Collection, Viability, and Storage of Ascospores of *Monilinia oxycocci*

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ABSTRACT

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Ascospores of *Monilinia oxycocci* were collected from apothecia onto membrane filters. Germination on potato-dextrose agar and fluorescence after staining with fluorescein diacetate were compared as methods for determining ascospore viability. Estimates of viability by the two methods were similar. Ascospores were stored for 24 mo with or without the desiccants $CaCl_2$ or $CaSO_4$ in four locations: a constant-temperature deep freezer at −20 C, a frost-free freezer at −20 C, a refrigerator at 4–6 C, and a laboratory drawer at room temperature (21–25 C). Ascospores survived best when stored desiccated in the deep freezer and did not survive at room temperature. A desiccant was necessary for ascospore survival in either of the freezers, but the type of desiccant made no difference. Viability of ascospores stored in the deep freezer over $CaSO_4$ was tested at 6, 12, and 24 mo and compared with that of freshly collected ascospores. The proportion of ascospores that was viable decreased by over 60% during the first 6 mo of storage; however, no further decrease was detected thereafter. Ascospores of *M. oxycocci* can be collected and preserved in a viable state for at least 24 mo if stored in a constant-temperature deep freezer at −20 C over a desiccant such as $CaCl_2$ or $CaSO_4$.

Additional keywords: cranberry cottonball, *Vaccinium macrocarpon*.

Monilinia oxycocci (Wor.) Honey is the causal agent of cranberry cottonball, the most important disease affecting cranberries (*Vaccinium macrocarpon* Ait.) during the growing season in Wisconsin. Pathogenesis by *M. oxycocci* on cranberry is similar to that by other species of *Monilinia* that attack ericaceous hosts (2,5,7) and is characterized by two distinct phases (1,13–15,19). Primary infection by ascospores results in a blight of shoots and flowers. Secondary infection of flowers by conidia results in fruit rot. *M. oxycocci* is one of four species of *Monilinia* with dimorphic (i.e., both large and small) ascospores, which occur in equal numbers in the ascus (2–4,19). Small ascospores have been reported to be nonviable and to degenerate shortly after release from asci (19).

To study primary infection of cranberry by *M. oxycocci* under controlled conditions, a ready supply of ascospores is needed. In Japan, apothecia of several species of *Monilinia*, including *M. oxycocci*, have been produced in vitro (5,6). To date, we have been unable to duplicate these results with isolates of the fungus from Wisconsin or to produce apothecia from ungerminated sclerotia collected in the field. However, apothecia do develop from sclerotia that germinate in the field. Consequently, inoculum would be available if viable ascospores could be collected from these apothecia and stored until needed. Ascospores of *Sclerotinia sclerotiorum*, a related fungus, have been collected from apothecia and remained viable for up to 24 mo (8,17). Similar methods may be useful for collecting and storing ascospores of *M. oxycocci*.

The purpose of this study was to determine whether ascospores of *M. oxycocci* could be harvested routinely from apothecia that developed from germinated, field-collected sclerotia and, if so, how they could be preserved most effectively.

MATERIALS AND METHODS

Collection of ascospores. Sclerotia of *M. oxycocci* that had germinated and produced rudimentary to immature apothecia were collected from a commercial cranberry field (cultivar Bain McFarlin) in April and May 1988 and May 1990 and were stored in a refrigerator (4–6 C) to retard development until needed. They then were placed in glass crystallizing dishes (90 × 70 mm) containing fine-texture vermiculite. Ten to 15 sclerotia were buried 1–2 cm apart in each dish with the upper surface just covered. The vermiculite was saturated with deionized water, and dishes were covered and sealed with Parafilm (American Can Co., Greenwich, CT). Dishes then were placed in a growth chamber at 16 C with a 12-h photoperiod (5,6,10) until apothecia matured.

A technique developed for *S. sclerotiorum* (8,17) was used to collect ascospores of *M. oxycocci* from mature apothecia directly onto 0.45-μm Millipore (Millipore Corp., Bedford, MA) or Nucleopore (VWR Scientific, Chicago, IL) membrane filters (47 mm in diameter). An inverted Millipore funnel was clamped to a ring stand and attached directly to a vacuum. Lids were removed and dishes containing apothecia were quickly placed under the funnel while a vacuum was applied. Dishes were removed after 1–2 min, and lids were replaced and resealed with Parafilm. Immediately after ascospores were collected, dishes were returned to the growth chamber. Each filter was used to collect spores from one to several dishes, depending on the extent of ascospore discharge (puffing) observed, and was considered an independent collection of a heterogeneous population of ascospores. Ascospores were collected three to four times per day, at 3- to 4-h intervals, until apothecia began to desiccate, which was usually 7–10 days after initial ascospore collection. Approximately 60 and 20 ascospore collections were made in 1988 and 1990, respectively.

Viability of ascospores. To test viability, spores on one-third to one-half of a filter were used to prepare a suspension. Filter sections were cut into small pieces (about 0.25 cm^2) and placed into 16-mm test tubes containing 2–3 ml of sterile, deionized water. Tubes were agitated vigorously for 1–2 min with a vortexing mixer. Viability of ascospores in the resulting suspensions was tested by two methods, germination on potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) or fluorescence of fluorescein after hydrolysis of the nonfluorescing vital stain fluorescein diacetate (FDA) (11,12).

In the first method, a 0.5-ml aliquot of spore suspension was spread onto each of two or three 9-cm-diameter petri dishes

containing PDA. Dishes were kept on a laboratory bench at room temperature (21–25 C) for 24–48 h, after which several drops of a 0.05% solution of aniline blue in lactophenol were placed on the agar surface to fix and stain ascospores. Dishes were examined directly with bright field microscopy (320×). On each dish, all germinated ascospores and ungerminated large ascospores were counted in successive traverses across the dish until a combined total of at least 100 spores had been observed. Germinated ascospores were assumed to be large ascospores, and the proportion of large ascospores that had germinated (i.e., produced a hypha more than twice the length of the spore) was calculated.

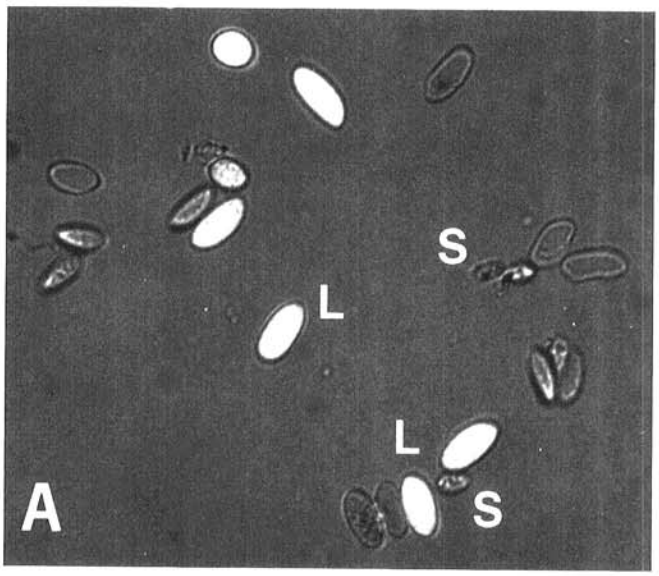
In the second method, a drop of freshly diluted FDA was added to a drop of spore suspension on a microscope slide. The mixture was covered with a cover glass and incubated for 10 min. Ascospores were observed with a compound microscope (250×) illuminated simultaneously with a xenon arc lamp with appropriate filtration and low-intensity bright field light so that both fluorescing and nonfluorescing spores could be counted. All large ascospores were counted in successive traverses across the slide until at least 100 spores had been observed, and the proportion that fluoresced was calculated. Results from two or three replicate aliquots of each spore suspension were averaged to estimate viability.

To compare the two methods, both were used to assess the viability of ascospores from the same filters with paired samples. The comparison was repeated nine times, each time with a different filter that had been stored for a period of time in one of several environments (see below). Mean proportions of ascospores determined to be viable by each method from all nine filters were compared with a paired Student's t test. The two methods also were compared across the range of observed mean values by regression analysis.

Storage of ascospores. An initial attempt to store ascospores of *M. oxycocci* was unsuccessful; none of the spores remained viable after 6 mo in a frost-free freezer (of a refrigerator/freezer) at −20 C when stored over $CaCl_2$, the desiccant previously used to store ascospores of *S. sclerotiorum* (8). It was suspected that the frost-free freezer impaired ascospore viability because these freezers cycle heating and cooling to prevent frost accumulation; for example, temperatures in containers holding petri dishes with membrane filters rose to −4 C for about 1 h during each 24-h period. In contrast, ordinary deep freezers maintain a relatively constant temperature (±2 C). To determine whether the type of freezer or desiccant affected viability of ascospores, these two types of freezers and two commonly used laboratory desiccants, $CaCl_2$ and $CaSO_4$, were compared.

Single filters with ascospores were placed arbitrarily in individual petri dishes with or without desiccants as ascospores were collected. All filters were placed on paper disks to prevent direct contact between filters and the desiccants or petri dishes. Dishes containing desiccant then were sealed with Parafilm and placed in large glass desiccators containing the same desiccant. Dishes without desiccant were left unsealed so they would be exposed to ambient relative humidities. Desiccators and unsealed dishes were placed in one of four locations: a laboratory bench drawer at room temperature (21–25 C), a refrigerator at 4–6 C, a frost-free freezer at −20 C, and a walk-in deep freezer at −20 C.

After 24 mo in storage, ascospores on at least two filters from each of the different environments were tested for viability with FDA. Because all combinations of storage location and desiccant were not included in this study, two subsets of treatments, in



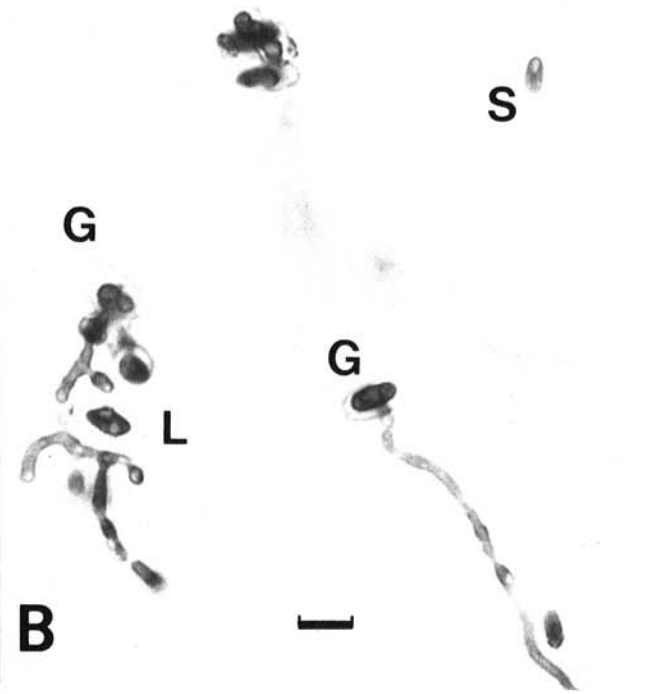


Fig. 1. Ascospores of *Monilinia oxycocci* washed from membrane filters. **A,** Ascospores stained with the vital stain fluorescein diacetate; large ascospores (L) fluoresced, whereas small ascospores (S) did not. **B,** Germinated (G) and ungerminated large (L) and small (S) ascospores after 48 h on potato-dextrose agar; spores were stained with 0.05% aniline blue in lactophenol. Bar = 10 μm.

TABLE 1. Comparison of germination on potato-dextrose agar (PDA) and fluorescence after hydrolysis of fluorescein diacetate (FDA) as methods for assessing viability of stored ascospores of *Monilinia oxycocci*[a]

Storage location[b]	Desiccant	Storage duration (mo)	Ascospore viability (%)[c]	
			PDA	FDA
NS	None	0	35.1	58.8
NS	None	0	76.6	84.7
DF	$CaSO_4$	6	28.4	32.7
DF	$CaSO_4$	6	28.2	24.3
DF	$CaSO_4$	6	13.9	13.3
DF	$CaSO_4$	24	30.9	46.8
FF	None	24	0	0
FF	$CaSO_4$	24	17.4	22.2
FF	$CaCl_2$	24	20.5	8.1
			$t^d = -1.04$	$P = 0.33$

[a] Ascospores collected on membrane filters were stored for 0, 6, or 24 mo with or without desiccant in either of two freezers at −20 C.
[b] Ascospores on nine separate membrane filters were: not stored (NS), i.e., were assessed immediately after collection; stored in a frost-free freezer (FF); or stored in a constant-temperature deep freezer (DF).
[c] Viability was determined by each method with paired samples from aqueous suspensions of ascospores.
[d] Paired Student's t statistic and its significance; proportions were transformed to arcsine-square root values before analysis.

which all levels of both factors were represented, were compared with separate two-way analyses of variance (ANOVA). In the first subset, the effects of freezer type and desiccant ($CaSO_4$, $CaCl_2$, or none) were examined. In the second subset, the effects of storage location and storage with or without a desiccant ($CaSO_4$) were examined. In addition, the effect of storage duration on viability was evaluated with ascospores that had been stored for 0, 6, 12, or 24 mo over $CaSO_4$ in the deep freezer. Mean proportions of viable ascospores were determined from individual membrane filters with FDA, and, again, at least two filters were used for each duration. Treatments were compared with a one-way ANOVA.

Data analysis. All proportions were transformed to arcsine-square root values (16) prior to analyses. Data were analyzed with MINITAB statistical software, release 7.2 (Minitab, Inc., State College, PA). If significant interactions were found in two-way ANOVAs, treatment main effects were not examined; instead, individual treatment means were compared. Means were separated by Fisher's protected least significant difference with $P = 0.05$.

RESULTS

Collection of ascospores. Germinated sclerotia collected in the field had to be handled carefully because rudimentary and immature apothecia became detached easily and then would not develop. In the laboratory, it was necessary to maintain the moisture content of the vermiculite in which the sclerotia were buried to prevent apothecia from desiccating. An incubation temperature around 16 C was favorable for normal development of apothecia in vitro. Immature apothecia incubated at room temperature (21–25 C) usually were swollen and distorted when they matured. The ascospores of mature apothecia were discharged in clouds within several seconds after the lids of crystallizing dishes were removed. However, apothecia did not discharge ascospores until several hours after growth chamber lights came on each day.

Viability of ascospores. Only large ascospores of *M. oxycocci* fluoresced when aqueous spore suspensions were treated with FDA, although small ascospores also were clearly visible (Fig. 1A). Ascospores that fluoresced did so 1–2 min after stain was added. Germinated ascospores on PDA often were swollen and distorted so that the original size of these spores (i.e., large or small) could not always be determined. However, ascospores of both types that had not germinated were distinguished easily (Fig. 1B). If all germinated spores were assumed to be large ascospores, the proportions of ascospores determined viable were not significantly different from those determined viable with FDA (Table 1). Linear regression of these same data, in which germination on PDA was designated the independent variable (X) and fluorescence after treatment with FDA was designated the dependent variable (Y), further demonstrated the equivalence of the two methods:

$$Y = -0.04 + 1.16X$$

with $R^2 = 88.9\%$. The intercept was not significantly different from 0 ($P = 0.66$), and the slope was not different from 1 ($P = 0.34$) based on Student's t tests of the estimated parameters.

Storage of ascospores. Both freezer type and the use of a desiccant significantly influenced ascospore viability after storage for 24 mo ($P = 0.004$ and $P < 0.001$, respectively). A significantly greater proportion of ascospores remained viable in the deep freezer (27.6%, 11 filters) than in the frost-free freezer (10.9%, nine filters). The proportion of ascospores that remained viable over $CaCl_2$ (29.5%, seven filters) was not significantly different ($P = 0.117$) from that over $CaSO_4$ (24.4%, eight filters). Ascospores did not survive in either freezer without a desiccant, and the no desiccant treatment (five filters) was significantly different from either the $CaCl_2$ or $CaSO_4$ treatment ($P < 0.001$).

When ascospores were stored for 24 mo in different locations either with or without $CaSO_4$, their viability was affected significantly (Fig. 2). The interaction between storage location and desiccant was significant ($P < 0.001$), indicating that desiccant affected spore viability differentially in the different locations. Ascospores did not survive storage without a desiccant in any location except the refrigerator. Significantly more ascospores remained viable in the deep freezer with desiccant than in any other treatment. The proportion of ascospores that were viable in the frost-free freezer with desiccant was not significantly different from that in the refrigerator with or without desiccant. No ascospores remained viable after storage at room temperature.

Viability of ascospores stored for 6 mo over $CaSO_4$ in a deep freezer was reduced significantly from that of freshly collected ascospores; however, viability did not decrease significantly thereafter (Table 2). An average of 38% of the ascospores that initially were viable remained so after storage over $CaSO_4$ at −20 C.

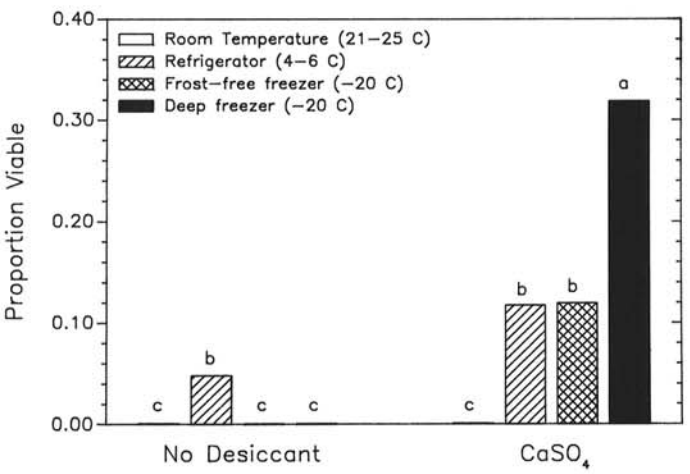


Fig. 2. Effects of four environments on survival of ascospores of *Monilinia oxycocci* after storage for 24 mo. Bars are mean proportions of ascospores that were determined to be viable by the vital stain fluorescein diacetate. Data were transformed to arcsine-square root values prior to analysis. An F statistic for the interaction between storage location and desiccant was significant ($P < 0.001$) in a two-way analysis of variance. Therefore, means of all treatments were separated by Fisher's protected least significant difference; bars with the same letter are not significantly different ($P = 0.05$).

DISCUSSION

Ascospores of *M. oxycocci* were collected on membrane filters from apothecia that developed in vitro from germinated, field-collected sclerotia. Apothecia developed and matured normally when germinated sclerotia were incubated in moist vermiculite at 16 C with a 12-h photoperiod. Similar conditions were found to be optimal for germination and maturation of apothecia of *M. vaccinii-corymbosi* (10). Ascospores could be collected several times per day, but visible clouds of ascospores were not observed until apothecia had been exposed to light for several hours. Asci

TABLE 2. Viability of ascospores of *Monilinia oxycocci* on membrane filters that were stored for various durations over $CaSO_4$ in a deep freezer at −20 C

Storage duration (mo)	Number of filters tested	Ascospore viability (%)[y]
0	2	71.7 ± 18.3 a[z]
6	7	27.4 ± 7.1 b
12	2	22.7 ± 1.6 b
24	3	31.8 ± 13.4 b

[y] Viability of spores from aqueous suspensions was determined with the vital stain fluorescein diacetate; data are means ± standard errors.

[z] Percents were transformed to arcsine-square root values before analysis. An F statistic from a one-way analysis of variance was significant ($P = 0.002$). Means followed by the same letter are not significantly different based on Fisher's protected least significant difference ($P = 0.05$).

of many Discomycetes are phototropic (9,18), and the delayed spore release that was observed may be due to a light-mediated process. Discharge of ascospores in the Discomycetes is associated with the accumulation and explosive release of ascus sap (18) and may occur as an immediate response to a number of stimuli, including a sudden decrease in temperature or relative humidity or a shock from mechanical or air pressure (9). Such stimuli would occur when the lids of dishes were removed.

Germination on PDA or fluorescence after staining with FDA yielded equivalent estimates of ascospore viability. Therefore, staining with FDA, a method not used previously, provided a very useful alternative for estimating viability of ascospores of *M. oxycocci*. This method has the advantage over spore germination on media in that an estimate of viability can be obtained almost immediately. FDA originally was developed as a vital stain for mammalian cells (12) and since has been used for determining viability of pollen and protoplasts of both plants and fungi (11). With both methods for assessing viability, small ascospores were clearly visible in samples from filter disks that had been stored for 24 mo, which is contradictory to Woronin's claim that small ascospores degenerate soon after being released from asci (19). However, they were not observed to fluoresce when stained with FDA nor did they appear to germinate on PDA. These observations support Woronin's additional claim that small ascospores are not viable (19).

Ascospores were preserved in a viable state for up to 24 mo; however, the environmental conditions under which ascospores were stored drastically affected survival. Ascospores survived best when stored desiccated in a constant-temperature deep freezer. In comparison, fewer ascospores remained viable when stored desiccated in a frost-free freezer at the same temperature. Consequently, frost-free freezers should not be used to store ascospores of *M. oxycocci* and may have adverse effects on survival of other living biological specimens. This deleterious effect of a frost-free freezer on inoculum viability has not been noted previously. Ascospores only survived in the freezers when a desiccant was used, and the two calcium salts evaluated as desiccants performed similarly. In contrast, the effect of a desiccant on survival of ascospores of *S. sclerotiorum* was inconsistent (8). Ascospores of *M. oxycocci*, like those of *S. sclerotiorum* (8,17), did not survive when stored at room temperature.

Viability decreased significantly when ascospores of *M. oxycocci* were stored after collection. The proportion of viable large ascospores decreased by 62% after 6 mo in storage over $CaSO_4$ at −20 C. In contrast, ascospores of *S. sclerotiorum* suffered almost no loss of viability after 24 mo of storage at either 2 or −19 C over $CaCl_2$ (8). Because no further loss of viability was noted in samples assayed at 12 or 24 mo, it may be possible to reduce the initial loss of viability by lyophilizing ascospores or by treating them with a cryoprotectant before storage. However, results from preliminary investigations (P. G. Sanderson and S. N. Jeffers, *unpublished*) suggest that it may be necessary to apply ascospores of *M. oxycocci* to cranberry plants in a dry state (e.g., directly from the filters) for inoculations to be successful. Consequently, the use of a cryoprotectant may adversely affect subsequent infection.

Until the routine production of fertile apothecia of *M. oxycocci* in vitro becomes possible, the methods described here can be used to obtain and maintain a supply of viable ascospores for research purposes. These methods also may be useful for collecting and preserving ascospores of other species of *Monilinia*, particularly those that attack ericaceous hosts. However, environmental conditions that optimize spore survival should be determined for each species before quantities of inoculum are stored for any length of time.

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