Soil Substances Inducing Chlamydospore Formation by Fusarium

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ABSTRACT

At least three, and possibly four substances in one Salinas Valley soil induced chlamydospore formation by one or more clones of *Fusarium solani* f. sp. *phaseoli*. The chlamydospore-inducing substances were separated by ion exchange and solvent precipitation techniques. Clones of the fungus responded differentially to each of the chlamydospore-inducing fractions isolated. Soil extracts made from soil samples collected at different times of the year varied independently in the capacity to induce three clones to form chlamydospores. The ability of clones of *Fusarium* to produce chlamydospores in a soil may be dependent on the presence of specific substances in that soil. Phytopathology 60:124-128.

Fusarium solani (Mart.) Appel & Wr. f. sp. *phaseoli* (Burk.) Snyd. & Hans. does not form chlamydospores rapidly nor in large numbers under the normal cultural conditions on agar (1, 3). This is in contrast to most of the saprophytic clones of *F. solani*, and most clones of *F. oxysporum* (Schlecht.) emend. Snyd. & Hans. and *F. roseum* (Lk.) emend. Snyd. & Hans., which produce chlamydospores in potato-dextrose agar (PDA) cultures or in distilled water. However, *F. solani* f. sp. *phaseoli* rapidly forms chlamydospores in large numbers when macroconidia are added to soil (12). These thick-walled spores enable the fungus to survive in the soil between host crops, while the thin-walled portions of the fungal thallus (hyphae and conidia) are rapidly lysed and disappear from soil (9, 11, 12). Warcup (14) found that most of the *Fusarium* colonies on soil dilution plates arose from chlamydospores.

Sterile aqueous extracts of soils induce rapid chlamydospore formation by *F. solani* f. sp. *phaseoli* (1, 2, 3, 8), *F. oxysporum*, and *F. roseum* (3). Bourret (3) reported that induction of chlamydospore formation in *F. solani* f. sp. *phaseoli* was a positive response to an unidentified, organic anion in the soil extracts. The possibility that more than one substance in soil extracts may induce chlamydospore formation in *Fusarium* was suggested by Alexander (1), Alexander et al. (2), and Bourret (3). This paper presents evidence supporting this hypothesis. A preliminary report of this work has been presented (7).

MATERIALS AND METHODS.—Three clones (S_{2a}, S_{2b}, and S_{2c}) of *F. solani* f. sp. *phaseoli* isolated from beans from the Salinas Valley, Calif., were used. The cultures were maintained in the laboratory on PDA and transferred every 3-4 weeks by single-spore techniques.

The soil was a fine sandy loam (overwash series) from the Salinas Valley in which beans are frequently grown. It was collected from one field at various dates throughout the course of the study (2 years), stored in galvanized garbage cans in the laboratory, and kept at approximately field capacity by periodically adding tap water.

Soil extracts were prepared by combining the desired ratios of soil and distilled water in a large beaker and mixing thoroughly. The soil particles were removed from the extract by filtering the mixture through two layers of Whatman No. 50 filter paper with the aid of a vacuum. The soil extract used in each experiment was designated by the ratio of the weight of soil to the volume of water used to extract it. The extraction ratios for different experiments ranged from 1 through 10.

The standard control solution for all experiments was a basal mineral salts medium (B.M.) prepared as a 20-fold concentrated stock solution and sterilized. Upon dilution, the basal medium had the following composition: KNO_3, 0.0252 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.0575 g; KH_2PO_4, 0.0136 g; K_2HPO_4, 0.0174 g; $MgSO_4 \cdot 7H_2O$, 0.2400 g; $MnSO_4 \cdot 4H_2O$, 0.0018 g; H_3BO_3, 0.0028 g; $(NH_4)_6Mo_7O_2$, 0.0020 g; distilled H_2O, 1 liter; pH 6.6.

This control solution was chosen on the basis of earlier work (3) which showed that chylamydospore formation in diluted soil extracts can be limited by the lack of essential mineral salts. The B.M. provided a nontoxic control in which the ability of the fungus to form chlamydospores was not limited by the lack of essential minerals.

Soil extracts were fractionated by passing them successively through columns of cation exchange resin (Amberlite IR 120, H^+ form) and anion exchange resin (Amberlite IR 45, OH^- form) at a flow rate of approximately 120 ml/hr. The resins were then washed with distilled, deionized water, and the wash water was combined with the effluent from the two columns.

The neutral fraction consisted of the effluent plus the wash water. This fraction was taken to dryness at 50°C in a vacuum evaporator. The residue was then dissolved in a volume of distilled water equivalent to one-twentieth the original soil extract volume, and its pH was adjusted 7.0 ± 0.1 with either 1 N HCl or 1 N KOH.

The cation fraction was prepared by eluting the cation resin with 2 N HCl. The eluate was taken to dryness at 50° C under vacuum, and the residue redissolved in a volume of distilled water equivalent to one-twentieth the original soil extract volume. The pH was adjusted to 7.0 ± 0.1 with 1 N KOH.

The anion fraction was prepared by eluting the anion exchange resin with 2 N NH_4OH. The eluate was taken to dryness at 50°C under vacuum. The residue was redissolved in a volume of distilled water equivalent to one-twentieth the original soil extract volume. The pH was adjusted to 7.0 ± 0.1 with 1 N HCl.

The anion fraction of soil extract was further separated into fractions soluble and insoluble in 90% ethanol. The concentrated anion fraction was acidified with 6 N HCl to pH 1.0. Nine volumes of absolute ethanol were slowly added to one volume of the anion fraction with continuous stirring. The 90% ethanol solution

was cooled to 4°C, and the fine precipitate was removed by filtration through a Millipore filter (pore size 0.22 μ).

The residue was washed with absolute ethanol, dried thoroughly, and then redissolved in a volume of water equivalent to that of the concentrated anion fraction. The 90% ethanol solution was taken to dryness at 50°C under vacuum. The residue was redissolved in a volume of distilled water equivalent to that of the concentrated anion fraction. The pH of both fractions was adjusted to 7.0 ± 0.1 with either 1 N HCl or 1 N KOH.

The control consisted of distilled water run through the same fractionation scheme as soil extract.

Each of the fractions was diluted with 8.5 parts distilled water and 0.5 part B.M. stock solution to a 2-fold concentration of the original soil extract volume and tested for its capacity to induce chlamydospore formation.

Chlamydospore formation was tested in vials containing 5 ml of the sterilized test solution. Each vial was seeded with 0.1 ml of an aqueous spore suspension of the test fungus, so that the final fungus concentration was approximately 2,000 macroconidia/ml. Each experiment consisted of three replications/treatment, and was repeated 2 or 3 times.

After 7 days' incubation, the number of chlamydospores produced per ml of test solution was determined using a modification of the technique described by Bourret (3). Three drops of a 0.1% phenol solution were added to each vial, and the vials were allowed to stand for 30 min. The fungal mat was then sonicated with a Bronwill Biosonik Model No. BP1 for 20 sec at full power. The mycelial fragments and intact chlamydospores in each vial were stained with three drops of 0.85% acid fuchsin in 85% lactic acid. One ml of the suspension was drawn through a disc (12.56 mm^2) of millipore filter (pore size: 0.45 or 0.65 μ), which was dried and then cleared with immersion oil. The number of chlamydospores in ten microscope fields (3.83 mm^2) at a magnification of ×205 was converted to chlamydospores/ml by multiplying this number by 12.56/3.83.

The significance of the difference between the number of chlamydospores produced in any treatment and the corresponding control was determined at the 99% level ($P = .01$) using the Students' t-test (13). Where a series of treatments in an experiment was compared, the modified Duncan's Multiple Range Analysis at the 99% level of significance was used (13).

RESULTS.—*Clones differ in response to extracts of different soil samples.*—Soil extracts made from different samples of the Salinas Valley soil differed in their capacity to induce chlamydospore formation by the three clones of *F. solani* f. sp. *phaseoli* (Table 1). Each extract was made from soil samples collected at different times of the year and/or after different lengths of storage in the laboratory.

Four distinct patterns of chlamydospore formation in the extracts were obtained. The extracts were arbitrarily placed into classes according to their capacity to induce chlamydospore formation in the three clones. Class-1 extracts induced all three clones to form significantly more chlamydospores than did the control. Class-2 extracts induced clones S_{2b} and S_{2c} to form chlamydospores, whereas Class-3 extracts induced chlamydospore production by only clone S_{2b}. Class-4 extracts did not induce any of the clones to form chlamydospores. No other chlamydospore induction pattern in crude soil extracts was observed during the study.

Clones differ in response to soil extracts of increasing extraction ratios.—Differences in chlamydospore formation by each clone occurred in both the numbers of chlamydospores formed by each clone at each extraction ratio and in the shape of the response curve for each clone as the extraction ratio increased (Fig. 1-A, B, C). Chlamydospore formation by clone S_{2a} in the extraction series followed a bimodal response curve (Fig. 1-A). Peaks in chlamydospore formation occurred

TABLE 1. Effect of extracts of different soil samples on chlamydospore formation by three clones of *Fusarium solani* f. sp. *phaseoli*

Soil extract no.	Chlamydospores/ml			Type of[a] response
	S_{2a}	S_{2b}	S_{2c}	
1	108 ± 8[b]	487 ± 24	112 ± 4	Class 1
	(0)[c]	(0)	(0)	
2	53 ± 13	220 ± 6	280 ± 50	
	(0)	(40 ± 4)	(39 ± 5)	
3	0	360 ± 13	312 ± 4	Class 2
	(0)	(163 ± 14)	(121 ± 3)	
4	0	189 ± 6	82 ± 4	
	(20 ± 1)	(48 ± 5)	(20 ± 2)	
5	0	87 ± 3[d]	0	Class 3
	(0)	(0)	(0)	
6	0	69 ± 5	0	
	(0)	(15 ± 2)	(19 ± 5)	

[a] Extracts showing a Class-4 type of response are not shown as there was no chlamydospore formation.
[b] Each value gives the mean of three replications ± standard error of mean.
[c] Corresponding control in parentheses under each treatment.
[d] This value is the mean of two replications.

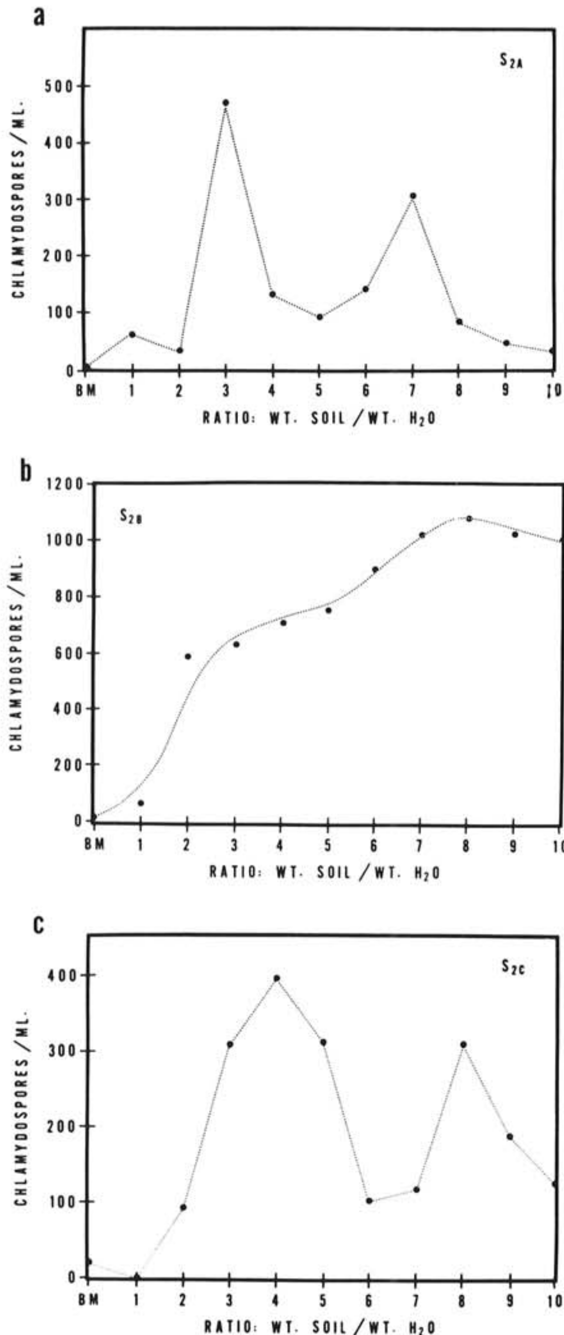

Fig. 1. Chlamydospore formation by three clones of *Fusarium solani* f. sp. *phaseoli* in sterile soil extracts of increasing extraction ratios. **a)** Clone S_{2a}. **b)** S_{2b}. **c)** Clone S_{2c}.

in extracts of extraction ratios of 3 and 7. The least significant difference between successive extracts with clone S_{2a} was 17 chlamydospores/ml. Chlamydospore formation by clone S_{2b} in the series of extracts increased as the extraction ratio increased (Fig. 1-B). The least significant difference between successive extracts with clone S_{2b} was 32 chlamydospores/ml. Chlamydospore formation by clone S_{2c} in the extraction series also followed a bimodal response curve (Fig. 1-C). In contrast to the curve obtained with clone S_{2a}, peaks in chlamydospore formation occurred in extracts of extraction ratios of 2 and 8. The least significant difference between successive extracts with clone S_{2c} was 72 chlamydospores/ml.

Similar results were obtained in three separate experiments using a different collection of soil. With all soil samples, clones S_{2a} and S_{2c} exhibited bimodal response curves with offset peaks in chlamydospore formation, and clone S_{2b} exhibited an irregularly increasing response curve.

Clones differ in response to ionic fractions of soil extract.—The three clones of *F. solani* f. sp. *phaseoli* differed in the production of chlamydospores in the ionic fractions of Class-1 and Class-2 soil extracts as prepared by ion exchange chromatography.

In the ionic fractions from Class-1 soil extracts (Table 2), chlamydospores were produced by one or more clones in the anion and neutral fractions. None of the clones formed chlamydospores in the cation fraction. The anion fraction of the soil extract induced all three clones to form significantly greater numbers of chlamydospores than the control. The neutral fraction induced only clone S_{2b} to form significantly more chlamydospores than the control. This indicated that there was a minimum of two chlamydospore-inducing substances, one in each the anion and the neutral fractions of Class-1 soil extracts.

In the ionic fractions from Class-2 soil extracts (Table 2), chlamydospores were also produced by one or more clones in the anion and neutral fractions. None of the clones formed chlamydospores in the cation fraction. The anion fraction of the soil extract induced clones S_{2b} and S_{2c} to form significantly greater numbers of chlamydospores than the control. The neutral fraction induced only clone S_{2b} to form significantly greater numbers of chlamydospores than the control. Clone S_{2a} was not induced to form chlamydospores in

TABLE 2. Disposition of *Fusarium solani* f. sp. *phaseoli*-chlamydospore-inducing substances from Class-1 and Class-2 soil extracts into ionic fractions

Treatment	Chlamydospore formation by clones		
	S_{2a}[a]	S_{2b}	S_{2c}
Class-1 soil extract			
Crude	+[b]	+	+
Cation fraction	—	—	—
Anion fraction	+	+	+
Neutral fraction	—	+	—
Class-2 soil extract			
Crude	—	+	+
Cation fraction	—	—	—
Anion fraction	—	+	+
Neutral fraction	—	+	—

[a] Clone designations S_{2a}, S_{2b}, and S_{2c} represent different isolates of *F. solani* f. sp. *phaseoli* from Salinas Valley, Calif.

[b] + indicates a significant increase in chlamydospore formation over B.M. control; — indicates no significant increase in chlamydospore formation over B.M. control.

either the crude soil extract or in any of the ionic fractions. Therefore, as with the ionic fractions of Class-1 soil extracts, it appeared that there was a minimum of two chlamydospore-inducing substances in Class-2 extracts, one in each the anion and neutral fractions.

No chlamydospores were produced by any of the clones in the ionic fractions of distilled water.

Separation of anionic chlamydospore-inducing substances.—The three clones of *F. solani* f. sp. *phaseoli* differed in the production of chlamydospores in the 90% ethanol soluble and insoluble fractions of the anion fraction of a Class-2 soil extract (Table 3). Clones S_{2b} and S_{2c} formed chlamydospores in the anion fraction of a Class-2 soil extract. This fraction was further separated into 90% ethanol soluble and insoluble fractions. The 90% ethanol soluble fraction induced only clone S_{2b} to form chlamydospores; whereas, the 90% ethanol insoluble fraction induced both clones S_{2b} and S_{2c} to form chlamydospores. This indicates that the anion fraction of this Class-2 soil extract contained a minimum of two chlamydospore-inducing substances, one soluble in 90% ethanol and one insoluble.

No chlamydospores were produced by any of the clones in the 90% ethanol soluble and insoluble fractions of distilled water.

DISCUSSION.—The failure of *F. solani* f. sp. *phaseoli* to form many chlamydospores in culture has raised the question as to the nature of the factors in soil that cause their abundant production. Investigations (1, 2, 3, 4, 8) have shown that soil extracts contain a substance(s) which induces this *forma specialis,* as well as *F. oxysporum* and *F. roseum,* to rapidly produce chlamydospores. Our results indicate that chlamydospore induction in soil extracts is dependent on several specific chlamydospore-inducing substances. Chlamydospore formation in soil extracts is a function of the genetic constitution of the clone as well as the particular chlamydospore-inducing substances in the extracts.

Consideration should be given to the possible presence of substances inhibitory to chlamydospore production when bioassaying a complex mixture such as soil extract. Therefore, extracts which do not induce chlamydospore formation may not necessarily lack chlamydospore-inducing substances. Alternatively, extracts which induce chlamydospore formation can be considered to contain chlamydospore-inducing substances. In spite of the limitations of the bioassay, it was possible to establish that there were several chlamydospore-inducing substances in soil, and that there was a clonal specificity to some of the chlamydospore-inducing substances.

TABLE 3. Chlamydospore formation by three clones of *Fusarium solani* f. sp. *phaseoli* in the anion portion of soil extract fractionated with 90% ethanol

Clone of *F. solani* f. sp. *phaseoli*	Chlamydospores/ml		
	B.M. control	90% Ethanol soluble	90% Ethanol insoluble
S_{2a}	0[a]	0	0
S_{2b}	38 ± 4	310 ± 3	558 ± 19
S_{2c}	44 ± 7	0	169 ± 11

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

The separation of one soil extract into several distinct chemical fractions which induced one or more of the clones to form chlamydospores provided evidence for the existence of several chlamydospore-inducing substances in soil. The capacity of some of the soil extracts and fractionated extracts to induce one or two of the clones to form chlamydospores, but not all of the clones, may be explained by either (i) a clonal specificity to the chlamydospore-inducing substances; (ii) a clonal specificity to inhibitors of chlamydospore formation which could be present in the extracts; or (iii) a combination of (i) and (ii). Clonal specificity can be established for the chlamydospore-inducing substances in the 90% ethanol soluble fraction (Table 3) and in the neutral fraction (Table 2) affecting only clone S_{2b}. Both of these fractions were prepared from solutions which supported chlamydospore formation by other clones, and therefore can be considered free from inhibitors of chlamydospore formation specific to those clones. While it was not possible to confirm the clonal specificity to the chlamydospore-inducing substances in the other fractions, it seems likely that there is a similar specificity to them. The chlamydospore formation response curves to soil extracts of increasing extraction ratios (Fig. 1-A, B, C) differ for each clone, indicating that the clones are affected by different factors.

No direct information exists on the relationship between chlamydospore formation in soil extracts and that in soil. For any one soil sample, the number of chlamydospores produced in sterile extracts of that sample is a function of the extraction ratio. Therefore, caution is necessary in relating the actual magnitude of chlamydospore formation in soil extracts to that in soil. One can, however, explore some of the possible parameters surrounding chlamydospore formation in soil by studying the process in soil extracts.

The existence of such materials in the soil prior to the introduction of the fungus is important in the establishment of this pathogen and other fusaria in soils. The phenomenon of soil mycolysis is well established for the fusaria (9, 11, 12). It is essential for the survival of the fungus in the soil that it enter the chlamydospore stage, which is resistant to lysis, as rapidly as possible. The presence of chlamydospore-inducing substances in soil would insure that the fungus will produce these spores.

It is conceivable that a soil may promote chlamydospore formation by one clone of the fungus, yet not by another clone. This situation would be brought about by the presence of an effective concentration of a specific chlamydospore-inducing substance to which only the first clone would respond. A comparable situation apparently occurred in extractable chlamydospore-inducing substances during the course of the present study (Table 1). Samples of soil collected at different times throughout the year differed in ability to induce chlamydospore formation as bioassayed using soil extracts. Since we did not collect soil at regular inter-

vals and take extensive data on the field conditions imposed on it prior to collection and extraction, no conclusions can be drawn on the causes of these fluctuations in the soil.

Burke (4, 5) described two soils from the bean-growing areas of Idaho on the basis of their ability to support bean root rot caused by *F. solani* f. sp. *phaseoli*. Losses are severe on one soil and mild on the other. The differences in disease incidence on these soils was traced initially to differences in their capacity to support high populations of the pathogens, and ultimately to differences in the ability of the fungus to produce chlamydospores in the soils. The differences in the two soils may be related to the presence or absence of specific chlamydospore-inducing substances.

Soil fungistasis is generally considered to be operative when fungus spores are unable to germinate in the soil. In certain fungi, one spore form may be relatively insensitive to soil fungistasis, while another spore form is highly sensitive. This is the case with *F. solani* f. sp. *phaseoli*. Macroconidia are insensitive to fungistasis, but chlamydospores are highly sensitive. The chlamydospore is the spore form of the fungus normally found in the soil; whereas, the macroconidia are usually produced on the surface of parasitized bean plants and serve as reproductive, dispersal units which are washed into the soil with rain, irrigation water, etc. Once in the soil, macroconidia germinate to produce a limited thallus, and then convert rapidly into chlamydospores prior to lysis of the thin-walled portions of the thallus (11, 12). The germination of the macroconidia and the conversion of the resultant thallus to chlamydospores in soil has been considered by Lockwood (10) to be initiated by the same stimuli responsible for soil fungistasis. We feel that this process is a part of fungistasis, in that the fungus is converting to a spore form that does not germinate in soil, and serves as a survival mechanism.

Previously (1, 2, 3, 8) and in the present study, chlamydospore formation by *F. solani* f. sp. *phaseoli* was promoted by the presence of different and distinct chlamydospore-inducing substances in soil. Hence, this form of soil fungistasis apparently is initiated by the presence of specific materials and is not due simply to the absence of carbon nutrition. In fact, in the presence of partly purified chlamydospore-inducing substances from soil, exogenous carbon nutrition has to be supplied for maximum chlamydospore production (Ford, *unpublished data*). Bourret (3) detected chlamydospore-inducing substances in 10 of 11 soils examined. Thus, like the widespread fungistasis described by Dobbs & Hinson (6), the presence of chlamydospore-inducing substances in soils appears to be widespread and may be a part of the same phenomenon.

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