Chlamydospore Formation in Fusarium in Sterile Salt Solutions

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ABSTRACT

Methods of inducing chlamydospore formation aseptically were developed for *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *melonis*, *F. solani* f. sp. *phaseoli*, *F. solani* f. sp. *pisi*, and *F. roseum* f. sp. *cerealis* 'Culmorum'. Chlamydospores were formed readily from washed, nongerminated macroconidia incubated on Nuclepore filters floated on 0.03 M Na_2SO_4, but not on water. Germlings, obtained by incubating macroconidia on filters placed on potato-dextrose agar, produced chlamydospores readily on acid-washed sterilized sand continually leached with water or 0.025 M phosphate buffer (pH 6.9). Fewer chlamydospores were formed without leaching. Germlings also produced large numbers of chlamydospores when incubated directly in 0.03 M Na_2SO_4; distilled water was slightly less effective. The results indicate that chlamydospores are produced when the environment is deficient in energy but contains appropriate mineral salts.

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Additional key words: lysis, nutrient deprivation.

Chlamydospore formation in *Fusarium* spp. has been induced by the presence of soil (11, 12) or certain bacteria (3, 14), by incubation in soil extracts (2, 4) or salt solutions (6, 8, 13) deficient or lacking in a carbon source, or by incubation in water (5). *Fusarium solani* f. sp. *phaseoli* is reported to be unique in rarely producing chlamydospores under cultural conditions which normally yield

chlamydospores in other Fusaria (2). Conditions favoring induction of chlamydospore formation in *F. solani* f. sp. *phaseoli* apparently are restricted to incubation in soil (11) or soil extracts (2, 4), or co-culture with certain bacteria (3). Such results have been interpreted as indicating the dependence of this fungus on specific chlamydospore-inducing factors of soil or microbial origin (2, 3, 4).

The purpose of the present work was to determine whether or not chlamydospore production comparable to that occurring in soil could be induced in *F. solani* f. sp. *phaseoli* and in other Fusaria in the absence of soil or soil microorganisms and their products. Conditions of nutrient stress were included since these are known to induce chlamydospore formation in some species of *Fusarium* (6, 8, 13) and to induce sclerotium formation from the mycelia of several fungi (1).

MATERIALS AND METHODS.–One isolate of each of the following fungi was used: *Fusarium oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) Snyd. & Hans., *F. oxysporum* Schlecht. f. sp. *melonis* (Leach & Currence) Snyd. & Hans., *F. roseum* (Lk. ex Fr.) emend. Snyd. & Hans. f. sp. *cerealis* (Cke.) Snyd. & Hans. 'Culmorum', and *F. solani* (Mart.) Appel & Wr. f. sp. *pisi* (F. R. Jones) Snyd. & Hans. Four isolates of *F. solani* (Mart.) Appel & Wr. f. sp. *phaseoli* (Burk.) Snyd. & Hans. were used. Three of these, designated as isolates 142, 150, and 151, were kindly supplied by W. C. Snyder and correspond to his isolates R-10-2, S_2d, and S_2e, respectively. Isolate 20 is an undesignated isolate obtained earlier from the same source. All fungi were maintained on potato-dextrose agar (PDA).

Macroconidia from agar slants were washed three times by centrifugation at 2 C and were used nongerminated or following germination on Nuclepore membrane filters (10 mm diam, 0.5 μ pore size) incubated on PDA for 24-36 hr. Membranes bearing macroconidia or germlings were incubated in petri dishes at 25 C in darkness (i) on the surface of 5 ml distilled water or various salt solutions, (ii) on acid-washed (H_2SO_4) silica sand saturated with distilled water or salt solutions, or (iii) on Conover loam soil. The soil has been previously described (10) and was used at 25% moisture content (moisture tension ca. 0.1 bars). In some experiments, germlings produced after 16- to 18-hr incubation directly in potato-dextrose broth (PDB) and washed by centrifugation were incubated in 1.5 ml water, salt solutions, or an extract of soil. The soil extract was prepared by shaking 500 g soil with 500 ml distilled water for 30 min, then filtering through Whatman No. 1 filter paper followed by sterilization by filtration (0.22-μ Millipore filters).

In other experiments, the sand was leached with water or salt solutions to remove nutrients from the fungi. The apparatus used was a modification of one previously used (9). It was composed of a separatory funnel equipped with a sealed-in dripping tip (Kontes Glass Co., Vineland, N.J.) 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 silica sand. The sand and all glass components were acid washed. The separatory funnel was filled with distilled water or other leaching solution, and the rate of flow adjusted to 7-8 ml/hr with the needle valve.

The chlamydospores were stained with rose bengal. Those on Nuclepore membranes were stained by placing membranes on rose bengal-saturated sand; excess stain was removed by transferring the membranes to wet paper towels. The air-dried membranes were made transparent by mounting them in clove oil on glass slides. Chlamydospore production from macroconidia was expressed as the percentage of macroconidia forming one or more chlamydospores. Macroconidia which produced a short germ tube terminating in a chlamydospore were also scored as having produced a chlamydospore. Chlamydospore production from germlings was expressed as the mean number formed per low-power microscope field (field diam = 0.87 mm). Data are based on observation of at least 100 spores in each of duplicate samples.

Aseptic conditions were maintained except where otherwise indicated.

RESULTS.–*Chlamydospore production from nongerminated macroconidia.*–In preliminary experiments, macroconidia of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* isolates 20 and 150 formed chlamydospores readily when incubated on Nuclepore membranes on acid-washed sand, though none formed on membranes floated on water. Therefore, chlamydospore formation from macroconidia floated on filter-sterilized aqueous extracts of sand (2:1, v/v and various autoclaved salt solutions was compared with that on moist sand

TABLE 1. Chlamydospore formation from macroconidia of *Fusarium solani* incubated on Nuclepore filters floated on salt solutions for 7 days[a]

Salt	Molarity	Chlamydospores formed by the indicated form species[b]		
		phaseoli (isolate 20)	*phaseoli* (isolate 150)	*pisi*
NaCl	0.01	18	20	43
Na_2SiO_3	0.01	12	23	56
Na_2CO_3	0.01	15	19	40
$NaHCO_3$	0.01	0	0	48
$CaCO_3$	0.01	45	20	41
Na_2SO_4	0.003	71	89	18
Na_2SO_4	0.01	74	87	22
Na_2SO_4	0.03	73	88	23
$MgSO_4$	0.01	0	11	8
$ZnSO_4$	0.001[c]	23	59	52
Natural soil control		78	80	85

[a] Filters were floated on 5 ml of the salt solutions, all adjusted to pH 7.0.

[b] Percentage of macroconidia forming one or more chlamydospores.

[c] 0.01 M concentration was toxic.

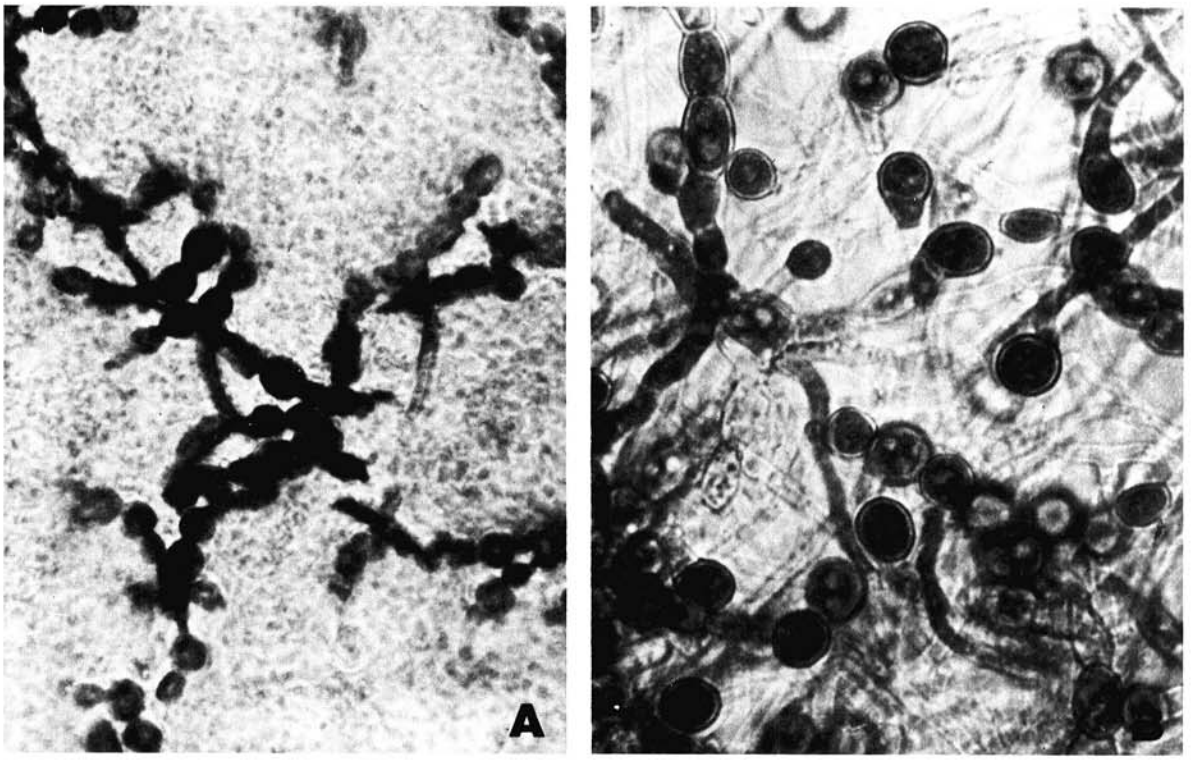

Fig. 1. **A)** Chlamydospore formation from macroconidia of *Fusarium solani* f. sp. *phaseoli* isolate 150 incubated on Nuclepore membrane filters on 0.03 M Na_2SO_4 for 7 days (ca. × 300). **B)** Chlamydospore formation from germlings of *F. solani* f. sp. *pisi* incubated directly in 0.03 M Na_2SO_4 for 3 days (ca. × 600).

and soil. On soil, 80% or more of the macroconidia of the three fungi formed chlamydospores in 7 days. On sand, more than 80% of the macroconidia of *F. solani* f. sp. *phaseoli* and 50% of those of *F. solani* f. sp. *pisi* formed chlamydospores in 7 days. In sand extract adjusted to pH 4.9, 6.0, and 7.0, chlamydospore production in both isolates of *F. solani* f. sp. *phaseoli* was ca. 80%, but in *F. solani* f. sp. *pisi* it was 10-20%. Sulfuric acid (0.03 M) adjusted to pH 4.7 gave results comparable to those in sand extract. At pH 6.0 and 7.0, chlamydospore production was 60-70% in *F. solani* f. sp. *phaseoli* and zero for *F. solani* f. sp. *pisi*. The mineral salts solution of Ford et al. (2) used at concentrations of 1X, 0.5X, 0.25X, and 0.1X gave poor production; maximum for *F. solani* f. sp. *phaseoli* was 14% and for *F. solani* f. sp. *pisi* was 24%. Fewer than 5% of the macroconidia of *F. solani* f. sp. *phaseoli* formed chlamydospores on Griffin's salt solution (7) at pH 5.7, and *F. solani* f. sp. *pisi* formed none. No chlamydospores of either fungus were produced in distilled water or in 0.025 M phosphate buffer at pH 7.0.

Residual sulfuric acid was implicated as a chlamydospore-inducing factor in the sand. Therefore, solutions of several single salts, all adjusted to pH 7.0, were evaluated for effectiveness in inducing chlamydospores of the same fungi. Of those used, only Na_2SO_4 at 0.003, 0.03, or 0.01 M resulted in chlamydospore numbers of *F. solani* f. sp. *phaseoli* equivalent to those formed on soil (Table 1; Fig. 1); NaCl, Na_2CO_3, $NaHCO_3$, $CaCO_3$, and Na_2SiO_3, each at 0.01 M, and $ZnSO_4$ at 0.001 M resulted in 41-56% formation of chlamydospores of *F. solani* f. sp. *pisi*, but none of these values was as high as the 85% formed on soil.

All eight fungi were tested for chlamydospore formation on Nuclepore membranes floated on 0.03 M Na_2SO_4, 0.01 M NaCl, and a mixture of the two (Fig. 2-A). In 7 days, chlamydospore production was 63% or more in conidia of all fungi, except for isolate 142 of *F. solani* f. sp. *phaseoli* in which production amounted to 38%. Sodium sulfate alone was generally less effective than the mixture, and NaCl resulted in least production.

A time course experiment was done using *F. solani* f. sp. *pisi*, *F. solani* f. sp. *phaseoli* isolates 20 and 150, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *melonis*, and *F. roseum* f. sp. *cerealis* Culmorum. Macroconidia on Nuclepore membranes were incubated on sand saturated with 0.03 M Na_2SO_4 under leaching and nonleaching conditions. Chlamydospore formation under nonleaching conditions was superior to that under leaching conditions for all fungi (Fig. 2-B). Figure 2-C gives time course curves for chlamydospore production in nonleaching conditions.

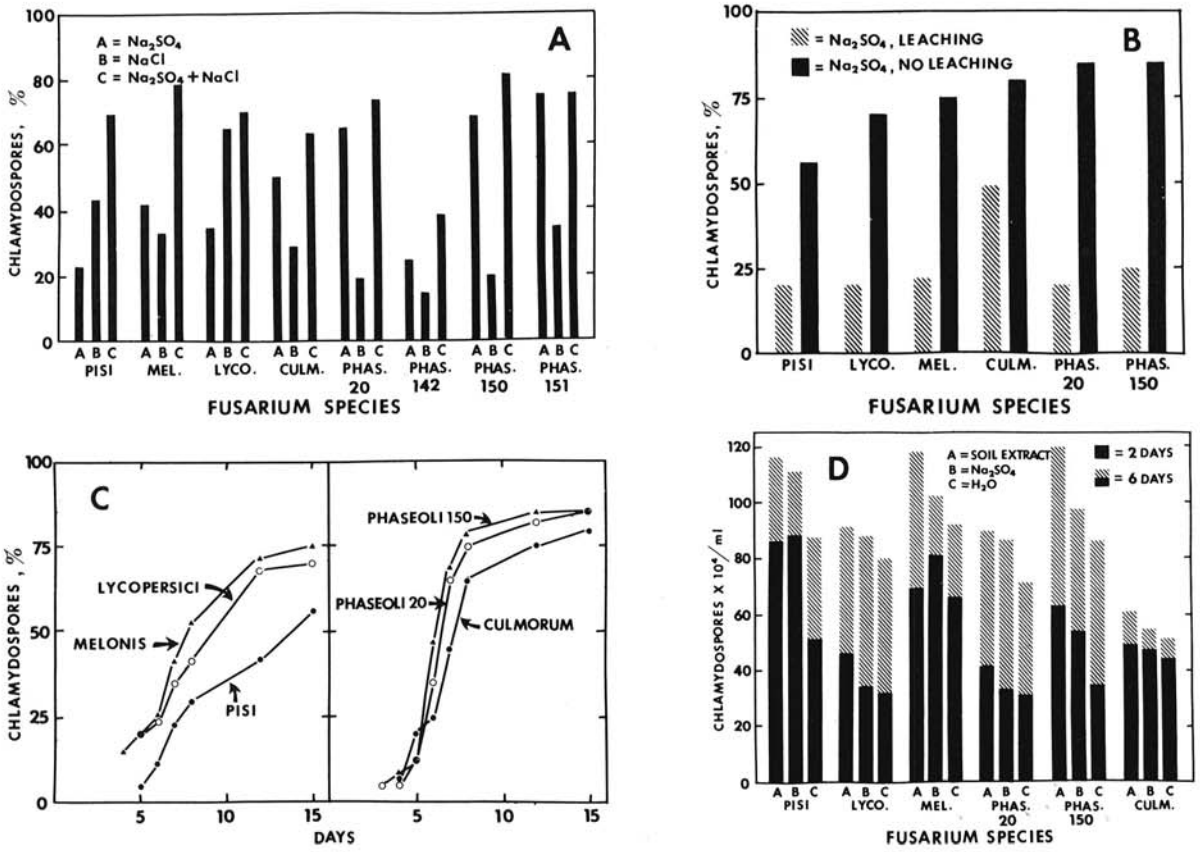

Fig. 2. Chlamydospore formation by *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *melonis*, *F. roseum* f. sp. *cerealis* 'Culmorum', *F. solani* f. sp. *pisi*, and *F. solani* f. sp. *phaseoli* isolates 20, 142, 150, and 151. **A)** Macroconidia were incubated 7 days on Nuclepore membranes floated on 0.01 M NaCl, 0.03 M Na_2SO_4, or a mixture of the two. **B)** Macroconidia were incubated for 15 days on Nuclepore filters on sand saturated with 0.03 M Na_2SO_4 under leaching and nonleaching conditions. **C)** Macroconidia were incubated on Nuclepore filters on sand saturated with 0.03 M Na_2SO_4. **D)** Germlings were incubated directly in 1.5 ml of 0.03 M Na_2SO_4, distilled water or an aqueous extract of natural soil.

Chlamydospores were first observed in 3-5 days. Their numbers increased rapidly until the 8th-12th day when the rate of formation decreased, except for *F. solani* f. sp. *pisi* which continued to increase until the experiment terminated at the 15th day. At least 70% of the macroconidia of all fungi had chlamydospores at the 15th day, except that for *F. solani* f. sp. *pisi* the value was 56%.

Chlamydospore formation from germlings.—Germlings of all eight fungi on Nuclepore filters were incubated for 11 days on sand saturated with phosphate buffer, pH 7.0, under leaching and nonleaching conditions, and on natural or autoclaved soil (Table 2). Large numbers of chlamydospores were formed by all species on natural soil, but few or none were formed on sterile soil. Incubation on buffer without leaching gave poor results, except for *F. roseum* f. sp. *cerealis* 'Culmorum' whose production on buffer equaled that on natural soil. However, leaching with phosphate buffer resulted in large numbers of chlamydospores, in most cases comparable to those formed on soil. In a separate experiment using germlings of *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *melonis*, and *F. solani* f. sp. *pisi*, leaching with water resulted in formation of large numbers of chlamydospores. In the leaching system, using either buffer or water, chlamydospore formation was accompanied by extensive hyphal lysis.

Germlings of *F. solani* f. sp. *pisi*, *F. solani* f. sp. *phaseoli* isolates 20 and 150, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *melonis*, and *F. roseum* f. sp. *cerealis* Culmorum were incubated directly in 0.03 M Na_2SO_4, soil extract, or distilled water. Counts of chlamydospores were made at 2 and 6 days (Fig. 2-D). Transformation of hyphae to chlamydospores was extremely rapid, with apparently mature chlamydospores present in high numbers in 2 days (Fig. 1). At 6 days, largest numbers were present in soil extract, but those in Na_2SO_4 were only slightly less. Large numbers of chlamydospores were also produced in water alone, their numbers lagging only slightly behind those in Na_2SO_4.

DISCUSSION.—Simple conditions were found which would induce chlamydospore formation in conidia and germlings of *Fusarium* spp. in amounts

generally comparable to those produced in soil or soil extracts. This was true for all the Fusaria used including *F. solani* f. sp. *phaseoli*, which was reported to require specific chlamydospore-inducing substances from soil (2).

Though our experiments with nongerminated macroconidia are not strictly comparable with those using germlings, conditions favoring chlamydospore production from the two types of propagules may differ. For example, more chlamydospores were formed from nongerminated macroconidia incubated on sand saturated with Na_2SO_4 under static conditions than under leaching conditions. Incubation in phosphate or water also gave poor results. By contrast, germlings formed chlamyspores more readily on buffer-saturated sand under leaching than under nonleaching conditions, and water alone was a favorable medium with or without leaching. The basis for these differences is not known. Chlamydospore formation from germlings under leaching conditions was associated with lysis of hyphae. Possibly, the beneficial effect of leaching was to induce partial hyphal autolysis and thus provide lytic products which were resynthesized into chlamydospores. Nutrient deprivation is known to induce autolysis in a number of fungi (10), and sclerotium formation at the expense of autolyzing mycelium has been shown in four fungal species incubated on soil or under artificial nutrient stress conditions (1). Formation of chlamydospores from germlings of *Fusarium* species incubated in carbon-deficient salt solutions has previously been reported (6, 8, 13).

Our findings may be related to those of Ford et al. (2) who obtained chlamydospores of *F. solani* f. sp. *phaseoli* from macroconidia incubated in fractionated extracts of soil, the anionic fraction in particular. They found evidence for clonal specificity of extracts from different soils and different fractions of the same soil extract (2), which was suggested to be due to a differential response to specific morphogenetic factors produced by soil bacteria (3). However, induction of chlamydospores by filtrates from bacterial cultures was less effective than the bacteria themselves (3). Chlamydospore formation in simple salt solutions in our research closely resembled that which occurred in soil and soil extracts, which suggests that the beneficial effects of soil extracts in inducing chlamydospore formation might be due to the presence of weak salt solutions in soil rather than to specific morphogens of microbial origin. The salt solutions used in the present work approximated the concentration range (0.08-0.20%) of salts occurring in soil solutions of fertile soils at field moisture capacity of 25% (9). The stimulatory effect of bacteria in co-culture with *F. solani* f. sp. *phaseoli* may be due to nutrient competition. An environment of low energy status is common to most of the conditions described which favor chlamydospore production (2, 5, 6, 8, 11, 13). Though Ford et al. (4) considered this factor as secondary in importance to specific morphogens, our results suggest that an environment deficient in energy but with an appropriate weak salt solution may be all that is required for chlamydospore formation in this as well as other Fusaria.

TABLE 2. Chlamydospore formation from germlings of *Fusarium* spp. incubated for 11 days on sand saturated with 0.05 M potassium phosphate buffer, pH 7.0; on sand leached with buffer; or on natural or autoclaved soil[a]

Fungus	No. of chlamydospores formed per microscope field[b]			
	Sand		Soil	
	Leached	Not leached	Natural	Sterile
F. oxysporum f. sp. *lycopersici*	182 ± 18[c]	0	192 ± 10	48 ± 17
F. oxysporum f. sp. *melonis*	102 ± 29	0	163 ± 14	0
F. roseum f. sp. *cerealis* 'Culmorum'	69 ± 9	70 ± 14	72 ± 6	0
F. solani f. sp. *phaseoli*, 20	67 ± 8	24 ± 5	182 ± 26	38 ± 6
F. solani f. sp. *phaseoli*, 142	154 ± 29	45 ± 7	68 ± 12	0
F. solani f. sp. *phaseoli*, 150	194 ± 17	0	186 ± 21	28 ± 13
F. solani f. sp. *phaseoli*, 151	178 ± 16	0	183 ± 28	0
F. solani f. sp. *pisi*	164 ± 23	38 ± 18	211 ± 11	0

[a] Germlings were produced by incubating conidia on Nuclepore membrane filters on potato-dextrose agar for 24-36 hr.
[b] Field diam = 0.87 mm.
[c] Standard error of the mean.

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