Germinability, Viability, and Virulence of Chlamydospores of *Fusarium solani* f. sp. *phaseoli* as Affected by the Loss of Endogenous Carbon

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ABSTRACT

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Chlamydospores of *Fusarium solani* f. sp. *phaseoli* progressively lost their endogenous ^{14}C during incubation on membrane filters on soil, maintained at –1 kPa for 70 days. The endogenous ^{14}C loss from labeled chlamydospores was comprised of residual ^{14}C in soil and evolved $^{14}CO_2$. Residual ^{14}C in soil ranged from 0.2 to 9.6% of the total label. $^{14}CO_2$ evolution from the respiration of chlamydospores and soil microbes accounted for 53.4% of the total label. Soil microbial respiration of exudates of chlamydospores accounted for 15 to 38% of the total residual ^{14}C (2 to 5% of the total label). Chlamydospore respiration represented 55 to 78% of the total ^{14}C loss and was the major factor contributing to the loss of endogenous carbon. Chlamydospores were highly germinable (above 90%) in the absence of an external source of nutrients (Pfeffer's salts solution) when their incubation period on nonsterile soil was 10 days or less, but germinability began to decrease when their exposure to soil exceeded 10 days. Germinability of chlamydospores dropped to zero and virulence significantly declined after incubation on nonsterile soil for 70 days. The linear depletion of ^{14}C was closely associated with the decline in germinability and virulence of chlamydospores. These results suggest that endogenous nutrient reserves are of importance in establishing a pathogenic relationship between a host and a soil-inhabiting pathogen.

Additional keywords: exudation, fungistasis, nutrient stress.

The failure of nutrient-independent fungal propagules to germinate in soil may be due to the imposition of nutrient stress by soil microbes (1,18,22,23,24). Extended exposure of fungal propagules to soil causes an increase in their nutrient requirements for germination, leading to a progressive loss of propagule viability (5,12) and decreased virulence (2,8,15). Such changes were thought to be associated with the loss of endogenous carbon from propagules through enhanced exudation (2,10,11,38). Recently, respiration of propagules, in addition to exudation, has been established as a major route leading to the loss of endogenous carbon (15,16). Hyakumachi and Lockwood (15) observed a strong relationship between the loss of endogenous carbon and decreased germinability and virulence of sclerotia of *Sclerotium rolfsii* during 50 days of incubation on soil. Loss of pathogenic aggressiveness of conidia of *Bipolaris sorokiniana* is associated with the depletion of endogenous reserves (2). Thus, the foregoing results implicate the importance of endogenous carbon for maintaining the vigor of conidia and sclerotia in soil. However, the significance of the carbon content of *Fusarium* chlamydospores in relation to their germinability, survival, and virulence during exposure to soil has not been investigated, although this propagule is described as the principal means of survival of this fungus in soil (6,27,28,35).

Some portion of the population of chlamydospores of many *Fusarium* spp. survive in soil more than a year in the absence of plants or plant parts (6,27,35). However, Wensley and McKeen (40) observed a rapid decline in the population of *F. oxysporum* f. sp. *melonis* in field soil during the interval between two successive crops. Stover (39) observed that the survival of chlamydospores of *F. oxysporum* f. sp. *cubense* declined within 40 days in nonsterile soil maintained at 50 to 100% water saturation (wt/vol). However, Oritsejafor (31) observed high survival of chlamydospores of *F. oxysporum* f. sp. *elaeidis* in nonsterile soil at lower levels of moisture (15 to 25% moisture content). In one study, chlamydospores of *F. solani* f. sp. *phaseoli* failed to germinate in potato-dextrose broth (PDB) after they were incubated on soil at high matric potentials (0 to –5 kPa), while the same pathogen showed more than 80% germinability when exposed to dry soil (–20 to –30 kPa) for 1 year (M. Hyakumachi, *unpublished data*). Differences in experimental procedures or incubation conditions probably can account for these contrasting results. Many studies have been conducted concerning the survival of *Fusarium* chlamydospores in soil (6,27,31,35,39,40). However, the loss of endogenous reserves from chlamydospores during their incubation on soil has not yet been investigated. Such knowledge could be significant for controlling the pathogen biologically. Therefore, the present study was conducted to estimate the endogenous carbon loss via respiration and exudation from chlamydospores of *F. solani* f. sp. *phaseoli* during incubation on soil and to investigate associated changes in chlamydospore germination and virulence.

MATERIALS AND METHODS

Fungal isolate and chlamydospore production. An isolate of *F. solani* f. sp. *phaseoli* (S-3), originally isolated from infected bean from Hokkaido, was maintained on homemade potato-

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dextrose agar (PDA) slants in the laboratory at 25°C and was subcultured monthly by single sporing. Root extract of bean plants was used to produce chlamydospores (25). Roots of 10-day-old bean seedlings were washed in running tap water and homogenized (7,500 rpm) in a blender (Type H, Teraoka Toyo Keisokuki Co. Ltd., Osaka, Japan) for 5 to 10 min in distilled water (1:10, wt/vol). The homogenized roots were filtered through two layers of cheesecloth and the filtrates were centrifuged (6,917 × *g*) for 10 min. The clear supernatant solution was dispensed into 100-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. Macroconidia were cultivated on PDA and harvested by dislodging the conidia (20- to 25-days-old) with Pfeffer's salts solution (10 ml per petri dish) (5). Conidia were washed three times in Pfeffer's salts solution by centrifuging (1,710 × *g*) for 5 min. A 1-ml aliquot of spore suspension (10^6 conidia) was aseptically dispensed into 9 ml of root extract supplemented with 2.5 μCi of uniformly labeled [^{14}C]glucose (specific activity = 180 μCi μmol^{-1}) to produce ^{14}C-labeled chlamydospores. The inoculated root extract was incubated on a reciprocal shaker (150 rpm) at room temperature (approximately 25°C) for 3 to 4 weeks. Chlamydospores that formed in the root extract were separated from the hyphal fragments by sonication for 1 to 2 min at 4°C and washed three to four times in Pfeffer's salts solution by centrifuging (1,710 × *g*) for 5 min at 4°C. The density of chlamydospores was adjusted to 10^4 chlamydospores per 10 μl of suspension, and the chlamydospores were kept on ice to prevent germination. The initial specific radioactivity of ^{14}C-labeled chlamydospores was 0.98 ± 0.3 disintegrations per min (dpm) per chlamydospore.

Soil. A sandy loam soil (pH 6.9 and 5.0% organic matter) from a noncultivated area of the Agricultural Farm, Gifu University, was collected, air-dried, passed through a 2-mm mesh sieve, and stored at 4°C until use. Subsamples were allowed to equilibrate at 24 to 26°C for 7 days prior to use. The soil was sterilized by autoclaving (approximately 500 g of soil) at 121°C for 50 min on each of two successive days. Sterility of the autoclaved soil was checked by plating a portion on PDA.

Incubation of chlamydospores on soil. ^{14}C-labeled chlamydospores at a density of 10^4 chlamydospores per 10 μl of suspension were deposited on Nuclepore polycarbonate membrane filters (1 by 1 cm, 0.2-μm pore size; Nuclepore Corp., Pleasanton, CA) by mild suction. Four membranes containing chlamydospores were placed between two circular Nuclepore polycarbonate membrane filters (25-mm diameter, 0.2-μm pore size). The edges of the filters were sealed with sterilized silicone vacuum grease (Dow Corning Corp., Midland, MI) to exclude microorganisms. The membrane filter envelopes bearing chlamydospores were placed on the surface of approximately 5 g of sterilized or nonsterilized soil contained in sterilized plastic planchets (25-mm diameter by 8-mm deep). The moisture content of the soil in the planchets was adjusted to −1 kPa following a standard moisture characteristic curve prepared by using a tensiometer (13). Eight planchets were placed on the surface of 300 g of water-saturated sand in a glass container (9.5-cm diameter by 7.5-cm high). The container was tightly closed with a rubber stopper fitted with two glass tubes which allowed the passage of air for collecting the evolved $^{14}CO_2$, but the tubes were connected by latex tubing and a plastic connector to maintain a closed system during incubation. The containers with sand were autoclaved twice, for 40 min each, on successive days prior to use. The stoppers and all other equipment needed to set up the experiment were sterilized prior to use.

^{14}C loss from chlamydospores of *F. solani* f. sp. *phaseoli*. ^{14}C loss from chlamydospores was assessed by measuring the evolved $^{14}CO_2$ and residual ^{14}C in soil. The amount of $^{14}CO_2$ that evolved due to respiration of chlamydospores and soil microbes was assessed by incubating the ^{14}C-labeled chlamydospores on nonsterile soil in a closed container at 25°C for up to 70 days. Moist air was passed, once daily, through the container at 180 ml/min and $^{14}CO_2$ was collected in 10-ml aliquots of ethanolamine cocktail (11) for 10 min per vial. Five aliquots, for a total sampling time of 50 min, were taken from each container. The concentration of $^{14}CO_2$ in the first sample was higher than that in subsequent samples. However, the values of the five samples were added together to represent $^{14}CO_2$ evolution per treatment during a 50-min period. The amount of $^{14}CO_2$ in the samples was determined in a Packard Tri-Carb liquid scintillation analyzer, Model 2500TR (Packard Instrument Co., Meriden, CT), and expressed as the percentage of the total label. Data for the evolved $^{14}CO_2$ were corrected, as necessary, by using the formula given by Jasalavich et al. (17) to account for the planchet removed during sequential sampling.

Estimation of chlamydospore respiration after removal from soil. The respiration of chlamydospores was estimated following their removal after different times of incubation on soil (15,16). The membrane filter envelopes were removed from the containers, and the inner filters bearing chlamydospores were separated and incubated aseptically on 300 g of water-saturated sand in a clean container. Measurement of $^{14}CO_2$ was done as described before, daily for 10 days and then at 20, 30, 50, and 70 days. The contribution of chlamydospore respiration to the total ^{14}C loss was estimated by the equation: [(Proportion of $^{14}CO_2$ evolution from chlamydospores during incubation on nonsterile soil to total ^{14}C loss) × (Proportion of $^{14}CO_2$ loss from chlamydospores during incubation on empty planchets to $^{14}CO_2$ evolution)] × 100.

Determination of residual ^{14}C in soil. During incubation of chlamydospores on soil, propagule exudates (^{14}C) remained unused in soil or became incorporated into the cells of other microbes. This residual ^{14}C was assessed by removing the membrane filter envelopes from the planchets and oxidizing the soil. The soil in each planchet was mixed thoroughly before taking a 500-mg sample. The sample was burned in a Biological Oxidizer, Model OX200 (Hervey Instrumental Corp., Hillsdale, NJ), and the evolved $^{14}CO_2$ was collected in glass vials containing 10 ml of Carbo-sorb and Permafluor cocktail (1:1, vol/vol) (Packard Instrument Co.). The concentration of $^{14}CO_2$ was determined as above. Since a portion of the radioactive $^{14}CO_2$ may have been lost in the period of preparing and burning the samples, a correction factor was applied: Corrected total dpm = (Total dpm for each unknown sample) × (Correction factor).

The correction factor was the ratio of the concentration of ^{14}C (dpm) in 50 mg of ^{14}C mannitol to the concentration of ^{14}C recovered after burning the ^{14}C mannitol in the biological oxidizer. The concentration of ^{14}C (dpm) was counted using the liquid scintillation analyzer. The correction factor in our experiments was 1.1. Residual ^{14}C in the soil of eight planchets was combined to constitute one replicate. The experiment was conducted twice, and each experiment had two replicates per treatment.

Determination of residual ^{14}C metabolized by soil microbes. After 3, 20, 50, and 70 days of incubation, the membrane filter envelopes bearing chlamydospores were removed from the planchets and placed on the surface of nonsterile soil in new planchets. The original planchets (after removal of chlamydospores) were placed in a fresh glass chamber containing 300 g of water-saturated sand and closed with rubber stoppers as described in the previous section. The $^{14}CO_2$ that evolved from the soil after the removal of chlamydospores was collected every day for 7 days in ethanolamine cocktail (10 ml per vial). The concentration of evolved $^{14}CO_2$ was determined. Data were expressed as the percentage of the total residual ^{14}C in the soil at the time of the removal of chlamydospores. Each container constituted one replicate. The trial was conducted twice, and each trial was comprised of three replicates per treatment.

Chlamydospore germination assay. The ability of chlamydospores to germinate in the absence of a C-source (Pfeffer's salts

solution) or in PDB was determined after incubation on membrane filters on soil. The large Nuclepore filter envelopes were removed from the planchets and aseptically opened. The inner filters bearing chlamydospores were placed in 5 ml of 1% Pfeffer's salts solution or in 3 ml of PDB in sterilized petri dishes (90-mm diameter) and incubated for 12 to 48 h at 25°C. Whenever chlamydospores showed a decline in germinability, the incubation period was extended up to 48 h to maximize germination. One hundred chlamydospores were randomly counted for germination using a light microscope (40×) with epi-illumination (20). Chlamydospores with germ tubes at least as long as half their diameter were considered germinated. In addition, germination of chlamydospores after exposure to nonsterile or sterile soil was checked immediately after opening the membrane filter envelopes. Chlamydospores on three filters were stained in an aqueous solution of 10% (wt/wt) lactic acid, 0.05% (wt/wt) cotton blue, and 25% (wt/wt) glycerol (19), and germination was recorded microscopically. Each circular filter bearing chlamydospores constituted one replicate. The experiment was conducted three times, and each experiment had three replicates per treatment.

Virulence of chlamydospores. Seeds of kidney bean (*Phaseolus vulgaris* L.) cultivar Hatumidori Nigou, susceptible to bean root rot, were surface sterilized with 1% sodium hypochlorite for 10 min and rinsed three times with distilled water. Disinfected seeds were transferred to glass petri dishes (90-mm diameter) lined with two moistened Whatman No. 1 filter papers. Seeds were incubated in the dark at 25°C for 2 days.

A glass culture tube (180 by 20 mm) filled with 5 g of sandy loam soil was autoclaved at 121°C for 50 min on successive days. Eight or more membrane filter envelopes per treatment were removed from the planchets and the inner circular filters bearing chlamydospores were placed on a moistened Whatman No. 1 filter paper. The edges of the filter was held with a fine forceps and the filter surface was rubbed with a moistened camel's hair brush to deposit the chlamydospores in a glass vial containing 15 ml of sterilized distilled water. The concentration of chlamydospores was adjusted to 2×10^4 m^{-1} by counting the number in 10 µl of droplets under the microscope. Counting was repeated three times. Chlamydospores were pipetted (0.5 ml) into a culture tube (10^4 chlamydospores per tube) and mixed thoroughly with soil. Control treatments consisted of chlamydospores from cultures started at the same time as those exposed to soil, but they were allowed to remain in the root extract on a reciprocal shaker at 25°C for the treatment interval. The concentration of chlamydospores in the control treatment also was adjusted to 2×10^4 ml^{-1}. The moisture content of the soil in the culture tubes was adjusted to −1 kPa. Sprouted bean seeds were placed into the culture tubes (one seed per tube) and covered by a layer (approximately 1 cm) of dampened sterilized river sand. The tubes were incubated at 25°C in darkness for 3 days and then incubated in a growth chamber at 25°C. The plants received 12/12 h of a light/darkness cycle (cool fluorescent daylight tubes, 300 µE $m^{-2}s^{-1}$). The moisture content of the soil in the tubes was maintained at −1 kPa by adding water aseptically. After 15 days, disease severity was estimated using a 0 to 4 scale, in which 0 = healthy or no symptoms; 1 = slight red to brown streaking or necrosis on the taproot; 2 = moderate brown streaking and necrosis on the taproot or slight hypocotyl necrosis; 3 = severe root and hypocotyl necrosis; and 4 = very severe root and hypocotyl necrosis, death of seedlings, or preemergence damping-off. Disease index (DI) was calculated by:

$$DI = \frac{\text{Sum of all individual ratings}}{\text{Number of plants tested}}$$

Nine culture tubes constituted one replicate. The experiment was conducted three times, and each experiment consisted of three replicates per treatment.

Statistical procedures. Each experiment had a completely randomized design with two or three replicates per treatment. Percentage data were transformed by angular transformation to stabilize the variance (37). Data of repeated experiments were pooled for analysis, because variances were homogeneous (two-tailed F test, $P < 0.05$). The treatment means were separated by Fisher's least significant difference test (LSD, $P = 0.05$). Regression analysis on the means of repeated trials were performed, when appropriate.

RESULTS

Evolution of $^{14}CO_2$ from ^{14}C-labeled chlamydospores during incubation on soil. Evolution of $^{14}CO_2$ from chlamydospores of *F. solani* f. sp. *phaseoli* exposed to nonsterile soil was greatest after 6 days. A high rate of $^{14}CO_2$ evolution continued for 10 days, and then declined to a lower but steady rate with little fluc-

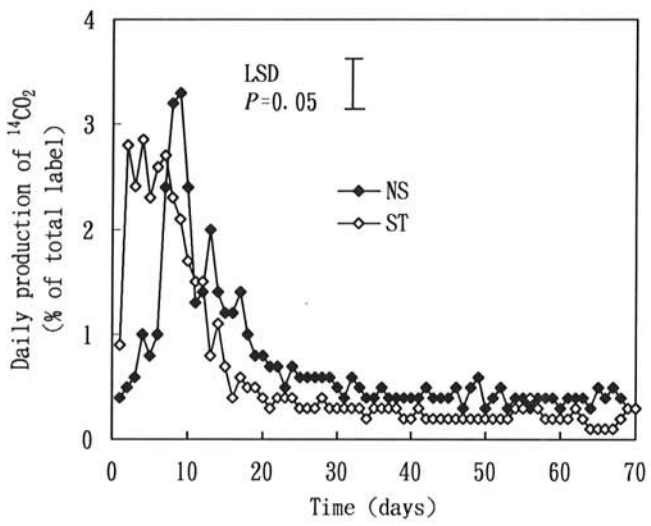

Fig. 1. Daily production of $^{14}CO_2$ from ^{14}C-labeled chlamydospores of *Fusarium solani* f. sp. *phaseoli* during incubation on membrane filters on nonsterile (NS) or sterile (ST) soil. Each point is the mean of two trials, each comprised of two replicates.

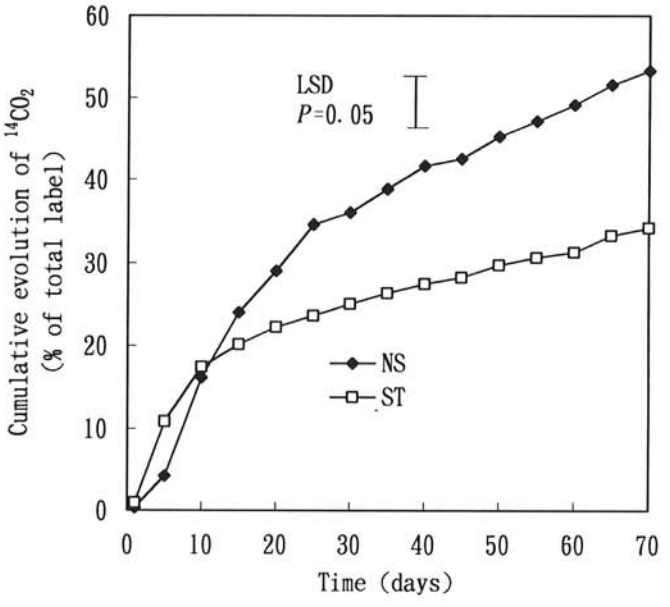

Fig. 2. Cumulative evolution of $^{14}CO_2$ from ^{14}C-labeled chlamydospores of *Fusarium solani* f. sp. *phaseoli*. Chlamydospores were incubated on membrane filters on nonsterile (NS) or sterile (ST) soil. Each point is the mean of two trials, each comprised of two replicates.

tuation until the experiment was terminated at 70 days (Fig. 1). $^{14}CO_2$ evolution reached a peak (3% of the total label) in nonsterile soil on day 9. During the first 6 days, the evolved $^{14}CO_2$ was significantly ($P = 0.05$) higher in sterile soil than in nonsterile soil. Daily evolution of $^{14}CO_2$ from chlamydospores exposed to sterile soil after 1 week was constantly at a lower rate than in nonsterile soil, although the statistical difference between the two soils became nonsignificant after 18 days.

Cumulative evolution of $^{14}CO_2$ in the initial 10 days was slightly higher in sterilized than in nonsterilized soil. Thereafter, the cumulative evolution of $^{14}CO_2$ in nonsterile soil exceeded that in sterile soil (Fig. 2). At 70 days, the total cumulative $^{14}CO_2$ evolved was 53.4% using nonsterile soil and 35% using sterile soil. Evolved $^{14}CO_2$ from nonsterilized soil was due to respiration of chlamydospores and soil microbes which metabolized propagule exudate. However, evolved $^{14}CO_2$ from sterilized soil was considered to be entirely due to respiration by *F. solani* f. sp. *phaseoli*, since soil microbes were killed by autoclaving the soil before starting the experiments. The sterility of the soil also was checked by plating a portion of sample on PDA at the end of each experiment. A few contaminants were present in some samples. However, the data from contaminated and noncontaminated replicates did not differ (data not shown).

Residual ^{14}C in soil. Accumulation of ^{14}C in nonsterile soil during the first 4 days was about half that for respired $^{14}CO_2$, after which the proportion due to ^{14}C remaining in the soil lagged behind $^{14}CO_2$ evolution (Table 1). By 70 days, the cumulative residual ^{14}C in the soil was 9.6% of the total label. Residual ^{14}C in sterile soil was less than 2% of the total label until 50 days; it had increased to about 4% at 70 days (data not shown).

Total loss of ^{14}C from chlamydospores during incubation on soil. The total loss of ^{14}C by chlamydospores was represented by the sum of $^{14}CO_2$ evolved and ^{14}C remaining in soil. In the first few days, the ^{14}C loss by chlamydospores was low, but thereafter the rate of loss accelerated until the end of the experiment (Table 1). At 70 days, the total loss of ^{14}C was estimated to be 63% of the total label from nonsterile soil and 39% from sterile soil. The evolution of $^{14}CO_2$ due to respiration by chlamydospores and soil microbes contributed the major portion of the total ^{14}C loss, which ranged from 60 to 93%. Chlamydospore respiration contributed 70 to 96% of the total $^{14}CO_2$ production and 55 to 78% of the total ^{14}C loss during incubation on nonsterile soil.

Metabolism of residual ^{14}C by soil microbes. The respiration of soil microbes which metabolized chlamydospore exudates was estimated by the removal of chlamydospores from soil (Fig. 3). Metabolism of residual ^{14}C by soil microbes was significantly ($P = 0.05$) higher when chlamydospores were removed at 20 days (38% of the total residual label) than after 3 (15%), 50 (26.9%), or 70 (22.8%) days. Soil microbes metabolized 2 to 5% of the total ^{14}C label from chlamydospores during the 70-day incubation period.

Germinability and virulence of chlamydospores. Germination of chlamydospores of *F. solani* f. sp. *phaseoli* in nonsterile soil in the first 20 days was 4 to 12%, and the corresponding value on sterile soil was 37 to 60% (Fig. 4). Chlamydospores were highly germinable (above 90%) in Pfeffer's salts solution, i.e., in the absence of a C-source when their incubation period on nonsterile soil was 10 days or less (Table 2), but germination was reduced after longer incubation periods. Germinability of chlamydospores in PDB significantly declined over the period of incubation on nonsterile soil. After 70 days of incubation on nonsterile soil, the fungus lost its ability to germinate in PDB, whereas germinability persisted after incubation on sterile soil (47%) or in the root extract (94%). ^{14}C loss was significantly negatively correlated with the germinability of chlamydospores in Pfeffer's salts solution ($r = -0.98$, $P = 0.01$) and in PDB ($r = -0.94$, $P = 0.01$) (Fig. 5). The slope of the regression of germinability versus ^{14}C loss was significantly different from zero ($P = 0.01$). Chlamydospores incubated on nonsterile soil became progressively less virulent as the period of incubation increased (Table 3). However, infectivity to bean seedlings did not decline after incubation on sterile soil or in the root extract. Bean root rot indices transformed by arcsine transformation and regressed against ^{14}C loss from chlamydospores yielded a slope significantly different from zero ($P = 0.01$). The correlation between ^{14}C loss and bean root rot indices was significant ($r = 0.97$, $P = 0.01$) (Fig. 6).

DISCUSSION

Inhibition or low germinability of propagules of soilborne pathogens in soil is well known (5,12,14,17,23), although the mechanism of fungistasis is still controversial (7,19,20,23,32,36). The results of the present study confirmed that chlamydospores of *F. solani* f. sp. *phaseoli* are subject to soil fungistasis. They also demonstrated that, although the chlamydospores were able to germinate in vitro in the absence of an external source of nutrients, their germinability began to decrease after 10 days incubation on nonsterile soil maintained at –1 kPa (45% moisture content). Longevity of chlamydospores in this system dropped from

TABLE 1. ^{14}C loss from ^{14}C-labeled chlamydospores of *Fusarium solani.* f. sp. *phaseoli* during incubation on membrane filters on nonsterile soil, the proportion of the total ^{14}C loss due to evolved $^{14}CO_2$, the relative contribution of chlamydospore respiration to the total $^{14}CO_2$ production, and the total ^{14}C loss[a]

Incubation time (days)	Cumulative ^{14}C loss (percentage of the total label)			Percentage of total ^{14}C loss due to evolved $^{14}CO_2$	Contribution of chlamydospore respiration[b] to	
	Evolved $^{14}CO_2$	Residual ^{14}C in soil	Total ^{14}C loss		Total $^{14}CO_2$ production (%)	Total ^{14}C loss (%)
1	0.3	0.2	0.5	60.0	90.8	54.5
2	0.9	0.5	1.4	64.2	95.5	61.3
3	1.5	0.7	2.2	68.1	80.0	54.5
4	2.8	1.0	3.8	73.6	90.0	66.2
5	4.2	1.3	5.5	76.3	80.4	61.3
6	5.3	1.7	7.0	75.7	85.0	64.3
7	7.1	1.3	8.4	84.6	79.2	66.9
10	16.7	1.3	18.0	92.7	84.2	78.0
20	29.0	2.2	31.2	92.9	81.2	75.4
30	36.0	5.2	41.2	87.3	78.9	68.8
50	45.2	8.5	53.4	84.1	69.6	58.8
70	53.4	9.6	63.0	84.7	80.0	67.7
LSD $P = 0.05$	5.7	2.0	6.4	...	...	...

[a] Data are means of two trials, each with two replicates.

[b] Contribution of chlamydospore respiration to the total ^{14}C loss was calculated based on the daily production of $^{14}CO_2$ following the removal of membrane filter envelopes from the soil. The filters bearing chlamydospores were incubated in sterilized empty planchets in a clean container to estimate their respiration (see the text for full explanation).

98 to 0% within 70 days. Wensley and McKeen (40) observed a rapid decline of *F. oxysporum* f. sp. *melonis* in field soil. However, Nash and Alexander (27) showed that chlamydospores of *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *cucurbitae* survived more than 1 year in natural, noncultivated field soil. There were two possible explanations for the decline in germinability of chlamydospores in our study.

First, chlamydospores were stressed by the activity of soil microbes when incubated at a high matric potential (–1 kPa). Chlamydospores showed a rapid decline in survival during incubation on nonsterile soil, but not in sterile soil. Stover (39) also observed shorter survival of chlamydospores of *F. oxysporum* f. sp. *cubense* in water-saturated (50 to 75% moisture content) nonsterile soil than in sterile soil; and conidia of *Cochilobolus sativus* (30), sclerotia of *S. rolfsii* (3), oospores of *Pythium aphanidermatum* (26), and chlamydospores of *F. oxysporum* f. sp. *elaeidis* (31)

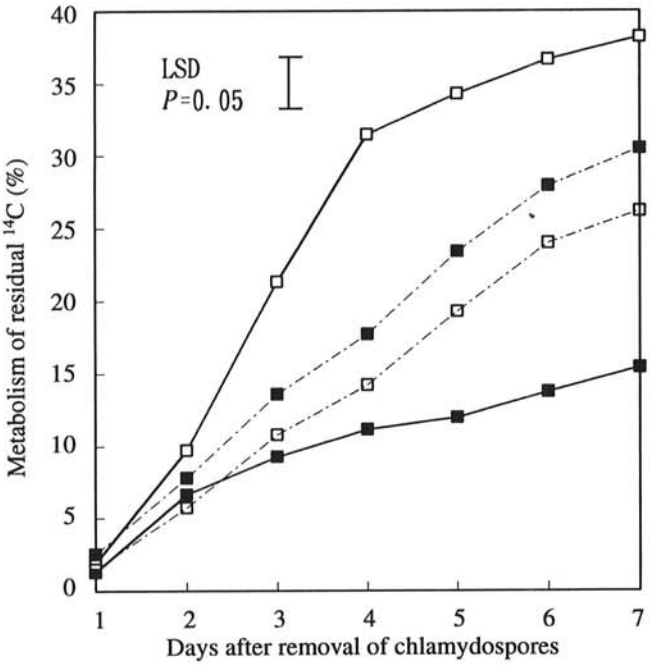

Fig. 3. Microbial metabolism of ^{14}C in soil following the removal of ^{14}C-labeled chlamydospores of *Fusarium solani* f. sp. *phaseoli* from nonsterile soil after 3 days (solid line with solid ■ symbol), 20 days (solid line with open □ symbol), 50 days (dotted line with solid ■ symbol), and 70 days (dotted line with open □ symbol) of incubation. Values are expressed as the percentage of the total residual ^{14}C in nonsterile soil at the time that chlamydospores were removed. Each point is the mean of two trials, each with two replicates.

TABLE 2. Germination of chlamydospores of *Fusarium solani* f. sp. *phaseoli* in Pfeffer's salts solution or in potato-dextrose broth (PDB) after incubation on nonsterile soil (NS), sterile soil (ST), or in an extract of bean roots (RE)

Incubation time (days)	Germinability in Pfeffer's salts solution			Germination in PDB		
	Incubation of chlamydospores[a] on/in			Incubation of chlamydospores on/in		
	NS	ST	RE	NS	ST	RE
1	92	ND[b]	95	96	ND	100
2	98	ND	90	98	ND	98
3	94	ND	100	95	ND	93
4	96	92	93	97	98	100
5	90	82	95	94	89	96
6	89	74	98	93	91	98
7	90	85	98	94	92	99
10	82	86	96	92	90	98
20	36	68	90	88	85	100
30	26	70	95	43	74	95
50	10	60	90	22	85	90
70	0	47	94	0	81	94
LSD $P = 0.05$	14	7	…[c]	14	…	…

[a] Chlamydospores were incubated in an extract of bean roots on a reciprocal shaker (25°C) corresponding to the time of their incubation on nonsterile or sterile soil. Data are means of three trials, each with three replicates.
[b] ND = not determined.
[c] … = not significant.

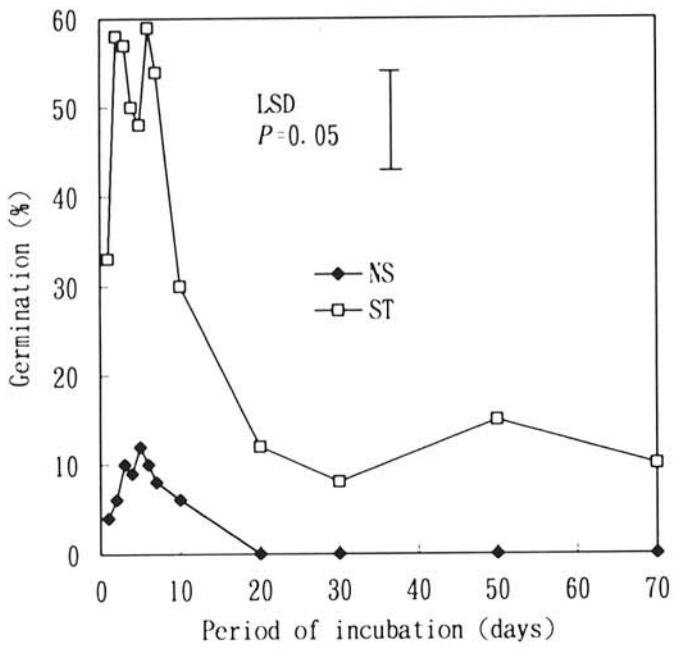

Fig. 4. Germination of chlamydospores of *Fusarium solani* f. sp. *phaseoli* exposed to nonsterile or sterile soil on a membrane filter. Filters bearing spores were stained in an aqueous solution of 10% (wt/wt) lactic acid, 0.05% (wt/wt) cotton blue, and 25% (wt/wt) glycerol immediately after removal from soil, and germinated spores were counted microscopically. Each point is the mean of two trials, each with three replicates.

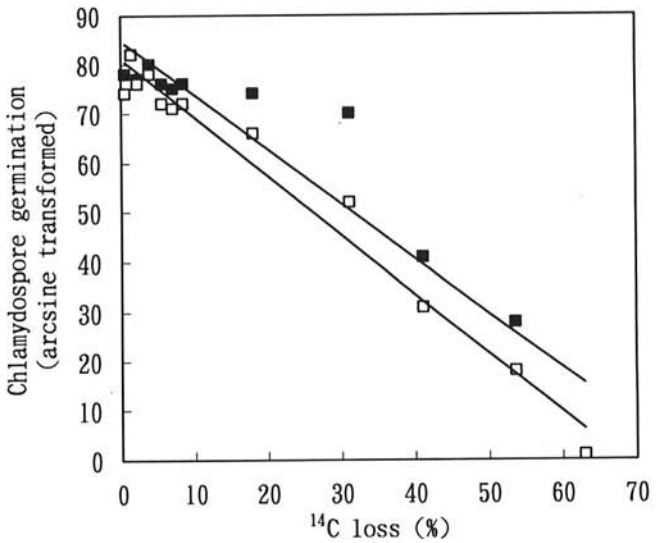

Fig. 5. The relationship between ^{14}C loss from ^{14}C-labeled chlamydospores of *Fusarium solani* f. sp. *phaseoli* and their ability to germinate in potato-dextrose broth (PDB) (solid ■ symbol) and in Pfeffer's salts solution (open □ symbol). Chlamydospores of different ^{14}C content were prepared by incubating them on nonsterile soil for various periods. Data from two trials were combined in the figure and analyzed by linear regression. There was a negative linear relationship between the ^{14}C loss from chlamydospores (x) and germinability of chlamydospores transformed by arcsine transformation (y): $y = 84.79 - 1.12x$, $r = -0.94$ for PDB; and $y = 81.1 - 1.18x$, $r = -0.98$ for the salts solution. The coefficients of correlation were significant ($P = 0.01$).

also had short survival times in water-saturated natural soil. The chlamydospores in our study lost endogenous carbon rapidly in nonsterile soil maintained at –1 kPa at 25°C. Other workers noted that conidia of *C. victoriae* lost 12% of the total ^{14}C label during incubation on nonsterile soil for 1 (11) or 5 (9) days. Loss of endogenous carbon was related to reduced germinability of fungal propagules. Death of sclerotia of *S. rolfsii* is noted when ^{14}C loss accounts for 48% of the total label (15). In our study, germinability of chlamydospores declined to zero when their endogenous carbon loss reached 63% of the total label.

Second, chlamydospores in our work were formed in root extract, prepared by sonication, and washed with Pfeffer's salts solution. This procedure might have decreased their survival in soil by promoting endogenous carbon loss. In other work, sclerotia of *S. rolfsii* produced in artificial media are more germinable and more readily killed by NaOCl (21) than those grown on natural substrates in soil (4). Couteaudier and Alabouvette (6) found little difference in the survival in soil of inocula of *F. oxysporum* f. sp. *lini* formed in nutrient rich culture and soil systems. In our own studies, chlamydospores of *F. solani* f. sp. *phaseoli* produced in root extract declined in germinability within 50 days after incubation on saturated soil (0 kPa), whereas chlamydospores obtained from the same source but incubated on soil at lower matric potentials (–20 to –30 kPa) survived about 1 year (S. Mondal and M. Hyakumachi, *unpublished data*). This suggested that soil moisture status may be more important than the method of chlamydospore production in their survival in soil.

Chlamydospores of *F. solani* f. sp. *phaseoli* in our study acquired an increased dependence on nutrients for germination after 20 days of incubation on soil. At this time, the viability of chlamydospores in PDB remained unchanged, implying that the nutrient reserves of the propagules were becoming exhausted. These results corroborate those of other researchers (5,12,15), who showed that conidia of *C. victoriae* and *C. sativus* and sclerotia of *Macrophomina phaseolina* and *S. rolfsii* incubated on soil or leached sand acquired an increased dependence on nutrients for germination; they also lost germinability on PDB, but at a slower rate than the loss of nutrient independence.

Virulence of chlamydospores was drastically reduced following incubation on nonsterile soil, and this was associated with large losses of ^{14}C from labeled chlamydospores (63% of the total label). Similar observations were made for sclerotia of *S. rolfsii* (15) and for conidia of *B. sorokiniana* (2). However, chlamydospores were not reduced in virulence after 70 days of incubation on sterile soil or in the root extract of bean plants. The propagules in sterile soil germinated and formed new chlamydospores during the period of incubation, thus allowing the fungus to maintain its inoculum potential. We expected no infection of bean seedlings by chlamydospores after 70 days of incubation on soil, because they failed to germinate in PDB. However, they were able to cause a low level of disease. Seed and/or root exudates must have stimulated germination of a few chlamydospores in the soil and, thereby, caused the infection of the bean seedlings (29,34). Lack of germination in PDB may not be an adequate criterion of viability.

Rapid evolution of $^{14}CO_2$ from chlamydospores in nonsterile soil occurred after 5 days of exposure and continued for up to 16 to 21 days, and then declined to a lower rate. Rapid loss of endogenous carbon as $^{14}CO_2$ in the initial days might be related to the increased metabolic events prior to germination in nonsterile soil, although chlamydospores germinated poorly in nonsterile soil. It was previously thought that early loss of ^{14}C from fungal propagules during incubation on soil or in a model system simulating diffusive stress in soil was due to exudation (11) and the role of propagule respiration was ignored (2,10,38). Hyakumachi and Lockwood (15) earlier demonstrated that respiration is the major route by which sclerotia of *S. rolfsii* loses endogenous carbon in soil. In the present study, chlamydospore respiration also was found as the major contributory factor to loss of endogenous carbon (55 to 78% of the total ^{14}C lost), and exudation accounted for a relatively small proportion of ^{14}C lost.

Loss of endogenous carbon from chlamydospores of *F. solani* f. sp. *phaseoli* during incubation on nonsterile soil does not necessarily indicate that the population will decrease, since many chlamydospores germinate in the rhizospheres of nonsusceptible plants or in crop residues, form new chlamydospores, and thereby renew the population (33). However, our results suggested that the loss of endogenous reserves from chlamydospores in soil might result in their debilitation. Factors that enhance endogenous carbon loss from propagules could have special significance

TABLE 3. Disease indices of bean seedlings inoculated with chlamydospores of *Fusarium solani* f. sp. *phaseoli* after incubation on nonsterile or sterile soil, or in an extract of bean roots

Incubation time (days)	Disease index[a]		
	Chlamydospores incubated on/in		
	Nonsterile soil	Sterile soil	Root extract
1	3.8[b]	3.8	3.9
2	3.6	3.6	3.9
3	3.6	3.7	3.9
4	3.6	3.7	3.8
5	3.5	3.6	3.8
6	3.6	3.6	3.8
7	3.4	3.7	3.6
10	3.1	3.3	3.8
20	2.6	3.3	3.5
30	2.5	3.2	3.6
50	1.4	3.1	3.7
70	0.7	2.6	3.6
LSD $P = 0.05$	0.6	...[c]	...

[a] Disease index was calculated on a 0 to 4 scale, in which 0 = healthy or disease-free seedlings and 4 = very severe root rotting or preemergence damping-off of bean (see the text for full explanation).

[b] Data are means of three trials. Each trial had three replicates, consisting of nine culture tubes.

[c] ... = not significant.

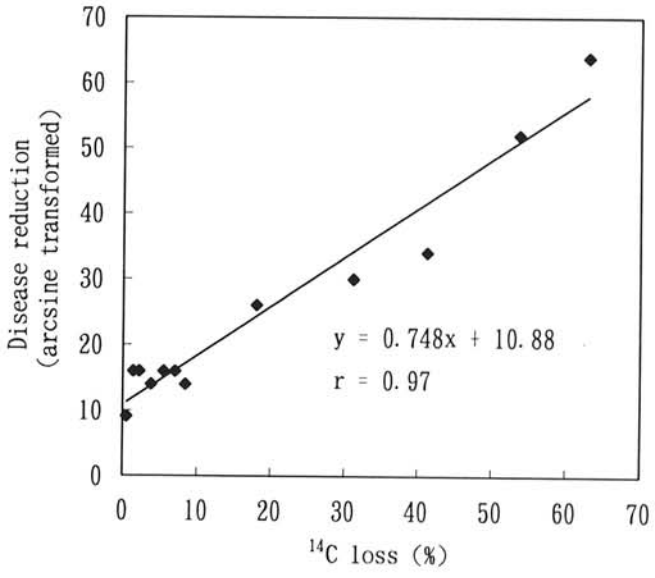

Fig. 6. The relationship between ^{14}C loss from ^{14}C-labeled chlamydospores and the reduction of bean root disease (◆) caused by chlamydospores of *Fusarium solani* f. sp. *phaseoli* after incubation on membrane filters on nonsterile soil. Chlamydospores of different ^{14}C content were obtained by incubation on nonsterile soil for various periods. Data from two experiments were combined in the figure and analyzed by linear regression. There was a positive linear relationship between the values for ^{14}C loss from chlamydospores (x) and reduction of bean root disease (y) transformed by the arcsine transformation. The coefficient of correlation was significant ($P = 0.01$).

for controlling soilborne plant pathogens biologically. In this regard, the influence of fluctuations of soil moisture, temperature, or pH seem worthy of special attention, since microbial cycles are influenced by such fluctuations in the soil (9).

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