Conditioning Sclerotia of *Sclerotinia sclerotiorum* for Carpogenic Germination

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ABSTRACT

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Sclerotia from one isolate of *Sclerotinia sclerotiorum* produced on cornmeal/vermiculite medium were subjected to seven different treatments at 8 C over an 8-wk period to overcome dormancy and promote carpogenic germination. The conditioning treatments of the sclerotia included 1) none (immediate use), 2) incubating dry in petri plates, 3) incubating on moistened filter paper in petri plates, 4) burying in masonry sand in clay pots and leaching with water 2–3 times per week, 5) suspending in bags of fiberglass screen in a pipette washer and constantly rinsing, 6) placing in flasks of water under constant aeration, and 7) placing in flasks of water with no aeration. Following treatment, the sclerotia were incubated on moist sand at 20 C for 4–6 wk. Sclerotia that were not conditioned or conditioned dry in glass petri plates did not germinate. Carpogenic germination increased as the conditioning duration increased, and was greatest after 8 wk of conditioning in a constant rinsing environment. Carpogenic germination of an additional 24 isolates was subsequently examined at conditioning temperatures of 4, 8, 16, and 24 C in flasks of water under constant aeration. Germination of New York isolates was almost eliminated at 24 C, but isolates from other locations varied in their response to conditioning at high temperature. The optimal temperature range for conditioning of all isolates was 8–16 C. As sclerotial size increased, the number of germinating sclerotia and apothecia produced per sclerotium also increased.

Sclerotinia sclerotiorum (Lib.) de Bary is an important pathogen with worldwide distribution, and attacks over 360 species of plants in 64 families (24). Diseases caused by *S. sclerotiorum* are difficult to control and may result in substantial yield losses. In some crops, a combination of protectant chemicals and cultural practices that minimize disease development are deployed (6,9,26,29). The fungus produces sclerotia that serve as survival structures and in most cases are the source of primary inoculum. These sclerotia germinate by production of mycelia or apothecia, and infections are initiated by mycelia arising from the sclerotia or by ascospores produced in the apothecia (1,31). Mycelial infections are usually initiated at the soil line resulting in basal stem rots, or by root contact with myceliogenically germinating sclerotia (10,11). Infections aboveground, especially in inflorescences, are initiated by ascospores (1,3,7,12,21,26).

The production of inoculum of *S. sclerotiorum* is of utmost importance in breeding plants for resistance. Mycelial inoculum consisting of colonized tissues has been used to screen for resistance in beans, soybeans, and sunflower (15,20, 27). However, disease resistance ratings obtained from experiments in the laboratory using artificial inoculation methods, and disease resistance ratings obtained from the field with natural inoculum, do not always correlate (20,27).

Ascospores are the preferred source of inoculum in resistance screening and fungicide testing programs involving crops that are naturally infected by ascospores of *S. sclerotiorum*. However, there are many factors affecting the ability of sclerotia of *S. sclerotiorum* to carpogenically germinate, and the lack of a consistent, reliable method of production has limited ascospore use. Sclerotia of most isolates must be conditioned for a prescribed length of time to overcome dormancy and carpogenically germinate (5,31). Previous research has examined the role of temperature, substrate, inhibitors, light and moisture as conditioning treatments (4,8, 14,17,22,25,28,30).

The objective of this study was to develop a fast and reliable method of inducing sclerotia of *S. sclerotiorum* to consistently germinate carpogenically and produce ascospores for use in ongoing research and breeding efforts on snap beans and cabbage in New York. Ideally, the method developed would be used to carpogenically germinate a large number of isolates obtained from different hosts, geographic locations, and climatic conditions.

MATERIALS AND METHODS

Isolates and production of sclerotia. Isolate #25, originally obtained from snap beans in Wayne County, NY, was used in experiments to study the influence of conditioning temperature, methods and duration on carpogenic germination. Following these studies, 24 additional isolates of *S. sclerotiorum* were conditioned and induced to carpogenically germinate (Table 1). All isolates were maintained as dry sclerotia at 4 C.

For experimental use, cultures were initiated by placing a dry sclerotium on Difco Bacto potato-dextrose agar (PDA) in 9-cm-diameter petri plates incubated at 20 C. A 5-mm-diameter agar plug with actively growing mycelium was subsequently used to inoculate a fresh petri plate of PDA. After 2 days at 20 C, the agar surface was nearly covered with mycelium. The agar from each petri plate was cut into approximately 5-mm squares and distributed evenly among six glass petri plates (9 cm diameter, 2 cm deep) that contained cornmeal/vermiculite medium as described by Nelson et al (19). After a 2-wk incubation at 20 C, the cultures were stirred to break up the medium and redistribute the inoculum. The cultures were incubated an additional 5 wk. Sclerotia were washed in a No. 10 soil sieve (2 mm mesh) to separate them from the medium, and dried overnight at room temperature (20–25 C) prior to conditioning.

Effect of conditioning methods and duration on carpogenic germination. Sclerotia of isolate #25 were produced as previously described, and divided in 6 groups of 250 sclerotia each. Each group was further divided to make 5 replicates of 50 sclerotia, and subjected to a different method of conditioning at 8 C. Seven treatments were used: 1) none, sclerotia were tested for germination immediately after production on cornmeal/vermiculite medium; 2) sclerotia incubated dry in 9-cm-diameter glass petri plates; 3) sclerotia incubated on moistened filter paper in 9-cm-diameter glass petri plates; 4) sclerotia buried in 10-cm clay pots filled with masonry sand (the sand/sclerotia mixture in the clay pots was leached with 40 ml of distilled water three times per week for the first 4 wk, and two times per week thereafter); 5) sclerotia incubated in bags of fiberglass screening suspended in a pipette washer and subjected to constant rinsing at the lowest possible flow rate; 6) sclerotia incubated in 250-ml flasks filled to 200 ml with distilled water and constantly aerated by an aquarium pump and bubble stone; 7) sclerotia incubated in a 250-ml beaker containing 200 ml of distilled water (no aeration). Sclerotia were removed from all treatments at 1, 2, 4, and 8 wk after the

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onset of the conditioning treatments. Fresh replacement water was supplied at each interval when the sclerotia were removed to test germination (treatments 6 and 7). The conditioning experiment was repeated once. Data from each experiment were analyzed separately by analysis of variance. The Waller-Duncan *k*-ratio *t* test was used for mean separation of treatments when the overall *F* test was significant.

Induction of carpogenic germination. To induce carpogenic germination, 10 conditioned sclerotia from each treatment were placed on the surface of sterilized masonry sand in a covered clear plastic box. The sand (1 cm deep) was kept saturated with distilled water to approximate field capacity. The plastic boxes containing sclerotia on sand were placed in a growth chamber at 20 C with 12-h cycles of fluorescent light (120–132 uE m^{-2} s^{-1}). The sclerotia were observed for 4 to 6 wk and the cumulative number of sclerotia with one or more apothecia present was recorded.

Effect of conditioning temperature on carpogenic germination. The sclerotia of isolates #5 and #25 were produced as previously described, and sclerotia of each isolate were divided into four groups of 250 each. The groups were further divided to make five replicates of 50 sclerotia each. Each of these groups of 50 was enclosed in a 400 cm^2 piece of cheesecloth. The sclerotia were conditioned by placing one cheesecloth bag of each isolate in a 400-ml beaker with 300 ml of distilled water. An aquarium pump provided aeration to each beaker. Five replicate beakers containing each isolate were placed at 4, 8, 16, and 24 C. The distilled water in the beakers was changed three times weekly with fresh distilled water of the same temperature. After 1, 2, 4, and 8 wk of conditioning, 10 sclerotia were removed from each cheesecloth bag and tested for carpogenic germination. The experiment was repeated once, and the data from each experiment were analyzed separately by analysis of variance.

Effect of size of sclerotia on apothecia production. Sclerotia of isolate #25 were produced as previously described, and sorted into three groups according to size. The group classified as large was retained by a #4 soil sieve (4.75 mm mesh). The medium group passed through a #4 soil sieve but not through a #6 soil sieve (3.36 mm mesh). The small sclerotia were trapped by a #12 soil sieve (1.68 mm screen). A sample of 50 sclerotia was taken from each size group, and conditioned by constant rinsing in a pipette washer at 8 C for 4 wk. After conditioning, five replicates of 10 sclerotia of each size were tested for carpogenic germination. In addition, the number of apothecia per sclerotium was determined. The experiment was repeated once, and the data from each experiment were analyzed separately by analysis of variance.

Carpogenic germination of 24 isolates of *S. sclerotiorum*. Twenty-four isolates of *S. sclerotiorum* from various hosts and worldwide locations (Table 1) were cultured as previously described and 50 sclerotia of each isolate were conditioned in water under constant aeration (same as method #6) for 4 wk at 4, 8, 16, or 24 C. The sclerotia were enclosed in cheesecloth and placed in a 5-L container of distilled water. There were three replicate cheesecloth pouches for each isolate and all isolates were placed in the same container at each temperature. The distilled water was continuously aerated by an aquarium pump, and was changed twice each week with fresh distilled water of the same temperature. After 4 wk, 10 sclerotia from each replicate were tested for carpogenic germination. The experiment was repeated once. Data from each experiment were analyzed separately by analysis of variance.

Table 1. Isolates of *Sclerotinia sclerotiorum* used to study conditioning requirements for carpogenic germination

Isolate	Host	Location	Year of isolation	Source
5	Tomato	San Diego, CA	ND[z]	K. Kimble
6	Lettuce	Elba, NY	1975	G. Abawi
10	Grape	Hammondsport, NY	1973	G. Abawi
11	Dry bean	Nebraska	1974	J. Steadman
12	Dry bean	Idaho	1974	L. Tourneau
13	ND	Israel	1976	I. Chet
15	ND	Colombia	1978	G. Abawi
19	Sunflower	California	1979	M. Manel
20	Sunflower	Minnesota	1980	M. Manel
25	Snap bean	Wayne Co., NY	1982	L. Aude
29	Snap bean	Allegheny Co., NY	1982	J. Hunter
32	Tomato	Florida	1983	J. Hunter
36	Bulb celery	Holland	ND	A. Ester
38	Lettuce	Minneto, NY	1988	G. Abawi
40	Pea	Genesee Co., NY	1989	D. Legard
41	Snap bean	Orleans Co., NY	1989	J. Ludwig
42	Snap bean	Oneida Co., NY	1989	J. Ludwig
43	Snap bean	Wayne Co., NY	1989	J. Ludwig
46	Snap bean	Monroe Co., NY	1989	K. Czymmek
48	Snap bean	Orleans Co., NY	1989	K. Czymmek
49	Cabbage	Hamlin, NY	1989	A. Cobb
50	Snap bean	Oregon	1989	M. Powelson
54	Snap bean	Ontario Co., NY	1989	L. Pedersen
56	Snap bean	Monroe Co., NY	1989	K. Czymmek

[z] ND = not determined.

Table 2. The effect of conditioning method and duration on carpogenic germination of *Sclerotinia sclerotiorum*[y]

Treatment	Number of weeks conditioned				
	0	1	2	4	8
Constant rinse	0[z]	1.2 ab	4.8 a	8.0 a	9.8 a
Aerated water	0	1.8 a	2.2 b	5.4 ab	7.2 b
Moist paper	0	0 c	1.6 bc	6.0 a	7.0 b
Nonaerated water	0	0 c	0.2 d	1.8 cd	6.6 b
Leached sand	0	0.4 bc	0.8 cd	3.0 bc	3.6 c
Dry	0	0 c	0 d	0 d	0 d
None	0	0 c	0 d	0 d	0 d

[y] Isolate #25. All treatments conditioned at 8 C.

[z] Data from two experiments were combined for analysis. Values represent cumulative carpogenic germination after 4 wk incubation on moist sand at 20 C. Values are the mean number of germinated sclerotia from 2 tests with five replications per treatment consisting of 10 sclerotia each. Means within a column followed by the same letter are not significantly different ($P = 0.05$) according to the Waller-Duncan *k*-ratio *t* test.

RESULTS

Effect of conditioning method and duration on carpogenic germination. Sclerotia that were not conditioned or were conditioned dry for 8 wk in glass petri plates did not germinate carpogenically (Table 2). Carpogenic germination increased as the conditioning duration increased in all other treatments, and was greatest after 8 wk of conditioning in a constant rinse environment. There was no statistically significant difference in carpogenic germination after 8 wk of conditioning in the aerated water, moist paper, or nonaerated water treatments.

Effect of conditioning temperature on carpogenic germination. More sclerotia of isolate #5 germinated when conditioned at 4 or 8 C than when conditioned at 16 or 24 C (Table 3). However, isolate #25 germinated better when conditioned at 8 or

16 C than when conditioned at 4 or 24 C. Carpogenic germination of either isolate was low when conditioned at 24 C.

Effect of size of sclerotia on apothecia production. A greater proportion of large and medium size sclerotia germinated than small size sclerotia (Table 4). The number of apothecia produced per sclerotium was significantly greater for large sclerotia and decreased as the size of the sclerotium decreased. Small sclerotia produced about five times fewer apothecia than large sclerotia.

Carpogenic germination of 24 isolates of *S. sclerotiorum*. Carpogenic germination among 24 isolates varied in response to the four conditioning temperatures tested (Figs. 1, 2). The optimum temperature for conditioning of New York isolates was 8 C (Fig. 1A, B). Germination of New York isolates was almost eliminated when sclerotia were conditioned at 24 C. Overall germination of New York isolates was greater in the second trial. Isolates from other locations varied in response to the four conditioning temperatures tested, and there were some with high levels of germination when conditioned at 24 C (Fig. 2A, B). Isolates #6 and #20 germinated poorly in both trials (Figs. 1, 2). Among all isolates, there was variability between trial runs. The optimal temperature range for conditioning of all isolates was 8–16 C.

DISCUSSION

Previous attempts at mass production of ascospore inoculum have been hampered by unreliable carpogenic germination of sclerotia of *S. sclerotiorum*. Sclerotia of most isolates of *S. sclerotiorum* exhibit constitutive dormancy (5,23,31). In some isolates, dormancy was released by incubating sclerotia at low temperatures (13, 14,25,28,31) or in sand or soil (17,22) for various lengths of time. Occasionally, isolates are found that carpogenically germinate without conditioning when taken directly from pure cultures on agar media and incubated in water (13,18).

Steadman and Nickerson (30) examined a large number of inorganic and organic compounds for their ability to inhibit germination of sclerotia. Several compounds were inhibitory and some were comparable in effectiveness to commercial fungicides. Some sugars (glucose, sucrose, trehalose) were also inhibitory at high concentrations. As sclerotia develop in cultures or on natural substrates, small droplets of exudate are formed on the surface that contain salts, carbohydrates, lipids, amino acids, proteins, and enzymes (31). We hypothesized that if inhibitors were present in the dried exudates on the surface of the sclerotia or in the sclerotia, then leaching these inhibitors could break dormancy and allow the sclerotia to carpogenically germinate. Sclerotia carpogenically germinated in all treatments in which water was used in the conditioning proce-

Table 3. The effect of conditioning temperature and duration on carpogenic germination of *Sclerotinia sclerotiorum*

Isolate	Temperature (C)	Number of weeks conditioned 1	2	4	8
5	4	1.2 a[z]	0.8 b	5.8 a	8.2 a
	8	1.2 a	4.0 a	5.8 a	6.4 b
	16	0.2 a	1.2 b	1.0 b	1.4 c
	24	0 a	0 c	0.2 b	1.6 c
25	4	0 a	0 b	1.0 bc	1.0 c
	8	0.4 a	1.4 ab	2.6 b	3.2 b
	16	0.2 a	2.2 a	5.8 a	4.8 a
	24	0 a	0 b	0.2 c	0.2 c

[z] Data from two experiments were combined for analysis. Values represent cumulative carpogenic germination after conditioning in aerated water for the time indicated and incubation on moist sand at 20 C for 6 wk. Values are the mean of 2 tests with five replications per treatment consisting of 10 sclerotia each. Means within a column followed by the same letter are not significantly different ($P = 0.05$) according to the Waller-Duncan k-ratio t test.

Table 4. The effect of size of sclerotia of *Sclerotinia sclerotiorum* on production of apothecia[x]

Size of sclerotia	Number of sclerotia carpogenically germinated	Number of apothecia per sclerotium
Large[y]	9.8 a[z]	7.78 a
Medium	9.8 a	3.18 b
Small	7.6 b	1.52 c

[x] Isolate #25. All treatments conditioned at 8 C by constant rinsing in a pipette washer for 4 wk.

[y] Large sclerotia were retained by a 4.75 mm mesh soil sieve. Medium sclerotia pass through a 4.75 mm mesh soil sieve but not a 3.36 mm mesh soil sieve. Small sclerotia pass through a 3.36 mm mesh soil sieve but not through a 1.68 mm mesh soil sieve.

[z] Data from two experiments were combined for analysis. Values represent cumulative carpogenic germination after a 5 wk incubation on moist sand at 20 C. Values are the mean of two tests with five replications per treatment consisting of 10 sclerotia each. Means within a column followed by the same letter are not significantly different ($P = 0.05$) according to the Waller-Duncan k-ratio t test.

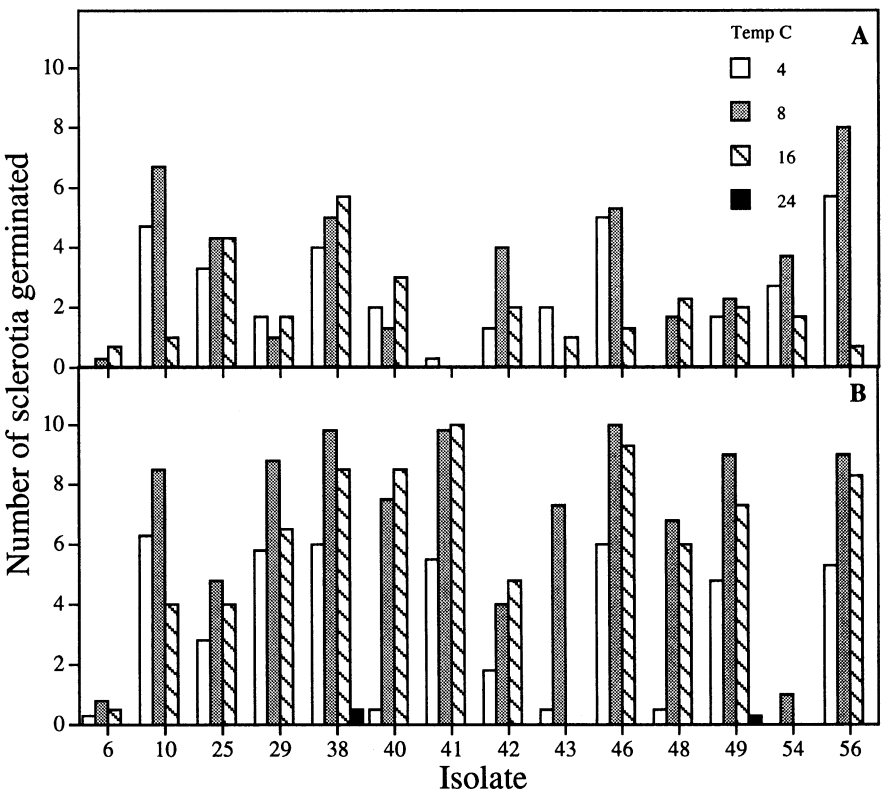

Fig. 1. The effect of temperature on carpogenic germination of isolates of *Sclerotinia sclerotiorum* from New York. All treatments were conditioned in water continuously aerated by an aquarium pump for 4 wk. Values represent cumulative carpogenic germination after 6 wk incubation on moist sand at 20 C. Values are the mean of three replicates consisting of 10 sclerotia each. **(A)** Trial 1. **(B)** Trial 2.

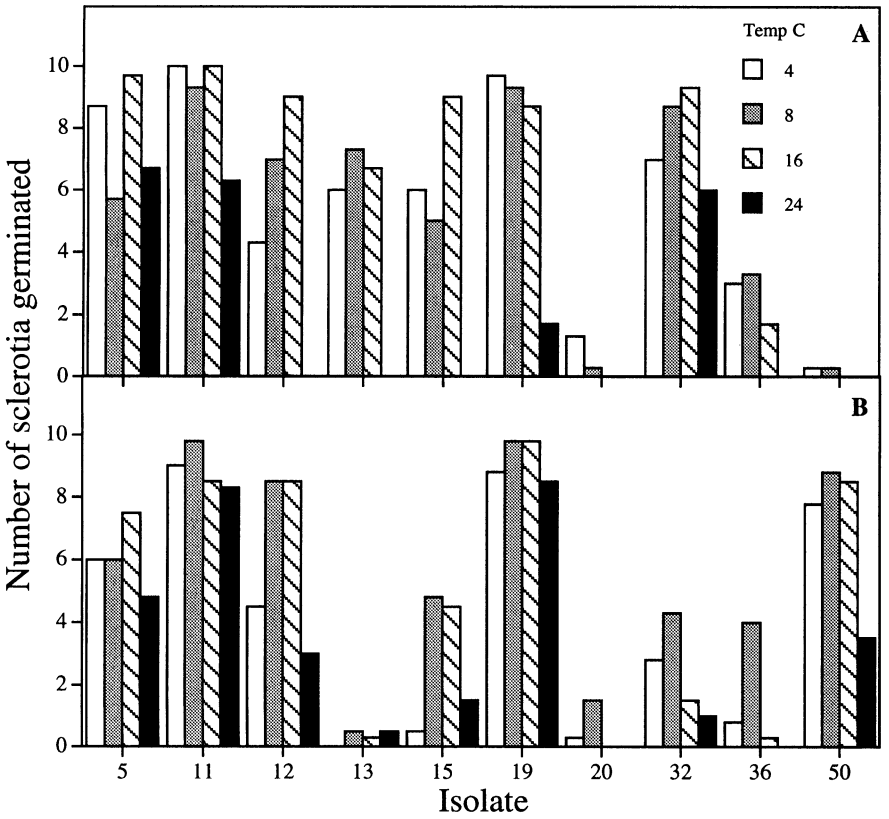

Fig. 2. The effect of temperature on carpogenic germination of isolates of *Sclerotinia sclerotiorum* from locations outside of New York State. All treatments were conditioned in water continuously aerated by an aquarium pump for 4 wk. Values represent cumulative carpogenic germination after 6 wk incubation on moist sand at 20 C. Values are the mean of three replicates consisting of 10 sclerotia each. **(A)** Trial 1. **(B)** Trial 2.

dure. Only sclerotia conditioned in a dry environment were unable to carpogenically germinate. These findings are consistent with those of Casale and Hart (4) who observed that pre-leached sclerotia carpogenically germinated earlier and in greater frequency (80%) than unleached sclerotia (10%).

The duration of the conditioning treatment may also influence the results. After 4 wk of conditioning, carpogenic germination was greatest in the constant rinse, aerated water, and moist filter paper treatments. After 8 wk of conditioning, additional sclerotia germinated in these treatments and in the nonaerated water treatment.

Although constant rinsing of isolate #25 for 8 wk resulted in a greater number of carpogenically germinating sclerotia, this method was not used to condition the 24 additional isolates of *S. sclerotiorum*. Alternatively, the aerated water method was used to condition the sclerotia for 4 wk for convenience and water conservation. As expected, no one conditioning temperature resulted in optimum carpogenic germination of all the isolates. The New York isolates either failed to germinate or germinated poorly when conditioned at 24 C whereas only 2 isolates from other areas (Minnesota, Holland) consistently failed to germinate after conditioning at 24 C. There was variability in germination between trial runs for which we have no explanation.

As sclerotial size increased, the number of germinating sclerotia and apothecia produced per sclerotium also increased. These results are in agreement with those of Ben-Yephet et al (2) who determined there was a significant increase in the number of apothecia produced per sclerotium with increasing sclerotial weight. Because of these results, we recommend avoiding the use of small sclerotia (<3 mm diameter) for ascospore production.

The aerated water method is currently used in our laboratory to condition sclerotia at 8 C and produce a year-round supply of ascosporic inoculum, some of which is stored on membrane filters as previously described (16). We adjust the duration of the conditioning from 4 to 8 wk, depending on the isolate, and apothecia are normally produced within 4 wk. A few isolates in our collection produce stipes during the longer conditioning period (6–8 wk), and subsequently bear apothecia within 2 wk upon exposure to light.

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