Fungistasis of Sclerotia of Sclerotium oryzae

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ABSTRACT

Sclerotia of *Sclerotium oryzae* germinate readily on distilled water, but not in rice-field soil. Soil inhibition of sclerotial germination apparently can only be effected by the presence of living cellular organisms, because it can be annulled by filtration (0.45 μm), heat sterilization, or by a 7-day period of soil microbial inactivity (dry at 1 C). Certain bacteria, actinomycetes, or fungi were found to produce inhibitory substances when incubated on potato-dextrose agar, while bacteria were found to do so apparently with only a substrate of sclerotial exudate. Organisms from a field soil with disease-free rice caused inhibition of sclerotial germination of higher magnitude than those from field soil endemic with stem rot disease of rice.

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Sclerotium oryzae Catt. causes stem rot disease of rice (13) which is endemic in parts of the northern Sacramento Valley of California (15). Sclerotia of the fungus are formed on infected tissues as the rice plants mature, and are scattered on the soil surface or remain in crop debris after harvest. They become incorporated into the soil at various depths and at various times depending upon tillage methods and serve as the primary inoculum for infecting young rice plants of a subsequent crop by floating to the surface of rice paddy water. In laboratory tests (5), the germination of cultured sclerotia extracted from wet soil was found to be strongly inhibited by a factor (believed to be biological) which was apparently retained in the surface structure of the sclerotia after recovery. This paper reports on various tests to characterize the nature of soil fungistasis of sclerotia of *S. oryzae*, and the inhibition of germination of sclerotia by certain soil-borne microorganisms.

MATERIALS AND METHODS.—Sclerotia of *S. oryzae* were produced according to the method described by Krause and Webster (7) and were stored at 1 C for future use. Liquids requiring sterilization were autoclaved 15 minutes at ca. 122 C. Soils or sands were autoclaved 90 minutes at ca. 122 C in open petri dishes, and the sterilization process was tested by plating subsamples of soil or sand on potato-dextrose agar (PDA) for fungi, and Medium 523 (4) for bacteria. All tests were incubated in the laboratory under continuous fluorescent light intensity of ca. 690 lx at room temperature (24 ± 2 C) unless otherwise indicated. Sclerotia were rated as having germinated on a test medium if characteristic colonies or conidia were produced within 14 days. Soil was obtained from a field on which rice had been grown many years without the occurrence of stem rot disease. Silica sand was no. 36 Ottawa sand from Amsco Division of Abex Corporation, Oakland, California. River-bottom sand was No. 2 mortar sand from Robertson Sand and Gravel Company, Sacramento, California.

Inhibition of germination of sclerotia of *S. oryzae* was determined by comparing the germination of sclerotia on assay media to germination of sclerotia from the same lot under identical conditions except for the absence of antagonistic organisms or their residual effects. Inhibition of germination was calculated by

$$\text{\% inhibition of germination of sclerotia} = \frac{\left(\begin{array}{c}\text{\% germination on}\\ \text{control medium}\end{array}\right) - \left(\begin{array}{c}\text{\%germination on}\\ \text{assay medium}\end{array}\right)}{\text{\% germination on control medium}} \times 100,$$

and expressed in the table as one of five general categories:

0 = No inhibition
1 = 1 to 38% inhibition
2 = 39 to 63% inhibition
3 = 64 to 99% inhibition
4 = Complete inhibition.

Sclerotia were surface disinfested by stirring in 0.5% sodium hypochlorite (ca. 500 mg per 125 ml) 3 minutes, drying on filter paper, and storing in sterile petri dishes at 1 C. Four replications of 50 to 100 sclerotia per petri dish were used in each test treatment.

RESULTS.—Sclerotia were first tested to determine the general nature of exogenous nutrition required to break their dormancy by placing them in petri dishes on water agar (1.5% Difco "Agar Flake"), distilled water or glass-distilled water. Germination of sclerotia was 100%, 85%, and 86%, respectively. The addition of glucose to distilled water (0.05% w/v solution) stimulated 100% of tested sclerotia to germinate, and activated charcoal blended with distilled water (0.1% w/v suspension) increased their germination to 96%.

Sclerotia were tested on the surfaces of rice-field soil, river-bottom sand, or silica sand, either autoclaved or unautoclaved, which were wet to saturation with sterile-distilled or distilled water. The results reported in Table 1 indicate that only the unautoclaved rice soil had an inhibitory effect on sclerotial germination, and that the inhibition could be annulled by autoclaving the soil before the addition of water.

Sclerotia were then tested on soil leachates prepared under various conditions from samples of the same soil which inhibited germination of sclerotia as reported in Table 1. First, the soil samples were incubated 7 days either dry at 1 C, or wet at 24 ± 2 C. Subsamples of each were either autoclaved or not autoclaved. Leachates were then prepared from each subsample by stirring 60 g of soil in 50 ml of distilled water, allowing the mixtures to settle, and decanting the supernatant liquids into sterile petri dishes. Subsamples of the leachates were either autoclaved or not autoclaved, and half of each of those were either filtered through a "Millipore" membrane filter (0.45 μm) or not filtered. The results reported in Table 2 show that an inhibitory factor was present in leachates of the soil which had been incubated wet at 24 ± 2 C, but not in soil incubated dry at 1 C. The inhibitory factor was annulled by autoclaving either the soil or the leachate, or by filtering the leachate through a "Millipore" filter (0.45 μm).

In another experiment, sclerotia were introduced into the rice field soil in petri dishes and incubated wet for 9 weeks at 24 ± 2 C. Some soils were autoclaved before adding sclerotia and water, and others were left unautoclaved. The sclerotia did not germinate in the unautoclaved soils, but in the autoclaved soils they germinated, colonized bits of rice straw, and increased their numbers fourfold.

Microbial effects on sclerotial germination.—Because the results thus far strongly indicated that microbiological activity was, at least in part, responsible for the inhibition of germination of sclerotia of *S. oryzae*, various microorganisms were tested individually or in groups for their influence on sclerotial germination. Fungi were isolated from two different soils: one a rice field soil endemic with stem rot disease, and the other a rice field soil free of stem rot disease. Actinomycetes were all isolated from the endemic field, and the bacteria isolates were all from the disease-free field. The method used was adapted from those described by Lockwood (10) and Mughogho (12). Cellophane cut to fit into petri

dishes was boiled to remove plasticizers, and then sterilized by autoclaving in distilled water at ca. 122 C. One piece was placed aseptically on the surface of PDA in each petri dish and an agar disk containing spores and mycelium of a fungus or actinomycete to be tested was placed on the surface of the cellophane. Bacteria were tested by smearing colonies on the surfaces of "Millipore" filter membranes (0.45 μm). The test organisms were incubated 2-4 days, depending upon individual growth rates; the cellophane or membrane filters were removed; and sclerotia of *S. oryzae* were placed on the agar sites colonized by the test organisms. Germination of sclerotia on precolonized media was compared to that on the same media not precolonized, and the results indicated that a mixed culture of two bacteria and a culture of *Trichoderma aureoviride* Rifai, from the disease-free rice field soil were the only organisms tested that completely inhibited sclerotial germination; while two isolates of bacteria from the disease-free soil, two isolates of *Penicillium*, and three isolates of actinomycetes from field soil endemic with stem rot disease moderately inhibited sclerotial germination. Other organisms tested from those soils that did not inhibit sclerotial germination were five isolates of *Penicillium*, six isolates of *Paecilomyces*, nine isolates of bacteria, three isolates of *Cephalosporium*, two isolates of *Chaetomium*, three unidentified fungi, one actinomycete, one isolate from each of four *Aspergillus* groups *(A. wentii, A. glaucus, A. versicolor, A. terreus)*, and one each from *Cladosporium, Phoma, Mucor, Trichoderma viride* Pers. ex S. F. Gray, *Epicoccum, Alternaria alternata* (Fries) Keissler, *Gliomastix, Humicola, Coniothyrium, Stachybotrys atra* Corda, *Mortierella, Helminthosporium pedicellatum* Henry, *Nigrospora, Cylindrocarpon*, and *Fusarium oxysporum* Schlecht ex Fr.

Washed bacteria.—Because of the possible dilution of test-organism metabolites on agar media which could greatly reduce their effects on sclerotial germination, and because the presence of nutrients in the media could be stimulatory to sclerotial germination, washed bacteria were tested with sclerotia on media without added nutrients. Results of tests reported by Keim and Webster (5) showed that sclerotia of *S. oryzae* may lose endogenous substances equal to 50% of their total weight in water. These substances could be a suitable substrate for the growth of washed bacteria in the epispheres of the sclerotia. The method reported by Lingappa and Lockwood (9) was used and it consisted briefly of the following: cultures of the same bacterial isolates reported above were grown in Medium 523 broth (4), harvested by centrifugation and washed three times with cold 0.01 M-phosphate buffer (pH 7.2). The washed cells were then suspended in buffer and adjusted to an optical density of 1.0 at 500 nm in a Bausch and Lomb Spectronic 20 colorimeter zeroed with a buffer blank.

Tests of viability of bacterial suspensions were all positive. Ten ml of each undiluted suspension and suspensions diluted 1/10, 1/100 and 1/1,000 were placed in sterile petri dishes and incubated with surface-disinfested sclerotia for 14 days at 24 ± 2 C. After incubation, the germination of sclerotia on the test media was compared to that on sterile buffer and reported in Table 3. The results indicate that two of the nine bacterial

TABLE 1. Germination of sclerotia of *Sclerotium oryzae* on rice field soil, river sand, or silica sand

Substrate treatment	Germination (%) Distilled Water	Sterile Distilled Water
Rice field soil		
Autoclaved	99	96
Unautoclaved	12	2
River sand		
Autoclaved	98	99
Unautoclaved	93	98
Silica sand		
Autoclaved	99	99
Unautoclaved	97	99

TABLE 2. Germination of sclerotia of *Sclerotium oryzae* on variously prepared and treated soil leachates

Soil treatment before preparation of leachate	Leachate treatment	Germination (%)
Maintained dry at 1 C, then autoclaved	Autoclaved	98
	Not autoclaved	97
not autoclaved	Autoclaved	100
	Not autoclaved	97
Maintained 7 days with 40 P_w[a] at 24 ± 2 C, then autoclaved	Autoclaved, not filtered	91
	Autoclaved, filtered (0.45 μm)	68
	Not autoclaved not filtered	90
	Not autoclaved, filtered (0.45 μm)	83
not autoclaved	Autoclaved, not filtered	91
	Autoclaved, filtered (0.45 μm)	83
	Not autoclaved not filtered	9
	Not autoclaved, filtered (0.45 μm)	85

[a]P_w is the percent of water in soil on oven-dry basis.

TABLE 3. Inhibition of germination of sclerotia of *Sclerotium oryzae* by washed bacteria suspended in 0.01 M phosphate buffer (pH 7.2) compared to germination of sclerotia on buffer without bacteria

Washed bacterial isolates	Bacterial dilutions and inhibition[a] of sclerotial germination 1/1	1/10	1/100	1/1,000
4	0	0	0	0
5a + 5 b	0	0	0	0
8	0	0	0	0
6a + 10	–	1	1	1
3	2	2	1	0
1	2	2	2	1
9	2	1	1	1
2a + 2b	4	0	0	0
10	4	1	0	0

[a]0 = no inhibition; 4 = complete inhibition.

cultures completely inhibited sclerotial germination and that they were the same cultures which were inhibitory in the PDA pre-colonization tests. Four others of the nine bacterial cultures shown in Table 3 inhibited sclerotial germination to some extent.

DISCUSSION.—Sclerotia of *S. oryzae* germinated well on distilled water, indicating that they do not require exogenous nutrients for germination. The same situation was reported by Hsu and Lockwood (2) for sclerotia of *Macrophomina phaseoli, S. cepivorum,* and *S. rolfsii.* The addition of glucose to distilled water stimulated all sclerotia of *S. oryzae* to germinate, while the addition of activated charcoal increased germination to 96%, suggesting that the distilled water used may have contained an impurity which slightly inhibited sclerotial germination, and which could either be inactivated by charcoal or overcome by the stimulatory effect of glucose.

Since sclerotia of *S. oryzae* require no nutrients to germinate, they should be expected to germinate in moist soil, but evidence reported by Keim and Webster (5) shows that they do not, and that when in soil they are under the influence of soil fungistasis, as originally described by Dobbs and Hinson (1) and reviewed by others (3, 11, 14). The data indicate that fungistasis occurred in a rice field soil, but not in either silica sand or in river bottom sand, and that it could be annulled by autoclaving the soil as reported by Lingappa and Lockwood (8) for fungal spores, which suggests that the source of fungistasis is biological.

The results with soil leachates indicate that after drying the soil and holding it at 1 C to keep microbial activity minimal, no factor which inhibited sclerotial germination could be leached from the soil. However, samples of the same soil incubated moist at 24 ± 2 C contained a factor which could be leached from the soil and which was capable of almost completely inhibiting sclerotial germination. The possibility that the factor was of biological origin, is further supported by the evidence that autoclaving the soil before adding water, or by autoclaving the leachate, annulled most of the inhibitive influence. The evidence appears quite conclusive that the inhibitory factor which was leached was not an antibiotic compound resulting from biological activity but instead a cellular form of life that could be removed from the leachates by filtration (0.45 μm). Since the presence of the cellular organisms in the vicinity of the sclerotia seems necessary for inhibition of sclerotial germination to occur, fungistasis should either be a result of the removal by the cellular organisms of exogenous circumstances favorable for sclerotial germination such as a nutrient sink (2, 6, 9), or the production of inhibitory metabolites by the cellular organisms. The latter has been postulated by Lockwood (11), Jackson (3), Watson and Ford (14), and others; however, evidence strongly supporting the latter was reported by Keim and Webster (5) which showed that the inhibitory factor was retained in the interstitial surface structure of sclerotia after their removal from wet rice field soil. It is therefore probable that the metabolites responsible for stasis of sclerotia are produced by active cellular organisms in the soil and that the metabolites are only effective when produced in the immediate microhabitats (14) of those sclerotia affected. Otherwise their apparent ineffectiveness can be attributed to dilution, adsorption by soil clays, or degradation by other organisms (3).

The evidence reported here conclusively shows that several organisms which were isolated from rice land soil were capable of causing the formation of substances inhibitive to sclerotial germination as a result of colonizing certain substrates, and that washed bacteria could do so with only the exudate of sclerotia as a substrate. This evidence, and that reported by Keim and Webster (5), which showed that nearly complete inhibition of sclerotial germination occurred in the soil within 48 hours, point to bacteria as the primary factor in the soil fungistasis of sclerotia of *S. oryzae* which is an essential natural phenomenon for the survival of sclerotia while in the soil. However, the different levels of inhibition of sclerotial germination caused by different microorganisms suggest that the degree of stasis of sclerotia may differ according to the native microflora in different soils. It may only be sufficient to prevent them from germinating while in soils such as those endemic with stem rot disease, or it may be of sufficient magnitude to prevent them from germinating not only while in the soil, but after they have floated free of the soil (5) which could prevent the disease from becoming established. These results suggest that the microorganisms exhibiting inhibition of sclerotial germination should also be tested while under the influence of fungistasis of native soils, and that disease conditions in different rice soils should be correlated with the inhibitive capabilities of the microflora of those soils to help in formulating methods of biological control.

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