

Relation of Carbon Loss from Sclerotia of *Sclerotium rolfsii* During Incubation in Soil to Decreased Germinability and Pathogenic Aggressiveness

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ABSTRACT

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Endogenous carbon from ^{14}C -labeled sclerotia of *Sclerotium rolfsii* was progressively lost during 50 days of incubation in soil at $\psi_m = -10$ mbar. The ^{14}C loss was mainly accounted for as residual ^{14}C in the soil (4.1–9.3% of the total label) and as $^{14}\text{CO}_2$ evolved from the soil (26.0–38.4% of the total label). $^{14}\text{CO}_2$ accounted for 45.9–88.4% of the total ^{14}C loss, and sclerotial respiration was estimated to account for 42.2–77.2% of

the ^{14}C loss during the same incubation time. Sclerotia incubated in soil became dependent on nutrients for germination and lost viability when the ^{14}C loss reached approximately 20 and 40%, respectively. Radish seedlings inoculated with sclerotia that had lost more than 20% of their ^{14}C had significantly longer shoots than seedlings inoculated with nonincubated sclerotia.

A potentially important aspect of the survival of fungal propagules in soil, which has not been investigated in any detail, is the effect of microbial nutrient (energy) stress on propagule vigor. The more rapidly propagule reserves are depleted by exudation and respiration during incubation in soil, the greater their debilitation would be expected to be.

The stress imposed by the activity of soil microorganisms has been shown to result in accelerated nutrient deprivation, which may lead to decreased germinability (1,4,8,10,13), attenuated virulence (2,5), and death (4). The debilitation of fungal propagules has been attributed mainly to the increased exudation of endogenous carbon compounds from the propagules, and the possible contribution of the respiration of the propagules to these losses has been ignored. Recently, we found that propagule respiration contributed more to energy loss from conidia of *Cochliobolus sativus* and *Sclerotium rolfsii* Sacc. than was previously assumed (11).

It is well known that the sclerotia of *S. rolfsii* can lose large amounts of nutrients in exudates, particularly following drying and rewetting, and that such losses can lead to their colonization and decay by soil microorganisms (18). In this research, we investigated the relationship between energy loss from sclerotia of *S. rolfsii* and their germinability and pathogenic aggressiveness during long-term incubation in soil under conditions that precluded their being colonized by other microorganisms. The relative contribution of sclerotial respiration to the energy loss was also estimated.

MATERIALS AND METHODS

Preparation of sclerotia. Sclerotia of *S. rolfsii* (ATCC accession number 44902), obtained from G. C. Adams, were produced on potato-dextrose agar (PDA) supplemented with 10 μCi of ^{14}C -glucose (specific activity 180 $\mu\text{Ci}/\mu\text{mol}$). The cultures were maintained at 23 ± 1 C under diffuse laboratory light. Sclerotia were harvested from 10- to 12-wk-old cultures with sterilized forceps and left to air-dry in sterile petri dishes for at least 1 day before use.

Soil. Capac loam (pH 5.4 and 5.3% organic matter) (12) was collected from a field on the Michigan State University farm, passed through a 2-mm-mesh sieve, and stored at 4 C until use.

The soil was sterilized, as required, by autoclaving for 40 min on each of two successive days.

Incubation of sclerotia in soil. Ten ^{14}C -labeled sclerotia of *S. rolfsii* were deposited on a 7- \times 7-mm piece of autoclavable adhesive tape (Professional Tape Co., Inc., Burr Ridge, IL) and placed inside each of eight Nuclepore filter envelopes (Nuclepore Corp., Pleasanton, CA) (11). Each envelope was buried in approximately 4 g of nonsterile or autoclaved soil at $\psi_m = -10$ mbar in a stainless steel planchet 2.4 cm in diameter. The eight planchets were placed on 300 g of water-saturated sand in a glass container (9.5 cm in diameter and 7.5 cm high). The container was tightly closed with a no. 14 rubber stopper fitted with two glass tubes, which allowed the passage of air. The sclerotia were not colonized by soil microorganisms, as was determined by incubating them in potato-dextrose broth (PDB) in germination tests conducted later.

Assessment of ^{14}C loss from sclerotia of *S. rolfsii* incubated in soil. To determine the amount of $^{14}\text{CO}_2$ derived from the respiration of sclerotia and soil microbes, the closed containers were incubated at 23 ± 1 C. Once daily, for 50 days, moist air was passed through each container at 200 $\text{ml}\cdot\text{min}^{-1}$ for 30 min, during which three samples of $^{14}\text{CO}_2$ were collected in 10 ml of ethanolamine cocktail for 5–10 min each (11). The concentrations of $^{14}\text{CO}_2$ in the samples were expressed as percentages of the total label, and the mean values were plotted (see Fig. 1, below). At longer intervals between 1 and 50 days (see Table 1, below), the residual ^{14}C in the soil was determined by oxidizing the soil in acid dichromate and measuring the radioactivity of the $^{14}\text{CO}_2$ evolved (6). Methanol (0.5 ml) was added to the samples to prevent phase separation, and the radioactivity was measured in a scintillation spectrometer (Packard Tri-Carb Liquid Scintillation Analyzer, model 1500, Packard Instrument Co., Downers Grove, IL). Data for the $^{14}\text{CO}_2$ evolved and for the residual ^{14}C were corrected as necessary to account for planchets removed during sequential sampling. A randomized complete block design was used, in which there were four or eight glass containers per experiment. One or two containers constituted a replicate; i.e., there were four replicates per treatment. Means and standard deviations of the measured data were calculated (see Table 1, below).

The contribution of sclerotia and soil microorganisms to $^{14}\text{CO}_2$ evolution was estimated by determining the amounts of $^{14}\text{CO}_2$ evolved from sclerotia and from soil separately following the removal of the sclerotia, which had been incubated in the soil

for different times (11). Tape pieces containing 10 sclerotia each were removed from each of four glass containers and placed in planchets on 300 g of water-saturated sand in four clean containers. Measurements of $^{14}\text{CO}_2$ were made daily for 10 days. These data showed that sclerotial respiration accounted for the following percentages of the $^{14}\text{CO}_2$ evolved: 91.6% (after 3 days of incubation), 84.0% (8 days), 76.8% (14 days), and 72.0% (26 days). The remainder was derived from the microbial metabolism of sclerotial exudates. These values were used to estimate the proportion of ^{14}C loss due to sclerotial respiration, on the assumption that 91.6% of the total $^{14}\text{CO}_2$ came from the sclerotia during 1–6 days of incubation, 84.0% during 7–13 days, 76.8% during 14–25 days, and 72.0% during 26–50 days.

The rate of utilization of ^{14}C in soil was measured after the removal of sclerotia from the soil. After 3, 14, 26, and 50 days of incubation, two tape pieces, each with 10 sclerotia, were removed from the soil in planchets in each replicate container. The planchets and the soil in them were transferred to fresh containers, as above, and the $^{14}\text{CO}_2$ evolved from the soil was measured daily over 4 days. The data were expressed as percentages of the residual ^{14}C in the soil at the time of the removal of the fungal propagules. Regression analysis was applied to the data.

Assays of sclerotial germination. To determine sclerotial germination, sclerotia that had been incubated in the soil were incubated in 0.3 ml of 1% Pfeffer's salts solution (4) or in PDB in a sterilized planchet (2.4 cm in diameter and 0.6 cm high) for 48 hr. The tape pieces bearing these sclerotia were cut in half, so that five sclerotia from each of four replicate incubation containers were counted per medium. Sclerotial germination was assessed visually after 48 hr by examination at 100 \times with a stereomicroscope. Fresh sclerotia always germinated at a rate of 95–100% in PDB. The relationship between carbon loss from sclerotia and their germinability in the salt solution and in PDB was determined by regression analysis of angular transformations of the data.

Assays of inoculum aggressiveness. Water-saturated sand (300 g) was placed in a glass container (9.5 cm in diameter and 7.5 cm high), and 40–60 glass tubes (0.5 cm in diameter and 3 cm high) were set vertically about 1 cm into the sand. One radish seed and one sclerotium were placed in each glass tube. Sclerotia of different carbon statuses were selected from among those that had been incubated in the soil for different lengths of time (see Table 1, below) and had been tested for ^{14}C loss. The control treatments used sclerotia from cultures started at the same time as those providing the stressed sclerotia and left on PDA for an additional period of time corresponding to the stress period. Sclerotia were removed from these cultures and air-dried for 1 or more days. After 7 days of incubation at 25 C, various amounts of seedling decay, including death, had occurred, accompanied by stunting of growth. The lengths of radish shoots were measured and expressed as percentages of the length of healthy (uninoculated) controls. The mean value of data from five sclerotia from the same tape piece was calculated, and the mean values were plotted (see Fig. 4, below). The relation between percent carbon loss and percent shoot length of radish was determined by regression analysis of logarithmic transformations of the data. The experiment was repeated several times with similar results.

RESULTS

Evolution of $^{14}\text{CO}_2$ from sclerotia of *S. rolfsii* incubated in soil. $^{14}\text{CO}_2$ evolution from the soil increased rapidly during the first 3 days and then declined sharply until about the ninth day, when a steady, low rate was maintained in both sterilized and natural soil (Fig. 1A). The peaks of maximum $^{14}\text{CO}_2$ evolution in sterilized and natural soil after 3 days of incubation represented 2.5 and 1.8% of the total label, respectively. After 6 days of incubation, the daily evolution of $^{14}\text{CO}_2$ was constantly higher in natural soil than in sterilized soil. The cumulative evolution of $^{14}\text{CO}_2$ in natural soil was approximately equal to or was slightly

lower than that in sterilized soil for the first 16 days of incubation, after which the evolution of $^{14}\text{CO}_2$ from natural soil exceeded that from sterilized soil (Fig. 1B).

Evaluation of total ^{14}C losses from sclerotia of *S. rolfsii*. As the amount of dissolved $^{14}\text{CO}_2$ in the soil was found to be negligible (11), the amount of ^{14}C lost from the labeled sclerotia was taken as the sum of the $^{14}\text{CO}_2$ evolved and the residual ^{14}C in the soil. The evolution of $^{14}\text{CO}_2$ and the amount of residual ^{14}C in the soil were determined at intervals during 50 days of incubation in two experiments.

The amount of residual ^{14}C in natural soil remained at a low and nearly constant level (1.7–2.9% of the total label) during incubation in both experiments, except on the 50th day, when

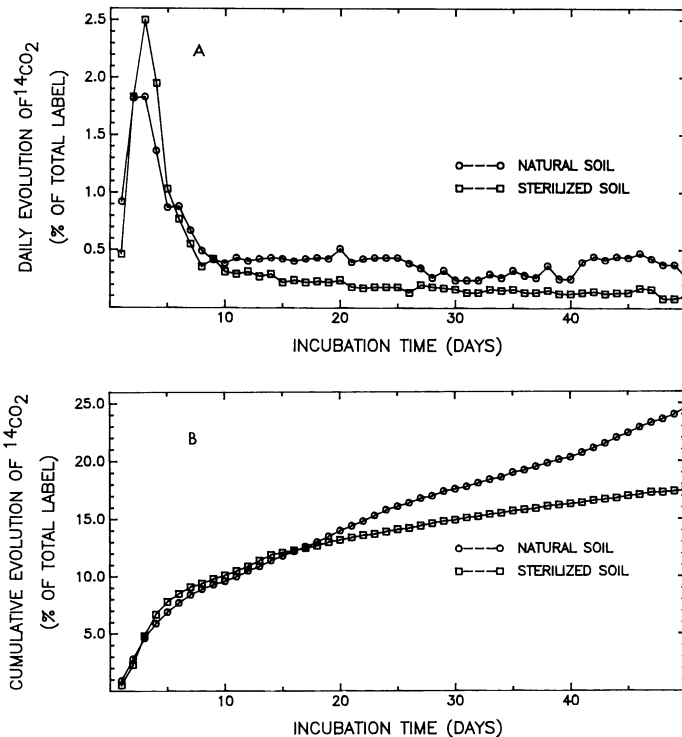


Fig. 1. Evolution of $^{14}\text{CO}_2$ from ^{14}C -labeled sclerotia of *Sclerotium rolfsii* incubated in natural or sterilized soil. Ten sclerotia were used per determination. A, Daily values. B, Cumulative values.

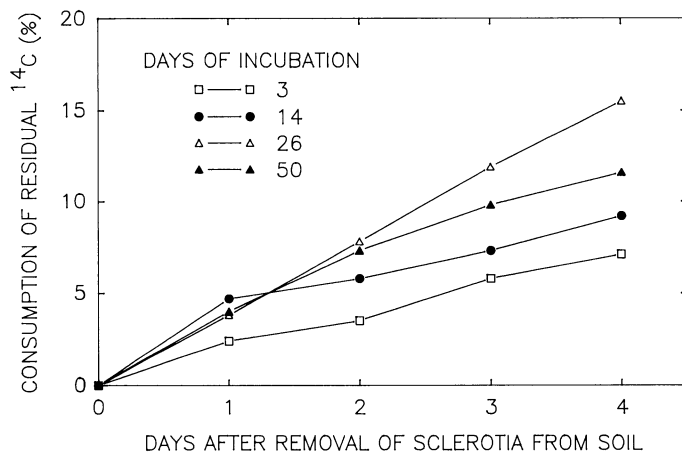


Fig. 2. Consumption of residual ^{14}C in soil during 4 days, following the removal of labeled sclerotia after 3, 14, 26, and 50 days of exposure to the soil. The values for the consumption of ^{14}C are expressed as percentages of the residual ^{14}C in the soil at the time the sclerotia were removed. The respective regression coefficients are $y = 0.60 + 1.64x$, $y = 3.0 + 1.50x$, $y = -0.05 + 3.92x$, and $y = 1.85 + 2.53x$. The correlation coefficients are 0.99 or 1.0.

it increased to 4.1 and 9.3% (Table 1). In sterilized soil, the amount of residual ^{14}C on the 50th day was 1.9% in one experiment and 2.6% in the other. The large increase in the amount of residual ^{14}C in natural soil on the 50th day was associated with drastically reduced sclerotial germinability (Table 1).

The cumulative amount of $^{14}\text{CO}_2$ evolved from sclerotia and natural soil combined increased from 1.7% after 1 day of incubation to 38.4% after 50 days in the first experiment (Table 1). The ^{14}C evolved from sclerotia incubated in sterilized soil was 20.4% after 50 days. The amount of total ^{14}C lost as $^{14}\text{CO}_2$ in natural soil was 45.9% after 1 day and more than 80% after 50 days. The proportion of the total ^{14}C loss ascribed to sclerotial respiration increased from 42.2 to 76.1% during the 50 days of incubation. In a second experiment done at different time intervals, these general trends were reproduced (Table 1). It is evident from these results that the portion of $^{14}\text{CO}_2$ emanating from sclerotia was greater than that derived from soil microorganisms utilizing sclerotial exudate, except on the first day of incubation.

These results show that the cumulative total ^{14}C loss increased with the time of incubation. For example, 10–12% was lost in 7–14 days, approximately 15% in 22 days, and 30–48% in 50 days (Table 1). The total loss of ^{14}C from sterilized soil was 19–23%.

Residual ^{14}C was metabolized slowly by the soil microflora after the removal of the sclerotia. For example, the cumulative evolution of $^{14}\text{CO}_2$ from soil in which sclerotia had been incubated for 3, 14, or 50 days represented only 2.4–4.0, 3.5–7.3, 5.8–9.8, and 7.1–11.6% of the residual ^{14}C 1, 2, 3, and 4 days, respectively, after the removal of the sclerotia (Fig. 2). The slopes of the lines in Figure 2 for soil with these incubation times do not significantly differ ($P < 0.05$) from each other. Somewhat larger losses were recorded from soil in which sclerotia had been incubated for 26 days; a maximum of 15.5% of the residual ^{14}C was evolved as $^{14}\text{CO}_2$ from this soil after the removal of the sclerotia. The slope of the line in Figure 2 for this treatment differs significantly ($P < 0.05$) from those for soil in which sclerotia had been incubated for 3 and 14 days.

Relation of ^{14}C loss from sclerotia to the germinability of sclerotia and to pathogenic aggressiveness. Sclerotia of *S. rolfsii* initially germinated in the absence of a carbon source but became nutrient-dependent when about 20% of the ^{14}C was lost (Fig. 3A). Sclerotia lost germinability when about 40% of the ^{14}C was

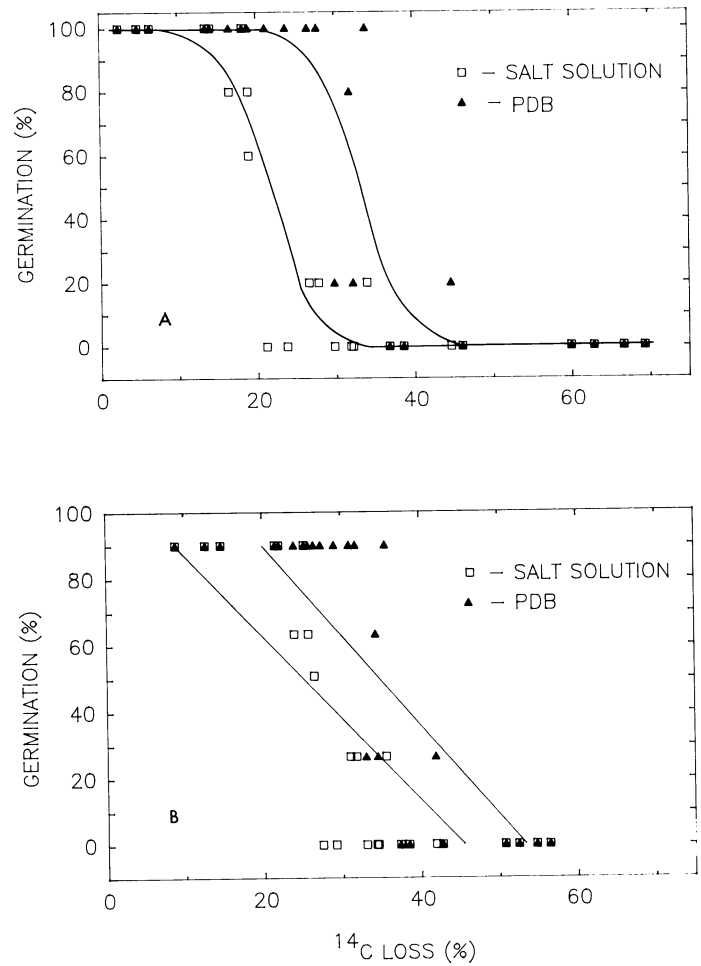


Fig. 3. Relationship of ^{14}C loss from labeled sclerotia of *Sclerotium rolfsii* to their ability to germinate in a salt solution and potato-dextrose broth (PDB). Sclerotia of differing ^{14}C contents were obtained by incubating them in soil for various periods of time. A, Arithmetic plot. B, Plot with angular transformation of data ($y = 111.8 - 2.46x$ for the salt solution, and $y = 143.9 - 2.70x$ for PDB).

TABLE 1. ^{14}C loss from sclerotia of *Sclerotium rolfsii* during incubation in soil, the calculated proportion of ^{14}C loss attributable to total $^{14}\text{CO}_2$ evolved and to $^{14}\text{CO}_2$ derived from sclerotial respiration, and germinability of the stressed sclerotia

Incubation time (days)	^{14}C loss (percentage of total label)			$^{14}\text{CO}_2$ evolved		Germinability of sclerotia ^c (%)	
	$^{14}\text{CO}_2$ evolved ^a	Residual ^{14}C in soil ^a	Total ^{14}C loss	Percentage of total ^{14}C loss	Percentage of total $^{14}\text{CO}_2$ loss ascribed to sclerotial respiration ^b		
Experiment 1							
Natural soil	1	1.7 ± 0.1	2.0 ± 0.8	3.7	45.9	42.2	100
	2	3.4 ± 0.1	1.7 ± 0.7	5.1	66.7	61.0	100
	3	4.7 ± 0.1	2.9 ± 0.6	7.6	61.8	56.7	100
	8	9.1 ± 0.2	2.5 ± 0.6	11.6	78.4	70.9	100
	14	10.0 ± 0.6	1.8 ± 0.4	11.8	84.7	76.1	100
	50	38.4 ± 2.0	9.3 ± 1.6	47.7	80.5	63.6	0
Sterilized soil	50	20.4 ± 1.6	2.6 ± 0.7	23.0	88.7	—	100
Experiment 2							
Natural soil	7	7.3 ± 0.1	2.3 ± 0.9	9.6	76.0	71.9	100
	22	13.0 ± 1.3	1.7 ± 0.3	14.7	88.4	77.2	100
	26	16.4 ± 1.5	2.3 ± 0.4	18.7	87.7	74.6	90
	50	26.0 ± 1.7	4.1 ± 1.1	30.1	86.4	65.1	10
Sterilized soil	50	17.5 ± 2.4	1.9 ± 0.3	19.4	90.2	—	100

^a Means plus or minus standard deviations of measured data.

^b The contribution of sclerotial respiration to the total $^{14}\text{CO}_2$ loss was estimated to be as follows: 91.6% during 1–6 days of incubation, based on data for the third day; 84.0% during 7–13 days, based on data for the eighth day; 76.8% during 14–25 days, based on data for the 14th day; and 72.0% for 26–50 days, based on data for the 26th day.

^c As determined in potato-dextrose broth.

lost. Regression analysis of angular transformations of the data gave straight lines, with correlation coefficients of 0.80 and 0.82, for germination in Pfeffer's solution and PDB, respectively (Fig. 3B). The positions of the lines in Figure 3 for these media differ significantly, but their slopes do not ($P < 0.01$).

The greater the ^{14}C loss from sclerotia was, the more the radish shoot lengths increased (Fig. 4A), i.e., the less pathogenically aggressive the sclerotia were. The decline in aggressiveness commenced when the ^{14}C loss was about 20% and then proceeded rapidly until the sclerotia had lost about 40% of the ^{14}C . Logarithmic transformation of radish shoot lengths indicated a linear relationship, with a high and significant correlation coefficient ($r = 0.88$; $P < 0.01$) between shoot length and ^{14}C loss (Fig. 4B).

DISCUSSION

Sclerotia of *S. rolf sii* are able to germinate independently of an external source of nutrients (17), but prolonged exposure to nutrient stress may cause them to become dependent on nutrients for germination (16). Loss of germinability of sclerotia of other fungi during incubation on soil also has been reported (8). Our results also show that sclerotia of *S. rolf sii* were able to germinate without exogenous nutrients until they lost about 20% of their endogenous nutrients, and this ability rapidly declined as more was lost. They were unable to germinate when about 40% of the ^{14}C was lost. The onset of the decline in pathogenic aggressiveness of sclerotia coincided with the onset of the decline in nutrient independence, i.e., when about 20% of the ^{14}C was

lost. Filonow and Lockwood (8) and Arora et al (2) observed that conidia of *Cochliobolus victoriae* and *C. sativus* incubated on leached sand or on soils acquired an increased dependence on nutrients for germination and a lengthened germination time on PDA, but viability was not decreased. However, O'Leary and Lockwood (15) recently reported that the decline in infectivity of conidia of *C. sativus* incubated in soil lagged more closely behind the loss in nutrient independence than it did in most previous studies. This tendency was also seen in sclerotia of *S. rolf sii* in our research.

Total $^{14}\text{CO}_2$ evolution during the incubation of sclerotia in soil increased rapidly during the first 3 days, then declined rapidly, and leveled off after 9 days. The reason for the initial peak of $^{14}\text{CO}_2$ evolution is not certain, but it could be related to increased metabolic activity preparatory to the germination of sclerotia (though the sclerotia did not germinate in natural soil) or to microbial metabolism of exudates from the sclerotia. An early burst of exudation has been reported in previous experiments in which other fungal propagules were incubated on soil or in a model system simulating diffusive stress in soil (2,7,11).

It was assumed in previous work that the respiration of pathogen propagules in soil is negligible and "exudation" could be taken as the sum of the residual ^{14}C in the soil and the $^{14}\text{CO}_2$ evolved (from microbial respiration of exudates) with little error. However, it seems more likely that the early $^{14}\text{CO}_2$ peak is due primarily to propagule respiration, on the basis of the following evidence: 1) the amounts of residual ^{14}C in soil were low and quite constant (1.7–2.9% of the total label) during incubation, except on the 50th day (4.1 or 9.3%), when the apparent mortality of sclerotia was high; 2) the rate of metabolism of residual ^{14}C following the removal of the sclerotia from soil was consistently low (e.g., when the sclerotia removed after 3 days of incubation, only about 2% of the residual ^{14}C per day was recovered as $^{14}\text{CO}_2$ during 4 days); and 3) the contributions of sclerotial respiration to the total $^{14}\text{CO}_2$ production and the total ^{14}C loss were relatively high (45.9–88.4% and 42.2–77.2%, respectively).

Our finding that the initial peak of $^{14}\text{CO}_2$ evolution in soil is more likely due to the respiration of pathogen propagules than to the respiration of propagule exudates by other soil microbes suggests that similar studies should be made with the leached sand model system (4,7). This system has been demonstrated to simulate many aspects of fungal behavior induced by energy stress in soil (2,4,5,8,10,13), including an apparent increase in exudation upon initial incubation of fungal propagules in it (4,6,7). Possibly, the peak of exudation might include a significant component of the $^{14}\text{CO}_2$ dissolved in the large volume of leaching solution.

The exudation losses from the sclerotia in our study may have been greater than those that would occur under more natural conditions. For example, sclerotia of *S. rolf sii* produced in artificial media were more germinable (3) and more readily killed by NaOCl (14) than those grown on natural substrates incubated on soil. Moreover, allowing sclerotia of *S. rolf sii* to air-dry, as we did, can result in increased exudation (9,18).

Our results indicate that the respiration of fungal propagules may contribute more to ^{14}C loss from propagules in soil than was previously assumed. Conditions that enhance fungal respiration by which endogenous carbon compounds from fungal propagules are exhausted, as well as factors increasing exudation, merit further study as a means of biological control of certain phytopathogenic fungi.

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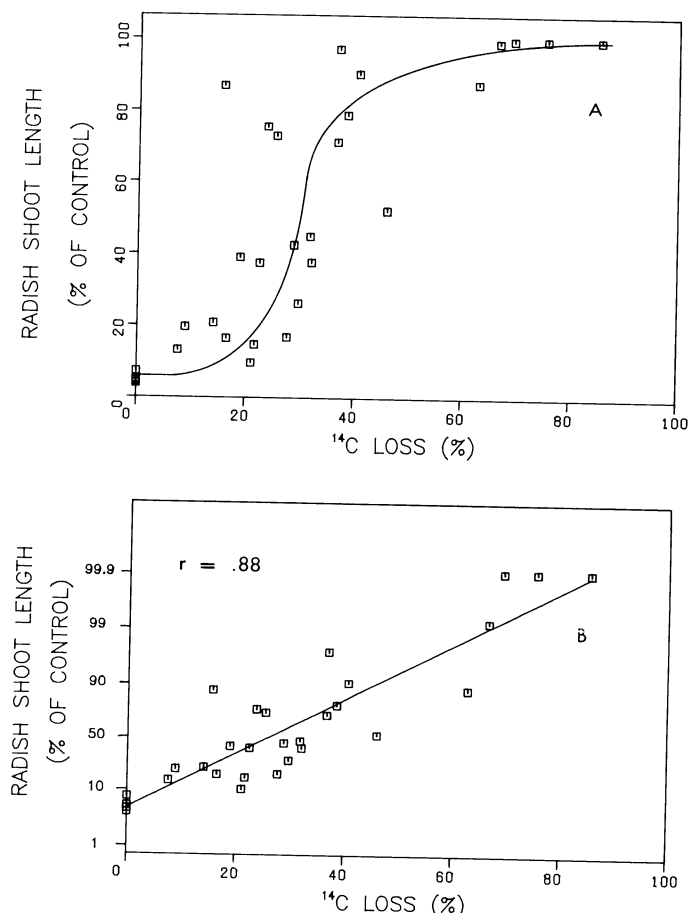


Fig. 4. Relationship of ^{14}C loss from sclerotia of *Sclerotium rolf sii* to pathogenic aggressiveness of the sclerotia on radish seedlings, as determined by shoot lengths (expressed as percentages of the length of uninoculated seedlings). Sclerotia of differing ^{14}C contents were obtained by incubating them in soil for different periods of time. A, Arithmetic plot. B, Plot with logarithmic transformation of shoot length percentages ($y = 3.35 + 0.115x$).

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