

Induction of Chlamydospore Formation in *Fusarium solani* by Abrupt Removal of the Organic Carbon Substrate

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Cooperative investigations, Plant Science Research Division, ARS, USDA, and the Experiment Stations of Idaho, Montana, Oregon, Utah, and Washington. Scientific Journal Paper No. 3776, College of Agriculture, Washington State University.

The authors thank Douglas Burke, Irrigated Agricultural Research and Extension Center, Washington State University, Prosser; Samuel Hess, Brigham Young University, Provo, Utah; and Shirley Smith, University of California, Berkeley; for providing the Washington, Idaho, and California clones, respectively, of *F. solani* f. sp. *phaseoli*. Photographs were taken by William Hawkins.

Accepted for publication 15 April 1972.

ABSTRACT

Sudden withdrawal of exogenous sucrose from the germlings of three clones each of *Fusarium solani*, *F. solani* f. sp. *phaseoli*, and *F. solani* f. sp. *pisi* in liquid culture resulted in rapid, synchronous chlamydospore formation. Eight of the nine clones formed chlamydospores within 24 hr; the ninth, a clone of *F. solani* f. sp. *phaseoli* from Washington, required 48 hr. Conversely, when sucrose utilization by the nine clones was gradual, and by the fungus itself, chlamydospores formed slowly, and took from 3 to 8 days, depending on the clone. Addition of 1% (w/v) of sterile or nonsterile Ritzville silt loam to the liquid cultures caused earlier and more chlamydospore formation under conditions of gradual sucrose depletion, and more under conditions of sudden

sucrose depletion. Adenosine 5' monophosphoric acid (AMP) or 3':5'-cyclic adenosine monophosphoric acid (cAMP) at 10^{-3} M also increased the rate and frequency of chlamydospore formation under conditions of gradual sucrose utilization.

We propose that depletion of organic carbon as the energy source is the major stimulus to chlamydospore formation in *Fusarium solani* in natural soil, where rapid growth of microorganisms on the nutrient-coated surface of the conidium causes its abrupt starvation. The possibility of interactions between AMP, cAMP, and energy depletion in chlamydospore formation is discussed.

Phytopathology 62:1148-1153.

The role of the chlamydospore in the life cycle of *Fusarium solani* (Mart.) Appel & Wr. and other *Fusarium* spp. is well established. Conidia are disseminated by splashing water from plant refuse on the soil surface, but they apparently are of little survival value in soil. As demonstrated by Park (23) for a saprophytic *F. solani*, and by Nash et al. (21) for *F. solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans., conidia in soil are converted into chlamydospores within a few days. Conidia in soil not converted to chlamydospores lyse.

The factors in soil responsible for the conversion of conidia to chlamydospores are less well known.

Depletion of exogenous organic carbon sources stimulates chlamydospore formation in pure cultures of *F. solani* 'Coeruleum', *F. roseum* (Lk.) emend. Snyd. & Hans. 'Sambucinum' (14, 15), and perhaps *F. solani* f. sp. *radicicola* (Wr.) Snyd. & Hans. as well (13). Similarly, in *F. oxysporum* (Schlecht.) emend. Snyd. & Hans., chlamydospores form abundantly within 7-10 days after mycelium in agar is suspended in sterile water (2), presumably because of depletion of available energy sources. *Fusarium solani* f. sp. *phaseoli*, on the other hand, rarely forms chlamydospores in pure culture, even in aging cultures (2), but does when grown in mixed culture

with soil bacteria (11, 25), in culture filtrates of *F. oxysporum* (9), or when placed as mycelia and conidia in contact with soil extracts (2, 10, 19). Chlamydospores also form when the fungus is grown in liquid media of low pH (6), incubated as large numbers of macrospores in water (6), or when agar colonies are leached (1). Thus far, however, there has been little direct evidence that exhaustion of organic carbon substrates per se causes chlamydospore formation in *F. solani*. Rather, substances of microbial origin or residual in soil are believed to be involved (12).

The *Fusarium* conidium presumably carries nutrients into soil as a slime coating originally from the sporodochium. This apparently supports conidial germination in soil since conidia washed free of this coating do not germinate unless supplied exogenous nitrogen and organic carbon (4, 5, 7). In soil, however, this nutrient coating is probably utilized rapidly by the other soil organisms, particularly by soil bacteria, with the result that conidial germings abruptly starve. Most research on chlamydospore formation in culture has allowed gradual nutrient starvation such as occurs in liquid or on agar culture with aging. There have been few attempts to duplicate in culture the abrupt nutrient deprivation of germings that probably occurs in nature. This paper reports the results of such experiments.

MATERIALS AND METHODS.—*Fungal cultures.*—Three clones each of *F. solani* saprophytes, *F. solani* f. sp. *phaseoli*, and *F. solani* f. sp. *pisi* were used. The saprophytic clones (A, B, and C) were isolated from Palouse silt loam near Pullman, Wash. The clones of *F. solani* f. sp. *phaseoli* were originally isolated from beans from California (S2d), Washington (W), and Idaho (I), respectively. Two clones of *F. solani* f. sp. *pisi* (W1 and W2) were isolated directly from eastern Washington pea-field soil (PSL) near Pullman. The third clone of *F. solani* f. sp. *pisi* (NZ) was originally obtained from New Zealand pea seed.

Isolations from soil were made using pentachloronitrobenzene (PCNB) selective medium (22). Single spore cultures were maintained on home-made potato-dextrose agar (PDA) slants at room temperature in diffuse daylight, and were subcultured monthly by single-sporing. Conidia used in the experiments were obtained from 2- to 3-week-old cultures.

The pathogenicity of the *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* isolates was confirmed by the nutrient-droplet technique (24), and by inoculation of pea or bean seedlings grown in autoclaved sand.

Soil sterilization.—A Ritzville silt loam (RSL) was adjusted to 37% water content, autoclaved at 121 C for 30 min, held at room temperature for 2 days, then reautoclaved for an additional 30 min. Other soil samples adjusted to 15% water content were exposed to 4.2 million rads gamma radiation from a cobalt 60 source. This dosage of gamma irradiation reportedly kills all microorganisms in soil (16) except possibly certain radiation-resistant bacteria (3, 8). No organisms were recovered when the sterilized soils were plated directly on PDA. In addition, a 1% glucose solution containing 1×10^3 conidia/ml of *F. solani* f.



Fig. 1. Chlamydospore formation following abrupt starvation in *Fusarium solani* f. sp. *phaseoli* clone S2d: (Above) at onset of starvation ($\times 470$); (Below) 5 days after onset ($\times 200$).

sp. *phaseoli* was added, as bacterial bait, to the sterilized soils, 1:1, w/w. The fungus was recovered in pure culture directly from the soil 1 week later. Because soil properties are changed less by irradiation than by autoclaving (16), most tests were with irradiation-sterilized soil.

Assessment of chlamyospore formation.—Chlamyospore formation was observed by direct microscopic observation of germlings and mycelium removed from the liquid growth medium. The time of onset of chlamyospore formation was arbitrarily set at that hour after start of the experiment when chlamyospores were first seen in samples of mycelial mats stained with 0.1% acid fuchsin in 85% lactic acid (Fig. 1). The relative speed of chlamyospore formation was defined as either synchronous (S) [i.e., most or all chlamyospores formed abruptly, rapidly, and simultaneously, much as occurs in germlings in soil (21)] or gradual (G) (i.e., slowly and with many stages of maturation visible in the culture at any given time).

RESULTS.—*Induction of chlamyospore formation.*—Both gradual and abrupt depletion of organic carbon were tested for their effects on chlamyospore formation. For gradual depletion, sucrose at 1%, w/v, and NaNO₃ at 0.3%, w/v, were added to 100 ml Czapek-Dox salts basal medium (BM) contained in nine 250-ml Erlenmeyer flasks for each clone to be tested. Three of the flasks received 1% (w/v) non-sterile soil as a source of microorganisms, three received 1% (w/v) sterile soil, and three no soil. Three additional flasks contained 100-ml BM plus sucrose at 0.25% and NaNO₃ at 0.075%, w/v; i.e., sucrose and

NaNO₃ at one-fourth the usual rate. Conidia of the test clones were introduced at 1×10^5 /ml final concentration and the flasks were then incubated on a rotary shaker (stroke 2.0 cm, 198 cycles/min) at room temperature. Conidia were added at 0, 12, or 24 hr after the start of the experiment to the three flasks of each treatment, respectively. Those flasks inoculated at zero time served for periodic inspections for chlamyospore formation; the flasks inoculated at 12 and 24 hr were saved for more accurate timing of chlamyospore formation. Thus, only those cultures inoculated at 0 hr were exposed to possible contamination due to repeated sampling. All treatments were repeated at least twice.

In general, each of the nine test clones was unique but consistent in the time required for chlamyospore formation with medium containing both high and low levels of sucrose and NaNO₃ (Table 1). In media containing the most commonly used concentrations of sucrose and NaNO₃ (1% and 0.3%, respectively), some clones began to form chlamyospores after only 63-64 hr; others required at least twice that much time. Use of sucrose and NaNO₃ at one-fourth the usual concentration shortened the time required for chlamyospore formation in all clones, but did not affect the relative speed of formation among the nine clones. The three saprophytic clones formed chlamyospores synchronously, within 3 days, whether supplied high or low sucrose and NaNO₃, and showed no clonal differences in relative speed of formation. In contrast, the six parasitic clones formed chlamyospores gradually over a 2- to 8-day period, depending on the

TABLE 1. Time (hours) of chlamyospore inception for *Fusarium solani* exposed to gradual depletion of nutrients in a complete medium (basal salts with sucrose and sodium nitrate) with and without natural or sterile soil amendments

Fungus clone	1% sucrose, 0.3% NaNO ₃	0.25% sucrose, 0.075% NaNO ₃	1% sucrose, 0.3 NaNO ₃ , 1% soil	1% sucrose, 0.3% NaNO ₃ , 1% irradiated soil ^a
<i>F. solani</i> A	64-68 S ^b	40-45 S	41-45 S	41 S
<i>F. solani</i> B	70 S	43-45 S	46-47 S	50-51 S
<i>F. solani</i> C	63-66 S	40-41 S	45-47 S	52-53 S
<i>F. solani</i> f. sp. <i>phaseoli</i> I	78-80 G ^c	52-55 G	54-56 S	54-56 S
<i>F. solani</i> f. sp. <i>phaseoli</i> S2d	168-188 G	120-125 G	63-68 S	80-88 G
<i>F. solani</i> f. sp. <i>phaseoli</i> W	120-125 G	96 G	75-80 S	100 G
<i>F. solani</i> f. sp. <i>pisi</i> W1	90-95 G	55 G	50-56 S	56 S
<i>F. solani</i> f. sp. <i>pisi</i> W2	74-76 G	44-48 G	56 S	62-63 G
<i>F. solani</i> f. sp. <i>pisi</i> NZF	120-125 G	78-82 G	52-58 S	73-75 S

^aOne clone each of *F. solani*, *F. solani* f. sp. *pisi*, and *F. solani* f. sp. *phaseoli* were also tested with autoclaved soil, and results were approximately the same as presented here for irradiated soil.

^bS = synchronous relative speed of chlamyospore formation; i.e., most or all chlamyospores formed rapidly and simultaneously.

^cG = gradual relative speed of chlamyospore formation; i.e., slowly and with many stages of maturation visible in the culture at any given time.

clone and amount of the sucrose and NaNO_3 in the medium. Clone S2d of *F. solani* f. sp. *phaseoli* was the slowest to form chlamydo spores.

Sterile and particularly nonsterile soil hastened the onset of chlamydo spore formation and considerably increased the number of chlamydo spores formed in all clones. Moreover, either treatment resulted in synchronous chlamydo spore formation in all clones except clones W and S2d of *F. solani* f. sp. *phaseoli* and clone W2 of *F. solani* f. sp. *pisi*; these three clones formed chlamydo spores synchronously when nonsterile soil was added, and gradually when sterile soil was added (Table 1).

For abrupt removal, macroconidia at a concentration of 1×10^6 /ml in 100 ml BM containing 1% sucrose and 0.3% NaNO_3 in 250-ml flasks were allowed to germinate for 15 hr with shaking; then the germlings were aseptically removed by membrane filtration and washed 3 times with sterile BM. The washed germlings were then resuspended at 1×10^5 germlings/ml in flasks containing 100 ml of either BM alone, BM with 1% sucrose, BM with 0.3% NaNO_3 , BM with both 1% sucrose and 0.3% NaNO_3 , or BM with 1% (w/v) nonsterile soil, and shaking was resumed. The growing germlings were thus abruptly removed from their complete media, then placed into media lacking sucrose, NaNO_3 , or both. Placement of washed germlings back into fresh BM with sucrose and NaNO_3 served as a control on the washing procedure, and addition of nonsterile soil to BM was a source of microorganisms. Each treatment was performed in duplicate, one flask for regular sampling, and the second for final assessments without the risk of contamination. All treatments were repeated at least twice.

Eight of the nine clones formed chlamydo spores synchronously, and within 24 hr when germlings were abruptly washed and resuspended in media lacking sucrose (Table 2). The ninth, *F. solani* f. sp. *phaseoli* W, required 48 hr and formed them gradually. The time of onset of chlamydo spore formation within a given clone was identical in all sucrose-deprivation treatments, whether or not NaNO_3 or soil was present. Chlamydo spore induction was extremely sparse and much delayed in BM containing sucrose, but not NaNO_3 . Those which did form possessed only a faintly staining strip of protoplasm lining the chlamydo spore wall. Germlings resuspended in BM containing NaNO_3 but not sucrose formed chlamydo spores synchronously and abruptly within 24 hr, like those resuspended in BM alone. Addition of nonsterile soil to BM lacking sucrose did not hasten the onset of chlamydo spore formation, but did result in more chlamydo spore formation in comparison to the other treatments.

During sustained growth in BM with sucrose and NaNO_3 , with or without soil, the pH increased from an initial value of 7.3 to 8.5 ± 0.2 at the time of chlamydo spore formation, regardless of the clone. In treatments involving abrupt sucrose removal, initial pH after washings was 7.3 and that at the time of chlamydo spore formation was 7.3 ± 0.2 for all clones. Chlamydo spore formation in all nine clones was accompanied by limited mycelial lysis. Mycelia and spores which did not lyse showed extensive vacuolization.

Tests with adenosine 5' monophosphoric acid (AMP) and 3':5' cyclic adenosine monophosphoric acid (cAMP).—In *Escherichia coli*, levels of the nucleotide, cAMP increase greatly in cells starved of

TABLE 2. Time (hours) of chlamydo spore inception for *Fusarium solani* following abrupt aseptic washing of germlings; i.e., germlings grown for 15 hr in complete medium (basal salts plus 1% sucrose and 0.3% sodium nitrate), then removed by membrane filtration, washed with basal salts solution, and resuspended in the salts solution without sucrose, sodium nitrate, or both; and with or without soil amendments

Fungus clone	Salts only	With 0.3% NaNO_3	With 1% soil	With 1% sucrose
<i>F. solani</i> A	10 S ^a	10 S	10 S	115-120
<i>F. solani</i> B	6-8 S	6-8 S	6-8 S	38-45
<i>F. solani</i> C	20-21 S	20-21 S	20-21 S	40-45
<i>F. solani</i> f. sp. <i>phaseoli</i> I	18 S	18 S	18 S	80-84
<i>F. solani</i> f. sp. <i>phaseoli</i> S2d	27-29 S	27-30 S	27-29 S	70-75
<i>F. solani</i> f. sp. <i>phaseoli</i> W	48 G ^b	48 G	48 S	140-144
<i>F. solani</i> f. sp. <i>pisi</i> W1	26-28 S	26-28 S	26-28 S	80-85
<i>F. solani</i> f. sp. <i>pisi</i> W2	19 S	19-20 S	20-21 S	75
<i>F. solani</i> f. sp. <i>pisi</i> NZF	20 S	20 S	20 S	65-70

^aS = synchronous relative speed of chlamydo spore formation; i.e., most or all chlamydo spores formed rapidly and simultaneously.

^bG = gradual relative speed of chlamydo spore formation; i.e., slowly and with many stages of maturation visible in the culture at any given time.

glucose (17). In *Dictyostelium discoideum*, cAMP is involved in the triggering mechanism for fruiting (17). The possibility exists that in *F. solani*, cAMP levels rise when carbon-based energy supplies drop, thereby triggering chlamydospore formation. The effect of cAMP and the related compound AMP (both from Sigma Chemical Co.) on chlamydospore formation was assessed in experiments with concurrent gradual depletion of sucrose. *Fusarium solani* f. sp. *psii* (NZ-1, a sporodochial isolate of NZ) was first introduced at 1×10^5 conidia/ml into complete media (BM with 1% sucrose and 0.3 NaNO₃) lacking either nucleotide, and germination was allowed to proceed for 15 hr. The germlings were then removed by membrane filtration and aseptically reintroduced, without washing, into the same medium, but containing one or the other nucleotide at 3×10^{-3} M, 1×10^{-3} M, 5×10^{-4} M, or 1×10^{-4} M. Germlings 15 hr old resuspended in complete medium lacking either nucleotide served as the control. We prepared nucleotide-supplemented medium by dissolving the nucleotides in distilled water, adjusting the pH to 7.3 with 1 N NaOH, and sterilizing the solutions by membrane filtration. Sterile basal salts, 1% sucrose, and 0.3% NaNO₃ were then added.

Chlamydospore formation occurred after 58-60 hr in media containing 3×10^{-3} M or 1×10^{-3} M AMP or cAMP; this was about 10 hr before chlamydospores started to form in lower concentrations of the nucleotides or in the controls. More strikingly, 5 to 10 times more chlamydospores were formed at these concentrations than in the controls or where lower concentrations of either nucleotide were used. The requirement of at least 1×10^{-3} M concentration may reflect poor penetration of the cell by these nucleotides.

DISCUSSION.—Sudden removal of sucrose led to rapid, abundant, and synchronous chlamydospore formation. Where the substrate was utilized gradually, and by the fungus itself, chlamydospore formation was slow and gradual, especially for the parasitic clones. With some clones, chlamydospore formation did not begin until 6, 7, or even 8 days after germination of the original conidia had occurred. In contrast, when sucrose was removed abruptly and immediately after conidial germination, germlings of eight of the nine clones formed chlamydospores synchronously and within 24 hr. The ninth, a clone of *F. solani* f. sp. *phaseoli* from Washington, formed chlamydospores gradually, but still within 48 hr, which was about one-third the time required with gradual substrate utilization.

The only treatment to induce chlamydospore formation that approached the effectiveness of the abrupt removal of sucrose was the addition of non-sterile soil to the complete medium. Possibly soil organisms introduced with the soil quickly utilized the sucrose and in so doing were the equivalent of an abrupt aseptic washing with basal medium. Depletion of carbon energy sources can also be the reason why mixed cultures of bacteria and *F. solani* (11, 25) caused increased chlamydospore formation by the fungus; through competition with the bacteria for the

substrate, the fungus may have been starved abruptly. Perhaps in soil as well, the utilization by bacteria of exogenous nutrients on the conidium is the stimulus which triggers abrupt, synchronous formation of chlamydospores so typical of the soil situation. Of all the methods reported to stimulate chlamydospore formation in *F. solani*, starvation as created in our study by abrupt removal of the exogenous organic carbon source is apparently the first to cause a rate of chlamydospore formation comparable to that which occurs in soil.

Lockwood (20) suggested that soil fungistasis is caused by lack of nutrients, especially at the immediate outside surface of the spore where other organisms keep the resting fungus in a more or less continual state of nutrient deprivation (Ko & Lockwood, 18). Spores which germinate in soil could also be prevented from germinating by slow, steady leaching with water (18). This was suggested (18) as somewhat comparable to the slow, steady erosive action of organisms operative near and on the spore in soil which thus maintain an extremely steep nutrient gradient from inside to outside the spore. Our suggestion that rapid depletion of organic-carbon energy supplies is a primary stimulus for chlamydospore formation is similar to that of Lockwood's for soil fungistasis. Similar mechanisms are involved: (i) The propagule before entry into soil has adequate nutrients for germination; (ii) subsequent rapid growth of soil organisms utilize nutrients of the propagule; and (iii) thereafter the maintenance of a near-continual state of nutrient deprivation causes death, dormancy, or resting spore formation.

Deprivation of energy substrates per se does not explain all aspects of chlamydospore formation in *F. solani*; it cannot explain why sterile soil extracts increase chlamydospore formation in *F. solani* f. sp. *phaseoli* as compared to sterile water alone (2, 10, 19), or why sterile culture filtrates of *F. oxysporum* stimulate chlamydospore formation in this fungus (9), or why in our own work, in all nine clones tested, additions of autoclaved or irradiated soil, apparently free of soil organisms, stimulated increased rates of chlamydospore formation. These observations strongly suggest that compounds, probably of biological origin, may act as stimulants to chlamydospore induction. Perhaps the compounds are nucleotides such as AMP or cAMP which are involved in gene regulation and morphogenesis (17), and which accumulate within cells starved of organic carbon sources, as shown for *E. coli* starved of glucose (17). In our experiments, AMP or cAMP both caused increased rate and especially frequency of chlamydospore formation that was concentration dependent. Some cells are known to excrete cAMP (17), which raises the possibility that microorganisms in proximity to *F. solani* germlings in soil do likewise.

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