An in Vitro Technique for Large-Scale Production of Sclerotia of *Sclerotinia sclerotiorum*

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ABSTRACT

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An in vitro technique for large-scale production of sclerotia of *Sclerotinia sclerotiorum* was developed and tested with 12 isolates. The growth medium consisted of 54 g of cornmeal, 3.5 g of vermiculite, and 37.5 ml of a solution of 1% casamino acids and 1% yeast extract. These ingredients were mixed in a 946-ml canning jar, and the water potential was adjusted to −25 bars. Production of sclerotia by the 12 isolates ranged from a mean of 1,404 to 2,926 per jar of medium with weights ranging from 22.6 to 35.8 g. The mean sizes of sclerotia varied from 4.3 to 5.7 mm. In 12 jars of medium, 17,768 sclerotia were produced with isolate ND21. The procedure, termed the cornmeal-vermiculite technique, appeared to have no adverse effects on the biology of sclerotia.

Sclerotinia sclerotiorum (Lib.) de Bary is an important soilborne pathogen that causes diseases on a wide variety of crops (18,20). The fungus produces sclerotia, which have a major role in the biology of the organism (4,20). Sclerotia are the principal survival structures under adverse conditions (4). Inocula sources for most diseases caused by *S. sclerotiorum* originate from sclerotia that germinate myceliogenically or carpogenically (1,12,20).

When one studies the biology of *S. sclerotiorum* it is often desirable or necessary to produce sclerotia with an in vitro technique. Although many researchers have used techniques for producing sclerotia of *Sclerotinia* spp., they usually only mention the composition of the medium, and the temperature and time required for sclerotia formation (2,3,5,7–9,13,15,16). There are no reports that provide detailed descriptions of in vitro techniques for large-scale production of sclerotia that include production data plus the sizes, germinability, and pathogenicity of the sclerotia. This paper describes such a technique.

MATERIALS AND METHODS

Inoculum. An isolate of *S. sclerotiorum*, ND21, originally cultured from a sclerotium sieved from soil of a sunflower field in Larimore, ND, was used for most of this study. This isolate was demonstrated to be pathogenic on sunflowers and drybeans. In addition to ND21, 11 other isolates were used. Their designations with host and geographic origin were as follows: ND3, field pea, Pembina, ND; ND13, soybean, Page, ND; ND19, dry bean, Northwood, ND; ND23, soybean, Emerado, ND; ND24, soybean, Kempton, ND; ND40, dry bean, Fort Totten, ND; ND42, potato, Conway, ND; S1, lupins, Park Rapids, MN; S10, dry bean, western Nebraska; S11, sunflower, Woodland, CA; S12, tomato, Homestead, FL. Sclerotia of ND21 were obtained from artificially inoculated sunflowers (Hybrid 894) growing in a greenhouse, while sclerotia of all other isolates were from the original hosts. All sclerotia were maintained in the laboratory at room temperature.

Three sclerotia of each isolate were hydrated for 30 min in running tap water, surface sterilized in a 1:1 (v/v) solution of 95% ethanol and 5.25% sodium hypochlorite for 30 sec then air dried in a laminar flow bench for 5 min. The three sclerotia were then placed separately on potato-dextrose agar (PDA) containing 0.15 mg of tetracycline and 0.15 mg of streptomycin per milliliter of PDA. These cultures were incubated for 5–7 days at 20 C under a light bank with two 20 W cool-white fluorescent lights on a 12-hr light-dark cycle until the sclerotia germinated myceliogenically and mycelium covered the agar surface. Subcultures were prepared by removing one 15-mm-diameter agar disk with mycelium from each culture and placing the three disks together on PDA in 100-× 20-mm petri plates containing 30 ml of medium. These subcultures were incubated at 20 C for 7–10 days.

Preparation of medium. The medium consisted of 54 g of cornmeal (Quaker yellow enriched degerminated), 3.5 g of vermiculite (Terra Lite vermiculite, grade 2, Grace Horticultural Products, Cambridge, MA), and 37.5 ml of a solution of 1% casamino acids (Difco, Detroit, MI) and 1% yeast extract (Difco, Detroit, MI) in distilled water. These ingredients were thoroughly mixed in a standard 946-ml (one quart) canning jar and the water potential of the medium adjusted to −25 bars (10) by adding 38 ml of distilled water. Water potential was determined by thermocouple psychrometry (17). The medium weighed 121.4 g, and the volume was 270 cm^3. The jar lid had a 17-mm-diameter hole plugged with cotton and was covered with aluminum foil. The medium was autoclaved for 20 min, cooled, then stirred with a 1-cm-diameter aluminum rod to break up the solidified medium into a uniform, granular consistency. The medium was then reautoclaved and shaken vigorously to break up the medium a second time. The pH of the medium was 5.9.

Inoculation of medium and incubation of culture. One 7–10-day-old subculture of *S. sclerotiorum* on PDA was cut into 2–3-mm squares and added to the autoclaved medium. The jar was rolled and shaken gently to distribute the inoculum throughout the medium and then placed on its side in the incubator with the medium evenly spread over the side of the jar. The culture was incubated at 20 C for 1–3 mo under a light bank with two 20 W cool-white fluorescent lights on a 12-hr light-dark cycle (14). Every 2 days for the first 7–10 days the jar was shaken to break up the medium and distribute the mycelium. The jar was rolled over twice weekly during the first 4 wk. Sclerotia production was repeated 12 times with ND21, and three times with all other isolates over 6 mo.

Harvesting of sclerotia. When sclerotia were ready for harvest, the jar was gently shaken to break up the mass of sclerotia. The jar lid was replaced with a sterile aluminum screen (1.5-mm openings) held in place by a sterile jar ring. Three hundred milliliters of sterile distilled water was added to the jar and the contents swirled. The jar was inverted and shaken to allow the water to drain into a container. If the jar was not shaken, the contents would plug the screen, obstucting removal of the water. This procedure was repeated four to six times until the wash water was visibly clear. Washing removed the remnants of the cornmeal and some vermiculite. In the final wash the sclerotia were poured into a sterile, metal kitchen strainer (15 cm in diameter with 1.5 mm openings), then placed on a flat surface in a laminar flow bench to be separated from the remaining vermiculite and PDA. Fifty sclerotia from each jar were chosen at random and measured to determine the average size. All sclerotia were dried for 4 hr in the

laminar flow bench at room temperature (23–26 C), counted, weighed, then stored at 4 C in sterile 100- × 25-mm test tubes.

Germinability and pathogenicity of sclerotia. Myceliogenic germination of sclerotia of all isolates was tested within 7 days of harvest. Fifty sclerotia chosen at random from each jar were hydrated for 30 min in sterile distilled water. Sclerotia were then placed individually on 1.5% water agar in 16- × 150-mm test tubes and incubated at 20 C under the previously mentioned light-dark cycle. Germination was evaluated after 7–10 days. To determine if mycelium was originating from germinating sclerotia and not from mycelial fragments adhering to the sclerotia, one half of the sclerotia of ND21 in five germination tests were surface sterilized in the ethanol-sodium hypochlorite solution for 60 sec after hydration, air dried for 5 min, then placed on the water agar.

Sclerotia harvested from two jars of ND21 and stored for 5 and 7 mo, respectively, were tested for carpogenic germination. Twenty-five sclerotia from each harvest, ranging in size from 4 to 15 mm in diameter or length, were soaked in sterile distilled water for 72 hr at 4 C and then placed in 100 × 20-mm petri plates containing 10 ml of sterile distilled water. These sclerotia were incubated at 16 ± 2 C under two 20 W cool-white fluorescent lights on a 12-hr light-dark cycle. When apothecia formed and matured, petri plates containing PDA were inverted above two different groups of apothecia to catch ascospores as they were forcibly ejected from asci. The ascospores were incubated at 22 C for 24 hr, then 200 ascospores from each group of apothecia were examined microscopically for germ tube formation as evidence of germination.

Pathogenicity of sclerotia of ND21 harvested from three jars and stored for 2, 4, and 6 mo was tested on sunflower Interstate 894 in the greenhouse. Plants were grown in a pasteurized potting mix (equal parts of Glyndon sandy loam, peat moss, and vermiculite) in 15.5-cm diameter clay pots and received 15 hr of lighting from high pressure sodium lamps (1,000 μE $m^{-2}sec^{-1}$). Greenhouse temperatures ranged from 19 to 25 C. Plants were inoculated at the R-2 growth stage (19). Sclerotia, 3–6 mm in size, were hydrated in sterile distilled water for 30 min then appressed to 3-mm-diameter punture wounds on the stems, one sclerotium per plant, about 4 cm above the soil surface. Sclerotia were held in place by tightly wrapped Parafilm strips. The stems were sprayed to runoff with sterile water before inoculation. Twenty sunflowers were inoculated in each of the three pathogenicity tests. Controls were treated similarly but without the sclerotia. Although infection of sunflower by sclerotia normally occurs on the roots (12), the stem inoculation technique was used because it is easier and results in more rapid disease development than root inoculations (Berlin Nelson, *unpublished*).

RESULTS AND DISCUSSION

Sclerotia of all 12 isolates of *S. sclerotiorum* were produced in large numbers with this technique (Table 1). Production ranged from a mean of 1,404 (S12) to 2,926 (ND44) sclerotia per jar with weights of sclerotia ranging from a mean of 22.6–35.8 g. The mean sizes of sclerotia varied from 4.3 to 5.7 mm, and most were either round or oblong. The large-scale production of sclerotia was demonstrated with ND21, where 17,768 sclerotia weighing 279.7 g were produced from 12 jars of media.

All sclerotia tested for germinability on water agar germinated myceliogenically within 4–7 days. Germination was hyphal as described by Adams and Tate (6), which is in agreement with other reports on in vitro myceliogenic germination of sclerotia of *S. sclerotiorum* (6,11). No eruptive germination was observed. Sclerotia produced in this study showed no dormancy for myceliogenic germination. This is in contrast to the report by Huang (11) who stated that sclerotia of three isolates of *S. sclerotiorum* that were produced on PDA showed marked dormancy for myceliogenic germination when placed on moist sand.

Sclerotia of ND21 that were surface sterilized in five of the germination tests germinated as well as nonsurface sterilized sclerotia, indicating that mycelium originated primarily from within the sclerotia and not from mycelial fragments on the surface that might have been carried over as debris from the medium. Surface sterilization appeared to stimulate germination because there was more profuse hyphal growth from surface sterilized sclerotia.

All sclerotia of ND21 inoculated onto sunflower were pathogenic. The 60 plants wilted within 30 days after inoculation, while the controls remained healthy. These results indicated that the production method did not affect pathogenicity of sclerotia and, furthermore, indicated that storage at 4 C for 2–6 mo did not reduce the high germination demonstrated immediately after harvest.

Apothecia were produced by 14 of 25 and six of 25 sclerotia of ND21 stored for 7 and 5 mo, respectively, following 55 days of incubation. Stipe formation began in 30 days, and the first apothecia with mature asci were observed in 40 days. New stipes were still forming at 55 days. Ascospores were trapped on PDA, and 91% germinated after 24 hr.

Cornmeal provided a nutritive medium for growth of *S. sclerotiorum*. Adams and Tate (5) used cornmeal mixed with sand to produce sclerotia of *S. minor*. The cornmeal in this study was supplemented, however, with the yeast extract-casamino acids solution to increase the growth rate of mycelium and reduce the C/N ratio. The vermiculite was an important component of the

TABLE 1. Production of sclerotia of *Sclerotinia sclerotiorum* with the cornmeal-vermiculite technique

Isolate	Incubation (days)	Sclerotia/270 cm^3 of medium[a]				
		Mean no. produced	Range of production	Mean wt (g)[b]	Mean size (mm)[c]	Mean (%) germination[d]
ND3	33	1,424	1,323–1,484	34.7	5.3	100
ND13	39	1,544	1,908–2,335	29.6	5.5	100
ND19	35	1,779	1,530–2,045	22.6	5.0	100
ND21	27–104	1,480	729–2,324	23.3	5.1	100
ND23	39	2,477	2,172–2,944	26.1	5.5	100
ND24	49	1,550	1,430–1,644	35.8	5.7	100
ND40	42	1,811	1,642–1,980	24.9	5.4	100
ND42	35	2,612	2,112–3,455	27.2	4.3	100
S-1	35	2,926	2,820–3,051	32.1	5.4	100
S-10	42	2,842	2,766–2,923	31.3	4.6	100
S-11	35	2,426	2,206–2,582	33.0	5.2	100
S-12	45	1,404	1,805–2,185	26.0	4.8	100

[a] The medium consisted of 54 g of cornmeal, 3.5 g of vermiculite, and 37.5 ml of a solution of 1% casamino acids and 1% yeast extract in distilled water. These ingredients were mixed in a 946-ml jar, the water potential was adjusted to −25 bars and the medium (270 cm^3) was autoclaved twice. The medium was seeded with a 7- to 10-day-old culture of *S. sclerotiorum* on PDA and then incubated at 20 C.

[b] Mean values represent three replications per isolate, except for ND21, which had 12 replications.

[c] Sizes are either the diameter or length, whichever was greatest. Based on 50 sclerotia per replication.

[d] For each replication, 50 sclerotia were evaluated for myceliogenic germination on water agar at 20 C.

medium because it helped maintain a friable consistency, which was essential for mixing during colonization by the fungus.

The procedure used to distribute the mycelium throughout the medium during the first 10 days of incubation facilitated colonization of the cornmeal and was necessary for uniformity in sclerotia morphology and the high production in numbers of sclerotia, especially of the 3–6 mm size. When there was no mixing, many large, odd-shaped sclerotia developed and parts of the medium were not colonized during the first 4–5 wk of incubation. Turning the cultures prevented formation of sclerotia with incomplete rinds, which occurred when sclerotia were in continuous contact with the glass jar.

Maximum numbers of sclerotia with well-developed black rinds were formed after 4–5 wk of incubation. After this, there was rarely any further sclerotia production. The medium, permeated with mycelium, eventually became a single mass of sclerotia. These sclerotia were ready for harvest when a gentle shaking of the jar broke up the mass of sclerotia. Delaying harvest for 1–2 mo appeared to have no adverse effects on sclerotia.

This procedure, termed the cornmeal-vermiculite technique, allowed for production of large quantities of sclerotia of *S. sclerotiorum* in small amounts of media and was tested with a wide variety of isolates of the fungus from different hosts. The technique did not appear to have any adverse effects on the biology of sclerotia and most of the sclerotia were a convenient size for handling and experimentation. The materials required were readily available and the procedures were easy to follow.

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