

## Influence of Volatile Compounds from Alfalfa Hay on Microbial Activity in Soil in Relation to Growth of *Sclerotium rolfsii*

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### ABSTRACT

Mycelial growth from sclerotia of *Sclerotium rolfsii* on natural soil is stimulated when exposed to vapors of alfalfa distillate (AD). This growth was indexed on soils which had been previously exposed to AD or water vapors. Vigor of mycelial growth from sclerotia was markedly decreased in soil previously exposed to vapors from natural or synthetic mixtures of AD. Inhibition of mycelial growth from sclerotia also occurred in soil previously amended with sclerotia or a sclerotial leachate. This self-induced inhibition was further enhanced by simultaneous exposure of the soil to AD vapors. Soil microbial population and respiration studies indicated

that growth inhibition of *S. rolfsii* may be related to the increased activity of soil microbes, primarily bacteria. Respiration rates of soils previously treated with water or AD, when exposed to AD vapors for 4 hr in Warburg flasks, were 12.3 and 22.9  $\mu\text{l}$  O<sub>2</sub>/g per hr, respectively. The same soils exposed only to water vapor in the Warburg flasks respired at only 3.1 and 3.9  $\mu\text{l}$  O<sub>2</sub>/g per hr, respectively. Greater respiratory activity in AD-treated soil over water-treated soil was attributed to the increased bacterial population, which was 3.5 times greater in the AD-treated than the water-treated soil.

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*Additional key words:* fungistasis, sclerotial germination.

We recently reported (7) that the relative number of microorganisms in soil near sclerotia of *Sclerotium rolfsii* Sacc. (primarily bacteria) was markedly higher than in soil not near sclerotia. We called this the "mycosphere (MS) effect", and demonstrated that it was induced by substances exuded from the sclerotia. Dickinson & Coley-Smith (4) recently reported a similar phenomenon with *Sclerotium cepivorum* Berk. We further demonstrated (7) that simultaneous exposure of soil and sclerotia (or sclerotial leachates) to volatile compounds from alfalfa hay enhanced the MS effect. Thus, the greatest increase in microbial populations occurred in soil adjacent to sclerotia exposed to alfalfa volatiles. Dickinson & Coley-Smith (4) stated: "The fact that sclerotia also influence the microbial activity of soil suggests that they may contribute to their own inhibition." We agree with this logic, and, since alfalfa volatiles also increase microbial activity in soil (6, 15), and especially in the MS soil (7), we proposed to study the growth response of *S. rolfsii* in soil that had been previously treated with alfalfa volatiles, sclerotia of *S. rolfsii*, or a concentrated sclerotial leachate. Our purpose here was to determine whether the increased microbial population would inhibit the growth of *S. rolfsii* in soil. A preliminary report was made (12).

**MATERIALS AND METHODS.**—*Soil characteristics.*—We used Warden soil from the vicinity of Prosser, Wash. It was a sandy clay loam with a pH of 7.3-7.5, organic matter content of 0.8%, cation exchange capacity of 120 meq/100 g, and a water-holding capacity of 12%. The soil was frozen in plastic bags in a moist condition, after collection in the field, until thawed for use. Thawed soil was refrigerated between experiments.

*Soil treatments.*—Three g of soil pressed into plastic petri dishes (50 × 12 mm) with sealable lids were exposed to vapors from drops of alfalfa distillate

(AD) hung from the lids of the dishes (1 drop = ca. 0.037 ml). Soil exposed only to water vapors was used as a control. In this system, only vapors diffusing from the drops across the air gap could affect the microorganisms in or on the soil sealed in the dish. Both natural and synthetic mixtures of AD were used. AD was prepared according to the method of Menzies & Gilbert (15). We prepared synthetic AD as per Owens et al. (16): methanol, 1.08%; acetaldehyde, 0.24%; isobutyraldehyde, 0.12%; and isovaleraldehyde, 0.26%. Except where indicated, treatment with natural or synthetic AD was always with four hanging drops (0.15 ml) for 1 week before the soil was assayed.

In some experiments, we treated soils by placing sclerotia (10/dish) or a sclerotial leachate (10 drops or 0.37 ml/dish) on the soil for 1 week. Sclerotia were either stimulated to germinate by placement of four drops (0.15 ml) of AD on the lid, or were not stimulated by placing water drops only on the lid. Where the sclerotial leachate was added, the soil was also exposed to vapors from either AD or water.

We prepared sclerotial leachate by leaching 0.2 g of 1-year-old culture sclerotia in 50 ml distilled water for 18 hr on a shaker. The sclerotial leachate volume was reduced to 5 ml at 30 C under vacuum.

Every treatment had two replications per experiment, and every experiment was repeated at least 3 times.

*Indexing *S. rolfsii* growth on soil.*—Mycelial growth from sclerotia placed on treated soil was compared to growth from sclerotia on control soil. Growth was stimulated by exposure of 10 sclerotia/dish of soil to vapors from AD as previously described (13). Any reduction in growth response to the AD stimulant on treated soil compared to control soil was considered due to the inhibitory action of the soil microorganisms whose activity was stimulated

by the treatment. Mycelial growth from each sclerotium was indexed after 4-6 days, using a subjective rating system on a scale of 0-10 (13). The rating was similar in principle to that of Emmatty & Green (5), who counted the number of germ tubes emerging from microsclerotia of *Verticillium albo-atrum* Rke. & Berth. in soil compared to filter paper controls or soil amended with nutrients. However, since the number of germ tubes emerging from sclerotia of *S. rolfii* was too high to count, we subjectively rated the relative amount of mycelial growth from each. Sclerotial germination was usually over 90%, even in the water controls. Without the AD stimulus, sclerotial germination was limited to one or two germ tubes. Sclerotia stimulated to germinate by AD vapors produced hundreds of germ tubes, and the entire contents of each sclerotium were exhausted (13). Thus, the latter degree of sclerotial germination was rated 10; no germination was rated 0. The ratings between were subjective and only relative. The growth index reported is the average of replicate dish ratings, and each dish rating was the average of 10 individual sclerotial ratings.

Sclerotia used in the assays were produced on

potato-dextrose agar (PDA) slants, air-dried, and stored in vials until used. Viability was over 90% on PDA even after 3 years of storage.

*Measurement of soil respiration and microbial populations.*—We determined soil respiration by measuring O<sub>2</sub> uptake manometrically according to the procedures of Menzies & Gilbert (15), except that 5 g soil/flask were used instead of 20 g.

Dilution plate colony counts of soil microorganisms were made on Difco plate count agar for bacteria (including actinomycetes), and on Littman's oxgall medium plus 100 ppm streptomycin for fungi. Counts were made after 4-5 days.

*RESULTS.—Effects of soil treatments with alfalfa volatiles, sclerotia, and sclerotial leachate on growth of S. rolfii on soil.*—Mycelial growth from sclerotia of *S. rolfii* placed on soil treated with vapors from AD was compared with growth on control soil treated with only water vapors. Mycelial growth in response to the AD stimulant was consistently less on the AD-treated soil than on control soil (Fig. 1).

We compared the natural AD mixture of volatiles with a synthetic mixture with respect to increasing the capacity of soil to inhibit *S. rolfii*. The two

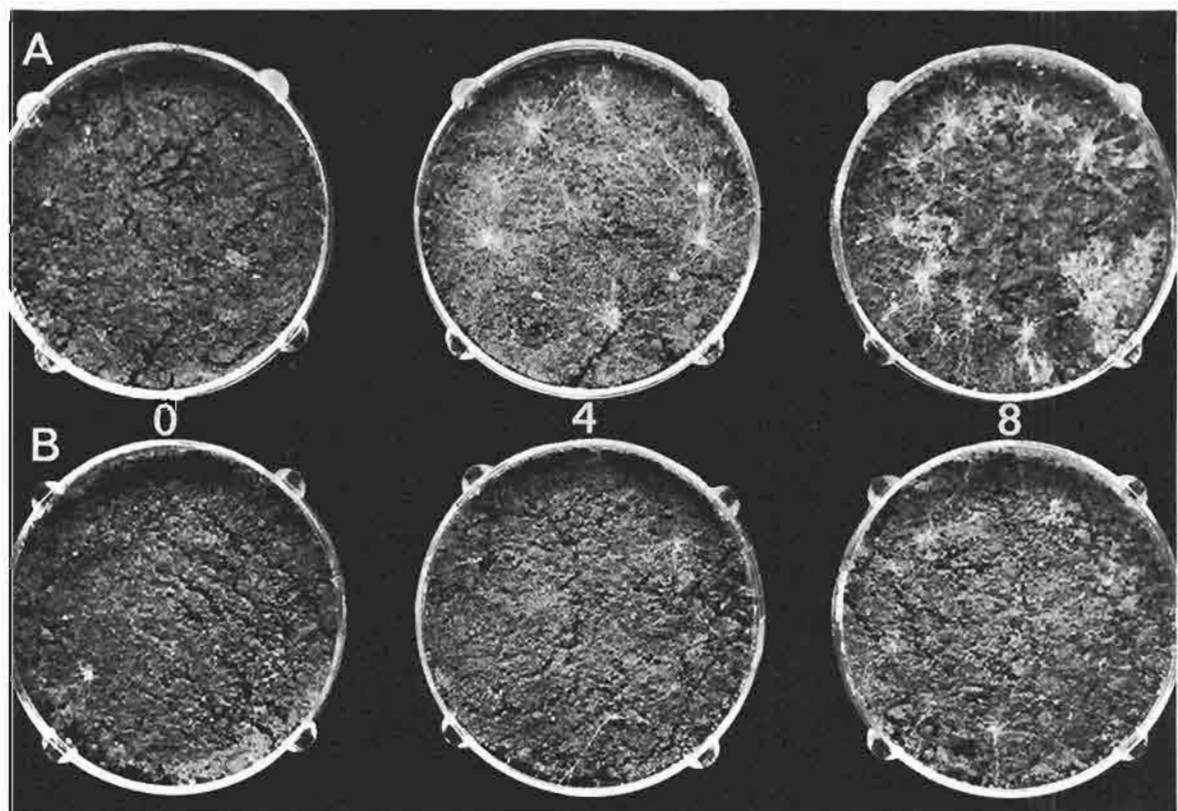


Fig. 1. Inhibition of mycelial growth from sclerotia of *Sclerotium rolfii* on soil treated with vapors from an alfalfa distillate (AD) compared to growth on untreated control soil. Soils were treated for 1 week with vapors from water (0) or four and eight drops of alfalfa distillate (AD) (0.15 and 0.30 ml, respectively). Sclerotia were then placed on the AD-treated soils (B) and water control soils (A) and exposed to vapors from water (0) or four and eight drops of AD as a growth stimulant.

TABLE 1. Index of mycelial growth from sclerotia of *Sclerotium rolfsii* on soil exposed for 1 week to combinations of alfalfa hay volatiles and sclerotia and mycelium (or sclerotial leachate) of *S. rolfsii*

Soil treatment <sup>a</sup>	Sclerotial growth rating <sup>b</sup>		% Growth reduction <sup>c</sup>
	Water	AD <sup>d</sup>	
Water control	0.4±.4	4.5±0.8	0
AD-natural	1.7±.4**	3.2±1.3**	29
AD-synthetic	1.2±.9	3.1±1.5**	33
<i>S. rolfsii</i> + water		3.5±0.6*	23
<i>S. rolfsii</i> + AD		1.6±0.8**	64
SL + water		4.2±0.8	7
SL + AD		3.1±0.9**	31

<sup>a</sup> Soil was exposed during the treatment period to water vapors (water control), vapors from volatile compounds in an alfalfa hay distillate (AD-natural), or a synthetic mixture of four volatiles known to be in the natural AD (AD-synthetic), sclerotia and mycelium of *S. rolfsii* (or sclerotial leachate-SL) combined with water or AD vapors.

<sup>b</sup> Following the soil treatments, mycelial growth from sclerotia placed on treated soil in response to water or AD vapors was indexed: 0 = no mycelial growth from sclerotia, 10 = maximum growth. \*\* and \* indicate significance in an analysis of variance compared to the water control treatment at the 1 and 5% levels, respectively. Data presented are mean growth ratings from 6 to 11 replicate dishes/treatment in two-four experiments.

<sup>c</sup> The percent growth reduction was derived from the growth ratings on treated soil compared to that on control soil.

<sup>d</sup> AD = alfalfa distillate.

mixtures were equally effective (Table 1).

We investigated the capacity of *S. rolfsii* to contribute to its own inhibition in soil by treating soil with amendments of sclerotia or a water leachate of sclerotia. Soils with these two treatments were simultaneously exposed to vapors from either 0.15 ml of water or AD to determine how the combination of *S. rolfsii* and AD influenced the growth response from sclerotia. Both sclerotia and the sclerotial leachate induced changes in the soil that contributed to the inhibition of mycelial growth of *S. rolfsii* (Table 1). The most striking inhibition of growth

occurred on soil exposed to both sclerotia and AD vapors.

*Influence of alfalfa volatiles on soil respiration and microbial populations.*—Soil was treated with vapors from 0.37 ml AD or water in dishes for 1 week and then transferred to Warburg flasks where O<sub>2</sub> uptake was measured manometrically for 4 hr. During that 4-hr period, the soils were exposed again to vapors from 2 ml of either AD or water held in the side arm. The fungal and bacterial numbers in these soils were determined following the 4-hr measurement period. The results (Table 2) show that exposure of soil to AD vapors for 1 week increased the numbers of bacteria 3.5-fold, but the numbers of fungi or actinomycetes remained relatively unchanged. During the 4-hr respiration measurement period, AD vapors increased the respiration of the organisms in water-treated soil 4-fold without a significant population increase, but the increase was 7-fold in the AD-treated soil, presumably due to the 3.5-fold increase in the bacterial population. However, the respiration of this increased bacterial population when exposed only to water vapors in the Warburg flask was only slightly greater than that in soil treated initially only with water vapors.

**DISCUSSION.**—Sclerotia of *S. rolfsii* were previously shown by us (7) to exude substances into the surrounding soil which selectively increased the population of certain bacteria in the MS soil. Lingappa & Lockwood (14) and more recently Dickinson & Coley-Smith (4) reported a similar rapid increase in soil bacteria when fungal spores or sclerotia of *S. cepivorum*, respectively, were added to soil. Our work with *S. rolfsii* (7), however, suggested that the bacteria that increased in the "mycosphere" (MS) were specifically selected by the sclerotia, since they exhibited not only a greater tolerance to streptomycin and bile salts in the plating medium than non-MS soil bacteria, but also a greater capacity to influence the in vitro growth of *S. rolfsii*. Furthermore, the increase of these specific bacteria was enhanced when the soil and sclerotia were exposed to volatile compounds from AD. Results of the present study show that the same soil treatment, i.e., AD, sclerotia of *S. rolfsii*, sclerotial leachates, or combinations thereof, which induced or enhanced the

TABLE 2. Influence of single and double exposures of Warden soil to vapors from alfalfa hay distillate (AD) or water on respiration and numbers of soil microorganisms

First exposure <sup>a</sup>	Second exposure	O <sub>2</sub> uptake in μliters O <sub>2</sub> /g per hr	Microbial populations		
			Fungi	Bacteria	Actino- mycetes
			× 10 <sup>-4</sup>	× 10 <sup>-6</sup>	× 10 <sup>-6</sup>
Water, 0.37 ml	Water, 2 ml	3.1	65.8	71.8	13.8
	AD, 2 ml	12.3	62.0	76.5	15.3
AD, 0.37 ml	Water, 2 ml	3.9	60.4	264.0	15.8
	AD, 2 ml	22.9	59.5	263.0	14.0

<sup>a</sup> Soil was treated in the first exposure for 1 week to vapors from 0.37 ml AD or water. Each treatment was subsequently exposed in the Warburg flasks to vapors from 2.0 ml AD or water. Respiration was measured for 4 hr; then microbial populations were immediately determined.

mycosphere effect, also increased the capacity of the soil to inhibit mycelial growth from sclerotia of *S. rolfsii* in soil. These results suggest that *S. rolfsii* contributes to its own inhibition in soil by inducing changes in the soil microflora, whose metabolism in turn influences the mycelial growth from sclerotia. Our respiration and population studies suggest that the bacterial segment of the microflora is specifically involved, and that the increased soil respiration induced by AD probably can be attributed to increased bacterial metabolism. By-products of their metabolism could be responsible for inhibiting mycelial growth from sclerotia of *S. rolfsii* in soil (2, 9, 11, 19).

The substance(s) exuded into soil by sclerotia of *S. rolfsii* have not been identified. However, Coley-Smith & Dickinson (3) recently reported that the major substances exuded by sclerotia of *S. cepivorum* into agar or water were trehalose, glucose, and mannitol, plus small quantities of a glucan. It is not known, however, whether these substances induce changes in the soil microbial activity that contribute to inhibition of mycelial growth from sclerotia.

If the sclerotia of *S. rolfsii* cannot maintain a sustained exudation of substances into the MS, the inhibitory capacity of the MS organisms may diminish with time. It seems logical that any treatment of the soil that would encourage and maintain those organisms may also maintain or even increase their inhibition of sclerotial germination and growth. Such may be the case where nitrogenous amendments, which are known to reduce the incidence of disease caused by *S. rolfsii*, reduced sclerotial germinability by increasing the numbers of antibiotic-producing organisms associated with the sclerotia at the soil-sclerotium interface (8). Others have shown that alfalfa amendments may have a similar effect of increasing the level of fungistasis to the pathogen propagule (1, 10, 17, 18). We are reporting for the first time that the volatile compounds emanating from alfalfa hay may induce changes in the soil microflora which result in the same inhibitory effect on the sclerotia of *S. rolfsii*. Whether this phenomenon is ephemeral and the volatiles do not induce permanent quantitative or qualitative changes in the bacterial population is not known. Nor is it known whether the greater inhibitory capacity of the soil on *S. rolfsii* induced by the alfalfa volatiles could reduce the incidence or severity of disease caused by this pathogen.

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