraction, however, which ultimately determined the number of chlamydospores formed at corresponding glucose levels. Thus, the potential for chlamydospore formation appears to be regulated by the concn of the chlamydospore-inducing fractions, although the actual number of chlamydospores produced is determined by the nutrient status. The regulatory nature of the chlamydospore-inducing fractions is illustrated further in Fig. 1-B and D, where chlamydospore formation was found to be highly dependent on the concn of the soil-extract fractions. The substance(s) present in each of the three fractions of soil extract apparently act as morphogens which stimulate chlamydospore production. Their effect is regulated by the nutritional status of the system. A limited amount of energy source allows chlamydospore production; excess nutrients overwhelm the influence of the chlamydospore-inducing substances and decrease chlamydospore production, while the absence or very low levels of nutrient limits chlamydospore formation by lack of sufficient energy.

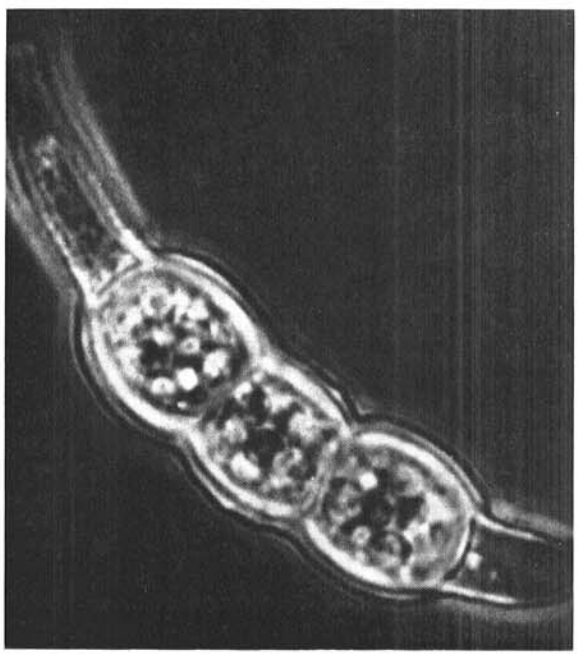

Fig. 2. Chlamydospores formed in the 2.5 times concn of the anionic 90% ethanol-insoluble fraction of soil extract supplemented with 50 ppm glucose (×4000).

Other workers have postulated various environmental factors, such as unfavorable conditions (8, 17), starvation (15), C:N ratio of the culture medium (6, 7, 21, 24), etc., for initiating chlamydospore formation by *Fusarium*. These environmental influences are not inconsistent with the morphogen theory. Endogenous production of chlamydospore-inducing substances under low nutrient conditions has been documented for several *F. oxysporum* isolates (9), and appears to be the case with *F. solani* 'Coeruleum' (13), which produces chlamydospores more quickly and in greater numbers when replacement cultures are used. In our study, the clone of *F. solani* f. sp. *phaseoli* used appeared to produce an endogenous chlamydospore-inducing substance when spores were taken from certain batches of PDA. It would appear that some fusaria are capable of synthesizing chlamydospore-inducing substances, such as those we isolated from soil, during mycelial growth. When these substances reach critical levels and environmental conditions are right, survival of the fungus is ensured by alteration of mycelial growth to chlamydospore formation.

Different genetic clones of *Fusarium* appear to vary in their ability to synthesize endogenous chlamydospore-inducing substances. Most clones of *F. oxysporum* will produce appreciable numbers of chlamydospores in a mineral salts medium, yet most clones of *F. solani* f. sp. *phaseoli* will not (W. C. Snyder, *personal communication*). There is variability in this property, even among the clones of *F. oxysporum*. Some clones may produce chlamydospores in 3 days, while others may take up to 30 days.

In nature, those clones of *Fusarium* which lack the ability to synthesize, or require specific nutrients to produce endogenous chlamydospore-inducing substances (6, 7, 21, 24), are placed in an environment which already contains these substances and compensates for this deficiency in their genetic makeup. It is well recognized that different soils may support widely different bacterial floras. Similarly, Burke (4, 5) reported that two different soils from the bean-growing regions of the Pacific Northwest support widely different populations of the bean root rot pathogen, *F. solani* f. sp. *phaseoli*. He related this difference to chlamydospore formation in the two soils. Perhaps the difference in survival of the bean pathogen in these soils may be related further to differences in quantity and/or quality of chlamydospore-inducing substances in the soils arising from different bacterial floras. At any rate, the knowledge that distinct chlamydospore-inducing substances occur in soils should be considered in studying factors affecting the survival of *Fusarium* clones in

different soils, or even in a single soil at different times of year.

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