Preservation of Ascospores of *Sclerotinia sclerotiorum* on Membrane Filters

J. E. Hunter, J. R. Steadman, and J. A. Cigna

First and third authors, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456; second author, Department of Plant Pathology, University of Nebraska, Lincoln 68583-0722.

Published as Journal Series Paper 6688, Nebraska Agricultural Experiment Station and Journal Series Paper 3378, New York State Agricultural Experiment Station, Geneva.

The technical assistance of Larry Einemann is gratefully acknowledged.

Accepted for publication 7 September 1981.

ABSTRACT

Hunter, J. E., Steadman, J. R., and Cigna, J. A. 1982. Preservation of ascospores of *Sclerotinia sclerotiorum* on membrane filters. Phytopathology 72:650-652.

More than 90% of the ascospores of *Sclerotinia sclerotiorum* collected from laboratory-produced apothecia, dried on Millipore membrane filters, and stored over calcium chloride desiccant in a closed vessel survived for 24 mo when refrigerated or frozen. A higher percentage of spores also survived without a desiccant in a freezer. With a desiccant, survival of freshly collected spores at 25 C was adequate for ascosporic inoculum to be shipped to workers who lack the facilities or experience to germinate sclerotia carpogenically. Isolates from different hosts and geographical locations survived similarly. Stored ascospores that were still germinable were as effective as freshly collected ascospores in inducing disease on beans (*Phaseolus vulgaris*). Germinability of stored ascospores was greater on a nutrient-rich medium, such as potato-dextrose agar, than in water.

Ascospores of *Sclerotinia sclerotiorum* (Lib.) de Bary are the primary inoculum for epidemics of white mold of beans (*Phaseolus* spp.) (1,6,12). To simulate natural infection for greenhouse fungicide and disease resistance tests, Abawi et al (2,3) produced apothecia in the laboratory, collected the ascospores in a vacuum-operated water trap, and sprayed a suspension of spores on blossoming plants. Inoculated plants were incubated in a greenhouse mist chamber. Ascospores were collected fresh for each test. To avoid wetting the spores, Steadman and Cook (11) discharged ascospores directly onto a membrane filter in a funnel attached to a vacuum pump. They stored the membrane filters with spores in petri dishes at 22 C, but within 3 mo the percentage of viable spores had decreased to 50% and after 10 mo to 0%.

Techniques to preserve germinability of a high percentage of spores have not been studied extensively, whereas cultures of numerous fungi have been preserved by drying, freezing, or freeze-drying (7,9). Slow drying of liquid cultures of aquatic fungi onto filter paper is one of the simpler methods used for long-term storage of cultures (8). Several workers preserved cultures by pipetting suspensions of conidia or mycelial fragments into tubes containing anhydrous silica gel particles and storing these at room, refrigerator, or freezer temperatures (4,10,13). Virus cultures have been preserved for several years over calcium chloride in closed containers (5).

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0031-949X/82/06065003/$03.00/0

Our objective was to use the simplest of these methods to preserve viable ascospores for 1–2 yr to ensure that ascosporic inoculum would always be available for experiments.

MATERIALS AND METHODS

Collection of ascospores in water. Ascospores were collected in distilled water by removing the cover of petri dishes containing apothecia and quickly covering the dish with a funnel attached to a vacuum-operated water trap (2). Within a few minutes the spores were removed from the water by using a Millipore membrane filter (type GS, 47 μm). After drying overnight in a laboratory at ~ 25 C and 10% relative humidity, the membrane filters with the spores were placed in standard 20–30 ml screw cap vials. Half of the vials were approximately half full of calcium chloride pellets that were covered with cotton to prevent direct contact between the spores and the $CaCl_2$. Equal numbers of vials with and without $CaCl_2$ were stored at room temperature (25 ± 2 C), refrigerator temperature (2 ± 2 C), and in a freezer at −19 ± 1 C. Immediately and after 1, 2, 4, 8, 12, and 24 mo, sectors of membrane filters stored under the various conditions were soaked briefly in water and the ascospores were removed by scraping the submerged membrane with a spatula. A 0.5-ml suspension of ascospores was spread over the surface of potato-dextrose agar (PDA) in a petri dish and incubated at 24 C. The percentage of spores that germinated was determined by counting 200 spores after 6 hr of incubation for the 0- and 1-mo samples, and after 16 hr of incubation for older spores. The experiment was conducted twice with ascospores collected at two dates. In both instances, sclerotia used to produce apothecia were

collected from naturally infected snap bean plants in New York.

Pathogenicity of ascospores collected in a water trap and stored for 18 mo was determined by spraying blossoming bean plants (*Phaseolus vulgaris* L.) with a suspension of spores. After determining the percentage of germination, spore counts were adjusted with the aid of a hemacytometer to a concentration of 2,000 germinable spores per milliliter. Inoculated plants were incubated for 7 days in a greenhouse mist chamber at ~24 C and then rated for disease. For comparison, plants were sprayed with a suspension of 2,000 freshly collected spores per milliliter.

Collection of dry ascospores. Ascospores were collected in a dry state directly on a Millipore filter (type HA, 0.45 μm) by inverting a modified Millipore funnel over an opened petri dish containing apothecia and applying a vacuum (11). The membrane filters with spores were held in a plastic petri dish at 22–24 C and ambient relative humidity until spores were collected on four to five filter disks (1–3 days). Each petri dish was placed in a desiccator containing calcium chloride, and refrigerated. Immediately, and after 0.5, 1, 9, 12, 18, 24, and 48 mo, small sectors of the membrane filters were soaked in water for a few minutes and the spores were dislodged with a camel's-hair brush. Subsamples of the spore suspension were held in water and also placed on PDA on microscope slides. The percentage of spores that germinated was determined after 6–18 hr of incubation at 23 C. Storage trials with spores collected dry were conducted with isolates of *S. sclerotiorum* from bean, lettuce, and soybean obtained from Nebraska, Arizona, and Colorado, respectively.

Pathogenicity of ascospores collected dry and stored for 24 mo was determined by placing bean blossoms in petri dishes containing ascospores suspended in a film of water. After colonization, the blossoms were placed on bean plants in a dew chamber at 24 C for 3-4 days. For comparison, blossoms were exposed to freshly collected ascospores and plants inoculated similarly.

RESULTS AND DISCUSSION

Ascospores survived the longest when stored over calcium chloride in the refrigerator or the freezer (Table 1), although the necessity for the desiccant at −19 C is uncertain. Without a desiccant, spores from the first collection date survived in the freezer for 12 but not 24 mo, whereas a high percentage of those collected on the second date germinated after 24 mo. Survival for 24 mo was excellent in the refrigerator, but only when a desiccant was used. The presence of the desiccant seemed to prolong spore survival at room temperature, but long-term survival at room temperature, even with a desiccant, was poor.

Spores collected in water on the second trial survived better than the first. This might have been due to lack of standarization of the drying conditions prior to placing the spores in the desiccator vials or to differences in spore maturity at the time of collection. Disintegration of dead spores is the most likely explanation for the apparent increase in percentage survival with increased time that occurred in some instances.

Survival of spores that had been collected dry and stored in a refrigerator was similar to those collected in water and stored under similar conditions (Table 2). Possibly the length of survival could be increased by storing dry-collected spores in a freezer. The percent germination was lower in water than on PDA; therefore, that is recommended for testing spore viability.

Bean plants inoculated with ascospores collected in water and stored for 18 mo developed the same severity of disease as those inoculated with freshly collected ascospores. Similarly, ascospores collected dry and stored for up to 24 mo readily colonized bean blossoms and subsequently infected bean plants.

Data similar to those in Table 2 were obtained for isolates collected from soybean in Colorado and from lettuce in Arizona. Thus, it appears that ascospores of various isolates of *S. sclerotiorum* can be preserved by a combination of drying and low temperature, regardless of the host plant or geographical location from which they are collected. Also, collection of spores in water or on a dry filter did not appear to affect their viability. However, clumping of spores occurred with the water-trap method, making it difficult to prepare a uniform suspension of ascospores for inoculation.

Unlike earlier studies (7,9) with other fungi in which long-term survival was based upon regeneration of cultures when transferred to a suitable environment, without testing survival of individual

TABLE 1. Effect of calcium chloride desiccant and temperature on the survival of stored ascospores of *Sclerotinia sclerotiorum* initially collected in water[a]

Trial number	Storage temp (C)	Survival of stored ascospores after: 0 Mo	1 Mo		2 Mo		4 Mo		8 Mo		12 Mo		24 Mo	
			D[b]	ND	D	ND	D	ND	D	ND	D	ND	D	ND
1	25 ± 2	100[c]	78	0	88	0	88	0	98	0	90	0	0	0
2	25 ± 2	91	49	17	49	9	53	0	14	0	0	0	0	0
1	2 ± 2	100	94	0	98	9	93	0	100	0	100	0	98	0
2	2 ± 2	91	100	58	92	44	95	86	89	8	82	3	93	80
1	−19 ± 1	100	99	91	99	98	98	52	100	99	100	100	72	0
2	−19 ± 1	91	100	97	98	88	96	96	93	86	87	69	90	88

[a] Sclerotia used to produce apothecia were collected from naturally infected snap bean plants in New York. Ascospores were collected in a water trap and recovered on membrane filters. After drying overnight, the filters were placed in screw cap vials with or without calcium chloride desiccant.
[b] D = desiccant; ND = no desiccant; desiccant in the bottom of the vial was separated from the membrane filters by cotton.
[c] Percentage of dried ascospores that germinated when suspended in water, spread over the surface of potato-dextrose agar and incubated at 24 C for 6 hr for the 0- and 1-mo samples and 16 hr for older samples.

TABLE 2. Survival of ascospores of *Sclerotinia sclerotiorum* collected dry on membrane filters and stored with calcium chloride desiccant in petri dishes at 4 C[a]

Germination medium	Survival of stored ascospores after: 0 Mo	1 Mo	9 Mo	12 Mo	18 Mo	24 Mo	48 Mo
Potato-dextrose agar	97 ± 3[b]	82 ± 10	84 ± 5	82 ± 8	63 ± 29	67 ± 23	48 ± 7
Water	65 ± 14	···	61 ± 15	63 ± 20	49 ± 33	44 ± 23	3 ± 3

[a] Sclerotia used to produce apothecia were produced from a bean isolate collected in Nebraska. Ascospores were collected by pulling a vacuum through a Millipore filter in a funnel placed over the top of a petri dish containing apothecia. Filter disks with ascospores were stored in petri dishes in a desiccator containing calcium chloride that was held in a refrigerator.
[b] Percentage of ascospores that germinated; each value ± one standard deviation is based upon the average of 7–15 trials.

propagules, results of our studies show that a high percentage of ascospores of *S. sclerotiorum* remained viable and capable of infecting plants for at least 24 mo when stored over calcium chloride in a refrigerator or freezer. Thus, this method can be used to ensure the availability of ascosporic inoculum, whereas dependence upon a continuous supply of fresh ascospores is less certain because the ability to consistently produce the sexual stage of *S. sclerotiorum* varies with different isolates and with unknown factors. Another important feature of this method is that it makes possible the shipment of ascosporic inoculum to workers who lack the facilities or experience to germinate sclerotia carpogenically, since spores dried over calcium chloride survived at room temperature for 1–12 mo.

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