Increased activity of soil microorganisms near sclerotia of *Sclerotium rolfsii* in soil

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Microbial activity of soil near sclerotia—"mycosphere" (MS)—increased markedly within 24 h after sclerotia of *Sclerotium rolfsii* were pressed into the soil, but no changes occurred in the microbial activity of soil not near the sclerotia—non-mycosphere (NMS). Increased microbial activity in the MS, called the "mycosphere effect," was attributed to increased bacterial activity, since numbers of fungi and actinomycetes remained relatively unchanged. The qualitative selection of specific bacteria in the MS by sclerotia was suggested by the increase in bacteria that were tolerant to streptomycin and oxgall bile salts in the plating medium. Exposure of the soil to volatile compounds from alfalfa hay before adding the sclerotia enhanced the rate of the microbial increase in the MS. The energy substrate for the increase in MS organisms was of sclerotial origin and not from culture nutrients adhering to the sclerotia. Addition of concentrated sclerotial leachate to soil, and exposure of soil to alfalfa volatiles also induced an increase in the MS organisms.

Bacteria from the MS affected *S. rolfsii* in vitro more than did bacteria from the NMS. Most of the MS bacteria were inhibitory to *S. rolfsii* on Difco plate-count agar, had no effect on potato-dextrose agar, and were stimulatory on Czapek-Dox agar. These results suggest that the nutritional status in the MS, as determined by sclerotial exudation or external nutrients, may be of primary importance in determining whether MS organisms will inhibit, will stimulate, or will have no effect on the sclerotia of *S. rolfsii*.

Introduction

The antagonistic or stimulatory effects of soil microorganisms on *Sclerotium rolfsii* Sacc. have been reviewed by Cooper (5) and more recently by Aycock (3). However, the significance of the soil microflora in disease development in the field is debatable. Anizohar-Hershenzon and Shacked (1) studied the saprophytic activity of *S. rolfsii* in soil with a baiting technique. They suggested that a microflora antagonistic to *S. rolfsii* reduced the incidence of bait colonization in natural soil, and that nitrogenous amendments contributed to disease control by suppressing the saprophytic activity of the pathogen in soil (2). Conversely, Henis and Chet (11) reported that nitrogenous amendments increased the number of antibiotic-producing organisms associated with sclerotia, and that these organisms at the soil–sclerotium interface inhibited sclerotial germination.

Gilbert *et al.* (10) reported that volatile compounds from alfalfa hay increased the microbial activity of treated soil. Linderman and Gilbert (13, 14) showed that the same volatile compounds stimulated sclerotial germination of *S. rolfsii* in soil, and increased the level of sclerotial fungistasis in treated soil. In addition, the volatiles, directly or indirectly, predisposed sclerotia to increased colonization by soil organisms and increased the rate and extent of lysis of mycelium. Thus, volatile compounds stimulated sclerotial germination in soil and simultaneously promoted the activity of other soil microorganisms. The net effect was reduced vegetative activity (and therefore fewer secondary sclerotia) of the fungus and an increased antagonistic potential of the soil.

In the present study, we examined more closely the qualitative and quantitative changes in microorganisms in soil adjacent to and surrounding sclerotia of *S. rolfsii* compared to the microbial status in soil away from sclerotia.

Materials and Methods

Mycosphere vs. Non-mycosphere Soil

We compared the microbial activity of the "mycosphere" (MS)—the soil surrounding sclerotia—to the activity of the "non-mycosphere" (NMS)—the soil away from or uninfluenced by the sclerotia. We pressed two groups of sclerotia (10 sclerotia/group) into 3 g of soil in the bottom of plastic snap-lid dishes, 50 × 12 mm (Fig. 2A). Water was placed on the lid as hanging drops to maintain a moist atmosphere. At various intervals of time within 72 h, we collected soil from the MS or NMS. Soil from the MS was removed from the soil dishes in cookie-cutter fashion with an 18-mm test tube and the rest was collected as NMS soil. Samples from five dishes were combined and duplicate 1-g subsamples were then weighed out from each for microbial population assays.

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Soil Microbial Assay Procedures

Populations of bacteria, actinomycetes, and fungi in MS and NMS soils were determined by the standard soil dilution-plate techniques. Duplicate 1-g (dry weight) subsamples of soil from the MS or NMS soil were suspended in 100 ml of sterile distilled water. These suspensions were then shaken on a reciprocating shaker for 15 min. Dilutions were then made from these suspensions, and 1 ml of each dilution was placed in petri dishes to which cooled agar of the appropriate medium was added. Fungi were assayed on Littman-oxgall agar (LOA) with 100 ppm streptomycin-sulphate (LOAS), pH 7.0. Bacteria and actinomycetes were assayed on Difco plate-count agar (PCA), pH 7.0. We also assayed the numbers of bacteria that were tolerant to streptomycin or bile salts in oxgall by counting bacterial colonies which grew on Littman-oxgall agar, with and without 100 ppm streptomycin-sulphate. The data are averages of at least three experiments.

Soil Treatments

A Warden, fine sandy loam from near Prosser, Washington, was used in our experiments (14).

In some experiments, we exposed soil dishes for 4 days to vapors from 4 drops of an alfalfa distillate (AD) placed as hanging drops on the lid. Then we removed the AD drops, pressed sclerotia into the soil, and placed water drops on the lid to maintain a moist atmosphere. Control soil dishes were handled the same way, but were treated with water only. The alfalfa distillate was obtained by distillation of a water slurry of alfalfa hay as described elsewhere (9).

Source of Sclerotia

Sclerotia of *S. rolfsii* were obtained from a culture isolated from soybean (*Glycine max.* (L.) Merr.) and grown on potato-dextrose agar (PDA) slants (pH 5.6). They were air-dried, stored at room temperature (ca. 23°C), and used after 12–14 months of storage (over 90% viable).

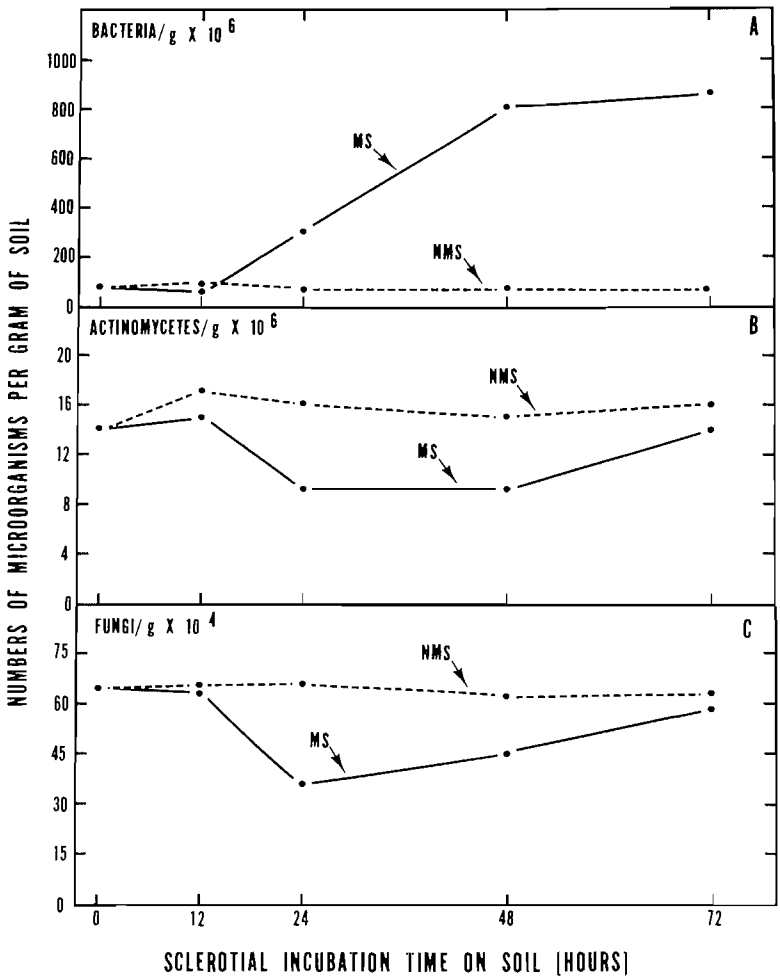

FIG. 1. The numbers of microorganisms in mycosphere (MS) or non-mycosphere (NMS) soils followed for 72 h after sclerotia of *Sclerotium rolfsii* were pressed into the soil.

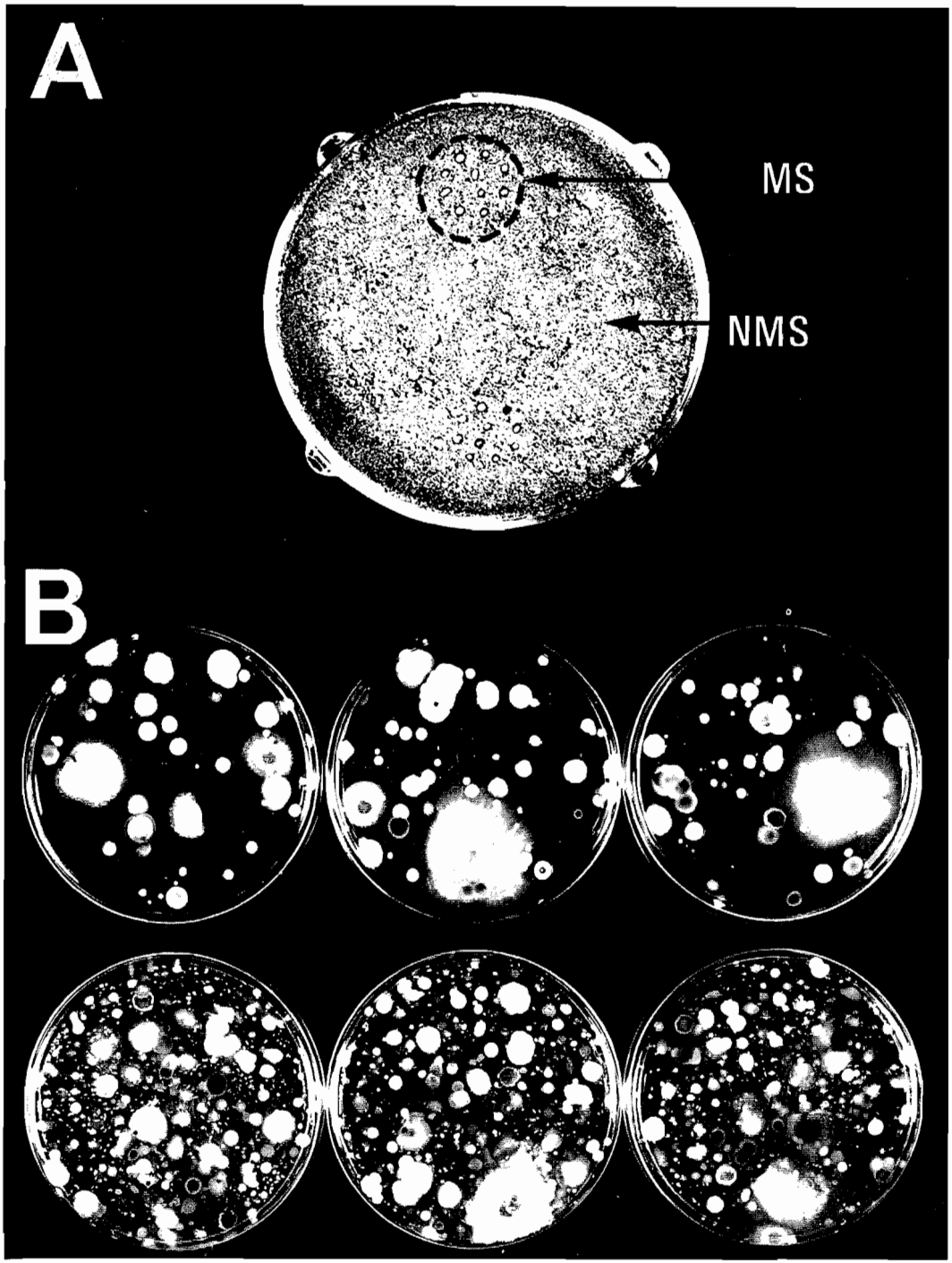

FIG. 2A. Illustration of soil dish "mycosphere" system. Soil collected from beneath and around the two groups of *Sclerotium rolfsii* sclerotia was called mycosphere (MS) soil. The remaining soil was called non-mycosphere (NMS) soil. FIG. 2B. Illustration of the "mycosphere effect" on Littman-oxgall agar with 100 ppm streptomycin. Sclerotia were incubated for 48 h on the soil. Mycosphere soil (bottom row) and non-mycosphere soil (top row) were plated on this medium at a dilution of 10^{-4} for fungi. Note the increase in the MS soil of bacteria tolerant of streptomycin and the bile salts of oxgall.

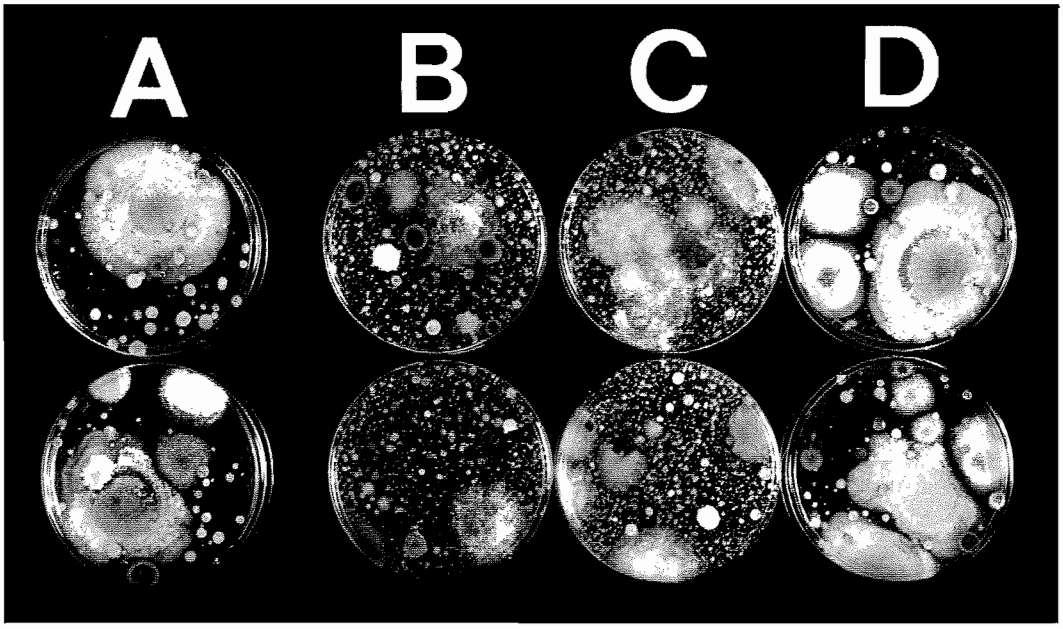

FIG. 3. The effect of washing on the capacity of sclerotia of *Sclerotium rolfsii* to induce the "mycosphere effect." Sclerotia were incubated for 48 h on the soil. Soil was plated at 10^{-4} on Littman-oxgall medium with 100 ppm streptomycin for fungi. Duplicate dishes illustrated are (A) non-mycosphere (NMS) soil, (B) mycosphere (MS) soil from unwashed sclerotia, (C) sclerotia washed 15 min, and (D) sclerotia washed 18 h.

Sclerotia were generally dry when placed in the soil dishes, but in some cases 0.2 g were washed in 50 ml of distilled water on a shaker for 15 min to 18 h before being placed on the soil.

Isolation and Characterization of Soil Bacteria

We randomly isolated bacteria from MS or NMS soil dilution plates of PCA, LOA, and LOAS. Thirty-seven different isolates were established based on the following characteristics: cell morphology, Gram-stain reaction, colony size, and colony color. All isolates were maintained on PCA slants.

Bacterial Antagonism Assay in vitro

We assayed the 37 bacterial isolates from MS and NMS soil for their effect on *S. rolfsii* as opposed colonies growing on three media: PCA, PDA, and Czapek-Dox agar (CDA), pH 7.3. Each plate was inoculated in the center with a single sclerotium of *S. rolfsii* and at four loci near the edge with bacterial isolates (either four different isolates or one isolate inoculated at four loci). The cultures were incubated for 2 weeks at 26°C. They were observed daily for inhibition or stimulation of *S. rolfsii*. All the bacterial isolates grew well on PCA and CDA; only half of them grew on PDA. Therefore, no data could be collected for some isolates on PDA. *S. rolfsii* alone grew better on PDA than on PCA. On CDA, however, the sclerotia germinated poorly, and vegetative growth either ceased or was abnormal.

Results

Microbial Populations of the MS vs. NMS

Soil from the MS and NMS was sampled at intervals up to 72 h after sclerotia were added.

TABLE 1

The effect of washing sclerotia on their capacity to produce the mycosphere effect in soil. The concentrated (10×) sclerotial leachate from the 18-h wash was also tested for its capacity to produce the mycosphere effect

	Numbers of microorganisms/g dry soil[b]			
Sclerotial washing time h[a]	Fungi × 10^4	Bacteria and actinomycetes × 10^6	Oxgall tolerant bacteria × 10^{6c}	Streptomycin tolerant bacteria × 10^{6d}
	Mycosphere soil			
0.00	40	955	108	35
0.25	37	1105	120	35
1.00	61	419	49	9
4.00	53	310	20	5
18.00	43	127	5	2
	Non-mycosphere soil			
Sclerotial leachate (10×)	33	281	44	11
Control	47	109	3	1

[a]Sclerotia washed in 50 ml sterile distilled water.
[b]Soils were plated 48 h after sclerotia or sclerotial leachate were added to the soil.
[c]Soil plated on Littman-oxgall medium without streptomycin.
[d]Soil plated on Littman-oxgall medium with 100 ppm streptomycin.

Bacterial populations in the MS were strikingly greater than in the NMS (Fig. 1). This effect, hereafter referred to as the "mycosphere effect," was detectable within 24 h, but was most striking after 48 h. Numbers of fungi and actinomycetes in the MS decreased during the first 24 h, but then increased to initial population levels after 72 h. Microbial populations in the NMS remained unchanged throughout the assay time period.

Dilution plates for assaying fungi in the MS were densely colonized by bacteria, but NMS dilution plates had very few bacterial colonies (Fig. 2B). Further investigation of MS and NMS microbial populations on Littman-oxgall agar with and without 100 ppm streptomycin-sulphate revealed that some of the bacteria that had been selectively increased in the MS were tolerant to the bile salts in oxgall and some were also tolerant to streptomycin.

Effects of Washing Sclerotia on the MS Effect

We attempted to determine the source of energy for the rapid increase in bacterial populations in the MS. We washed sclerotia for 15 min to 18 h in distilled water before pressing them into the soil. Culture nutrients were removed from the sclerotial surface by the short wash periods. The MS effect was not decreased by the 15-min wash; however, it was decreased considerably after 1 h of washing and was almost eliminated after 18 h of washing (Table 1 and Fig. 3). There was no significant change in fungal numbers after the sclerotia were washed.

We also added the sclerotial leachate from the 18-h wash period (concentrated 10× under vacuum at 30C) to soil and incubated the soil for 48 h. When the sclerotial leachate-treated soil was plated out on Littman-oxgall agar with and without streptomycin, we observed the MS effect (Table 1). We concluded, therefore, that the increase in MS bacteria, including those tolerant to streptomycin and oxgall bile salts, resulted from the use of substrates exuded by or originating from sclerotia.

The Effects of Alfalfa Volatiles on the MS Effect

The influence of volatile compounds from an alfalfa hay distillate (AD) on the microbial populations in the MS was determined. The bacterial population increase in the MS in AD-treated soil was nearly twice that of water-treated control soil (Fig. 4). This was especially

true for the streptomycin- and oxgall bile salt-tolerant bacteria. The same bacteria also increased to detectable levels in NMS and control soil exposed to the volatiles, but not in untreated soil. Numbers of fungi and actinomycetes were not affected.

Influence of MS Bacteria on S. rolfsii in vitro

In general, the results of our in vitro tests showed that MS bacteria affected *S. rolfsii* more than did NMS bacteria. The nature of this activity, however, depended on the culture medium. For example, most of the MS bacteria were very inhibitory and, in some cases, lethal to *S. rolfsii* on PCA. The MS bacteria had no effect on *S. rolfsii* when grown on PDA, and were very stimulatory to the fungus when plated on CDA (Fig. 5). Many MS bacterial colonies on CDA had a chemotactic effect on the vegetative growth of *S. rolfsii*. By contrast, NMS and control soil bacteria had little or no effect on the growth of *S. rolfsii* on any of the media.

Discussion

Our prime consideration was to examine more closely the relative activity of the major group of soil microorganisms that would come

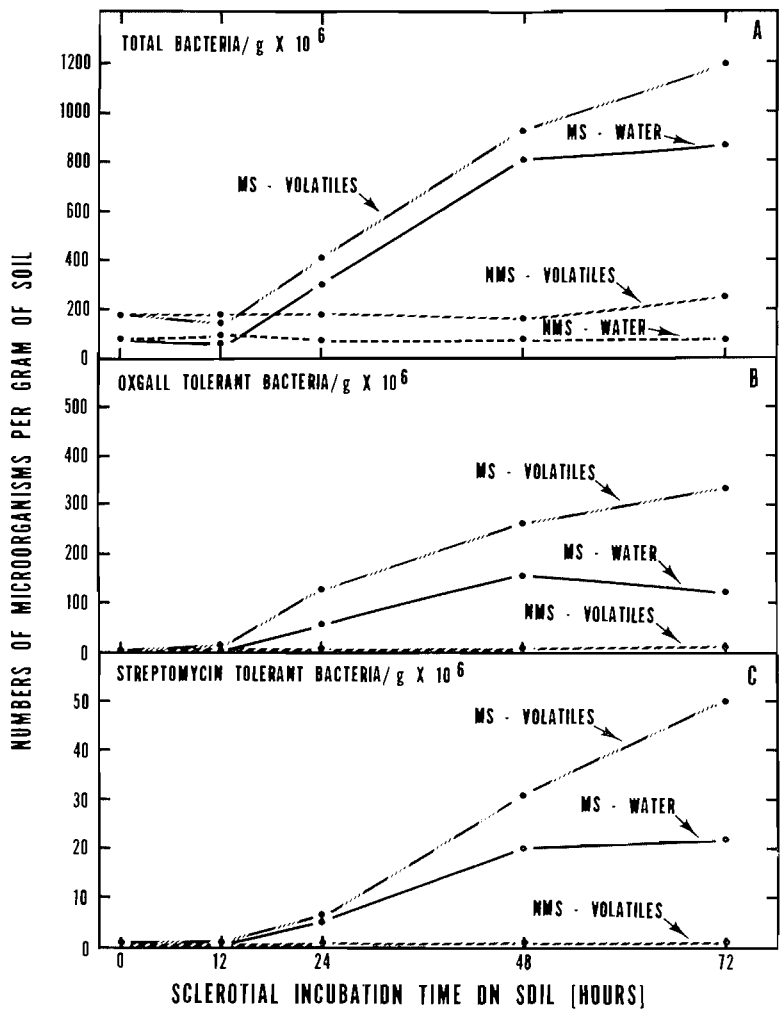

FIG. 4. The influence of volatile compounds from alfalfa hay on the bacterial numbers of mycosphere and non-mycosphere soil of *Sclerotium rolfsii*. Soils were exposed to vapors from 4 drops of alfalfa distillate for 4 days before sclerotia were added to the soil.

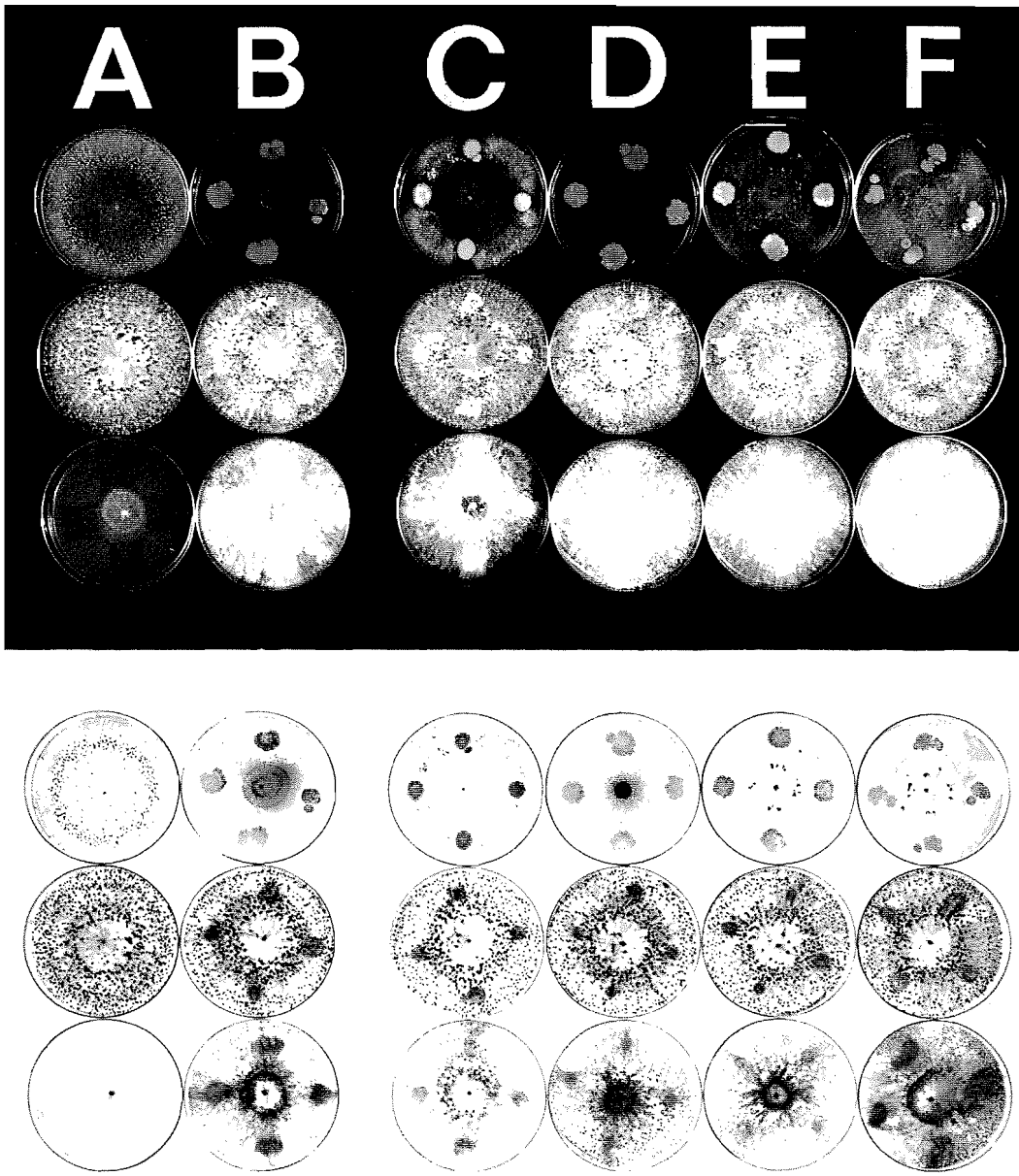

FIG. 5. Influence of mycosphere bacteria on the growth of *Sclerotium rolfsii* in vitro. Both illustrations are the same but with different backgrounds to emphasize mycelial (dark background) or sclerotial (light background) characteristics. The three media used, from top to bottom were plate count agar (PCA), potato-dextrose agar (PDA), and Czapek-Dox agar (CDA). From left to right: *S. rolfsii* alone (A), *S. rolfsii* plus four different mycosphere bacteria (B), and each of the four bacteria in B assayed separately (C–F). The culture plates were incubated for 14 days. Note that the mycosphere bacteria inhibit *S. rolfsii* on PCA, have no effect on PDA, and stimulate on CDA.

into direct contact with specific propagules of *Sclerotium rolfsii*. Our evidence suggests that sclerotia, when placed into natural soil, stimulate microbial activity in their immediate vicinity. This increased activity near the sclerotia, termed the "mycosphere effect," is similar to the rhizosphere effect, wherein microbial activity of soil in the immediate vicinity of roots increases because of the use of exuded nutrients by the soil microflora. Also, it has been reported that rhizosphere bacteria are more tolerant to antibiotics than non-rhizosphere bacteria (4, 18). We are reporting a comparable phenomenon by sclerotia of *S. rolfsii*. Our evidence suggests that nutrients, originating from sclerotia, are exuded or released into the MS. Specific components of this exudate are preferentially used by certain bacterial groups, such as those tolerant to streptomycin or bile salts of oxgall. While the detection of antibiotic- and oxgall-tolerant bacteria in the MS may be incidental, we present evidence that organisms from the MS have a greater potential to influence sclerotia of *S. rolfsii* than those organisms which were not selectively favored by the sclerotial exudate. Viable sclerotia have the inherent capacity to resist colonization by other soil organisms (8). It is possible that antibiotics are released by sclerotia which, in combination with specific nutrients, may cause the specific microbial changes in the MS.

We wish to draw further attention to the potential significance of the metabolic exchanges which may be taking place in the MS and to emphasize the reciprocal nature of those exchanges. Our evidence suggests that substances of sclerotial origin are preferentially used by certain groups of soil bacteria which selectively build up in the MS. Other workers (6, 7, 17) have reported that some soil microorganisms may stimulate and that others may inhibit *S. rolfsii*. But, our studies in vitro suggest that the same MS organism may respond to different nutrient regimes by producing metabolites that either inhibit or stimulate *S. rolfsii*. The subsequent qualitative nutritional changes which take place in the MS, either from nutrients in sclerotial exudate or from external nutrient sources, may determine what metabolic message the MS organisms give to the sclerotium. This may mean that MS organisms make *S. rolfsii* more responsive to its environment.

If, as our studies suggest, MS organisms stimulate *S. rolfsii* under one set of nutritional conditions and inhibit under another, then nutrition is likely to be of prime importance in determining whether sclerotia germinate in soil or remain quiescent. In another study (Linderman and Gilbert, unpublished), addition of concentrated sclerotial leachate to soil and exposure of soil to volatile compounds from alfalfa hay, or combinations of sclerotia or sclerotial leachate with alfalfa volatiles, increased the level of soil fungistasis to sclerotia. The present study shows that these same materials, when added to soil, selectively increase MS bacteria which are more able to stimulate or inhibit than NMS soil organisms. Lingappa and Lockwood (15) reported that microbial activity increased around fungus spores that had been added to soil. They proposed that the spores acted as nutrient substrates used by microorganisms which produced antibiotics responsible for keeping the spores fungistatic. Since then, however, Lockwood (16) and Ko and Lockwood (12) have emphasized the importance of the lack of available nutrients as the factor preventing spore germination in soil. They propose that the increased number of organisms near fungal spores compete for and deplete the supply of available nutrients needed for the spore to germinate. Our work, however, suggests the possibility that the microorganisms in the MS might use the added nutrients, and byproducts of their metabolism may stimulate spore germination.

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