Influence of Surface-Disinfestation Procedures and Tissue Storage on Isolation of *Cylindrocladium* spp. from Leatherleaf Fern Fronds

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ABSTRACT

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The effects of surface-disinfestation, exposure time, vacuum-infiltration, and preisolation storage conditions (moisture and temperature) on isolation of *Cylindrocladium heptaseptatum* and *C. pteridis* were determined. Increasing the surface-disinfestant (NaOCl) concentration from 0 to 2.6% decreased saprophyte recovery exponentially and increased recovery of *Cylindrocladium* spp. Exposure time (1–10 min) and vacuum-infiltration (81.3 kPa) did not influence recovery of *Cylindrocladium* spp., although higher exposure times sometimes decreased recovery of saprophytes. Storage of tissue in plastic bags increased recovery of *Cylindrocladium* spp. and decreased saprophyte recovery compared with storage in paper bags. Storage of tissue at 5–6 C reduced recovery of the pathogens but increased recovery of saprophytes compared with storage at 23–25 C. *Cylindrocladium* spp. recovery decreased exponentially ($y = 5.4x^{-0.24}$) as storage time increased from 0 to 14 days.

Fungal isolation from diseased plant material is an integral part of disease diagnosis. In many cases, the accuracy of the diagnosis depends on isolation of the pathogen on culture media. The number and growth rate of colonies affect the accuracy and speed with which the diagnosis and recommendations are made. Selective media (6,7) and specific techniques for pathogen isolation (2,4) from specific hosts (1,3,5) have been the bases for diagnostic methods. This is the first in a series of papers that attempts to elucidate the role of surface-disinfestation parameters on recovery of fungi from leaf tissue. The purpose of this research was to investigate the roles of surface-disinfestant concentration, vacuum-infiltration, exposure time, and tissue storage conditions on recovery of *Cylindrocladium* spp. from leatherleaf fern (*Rumohra adiantiformis* (G. Forst) Ching) because this pathogen-suscept system was readily available and could serve as a model for future testing.

MATERIALS AND METHODS

Lesions for all tests were collected from leatherleaf fern plants naturally infected with *Cylindrocladium heptaseptatum* Sob., Alf., & Knauss and *C. pteridis* Wolf. Previous studies indicated that only these pathogens were present and that the ratio of the two species was about 1:1. Lesions about 1–2 mm wide with a border (1–2 mm) of green tissue were excised before surface-disinfestation treatment (immediately before use or as indicated). Lesions in each test were bulked and mixed thoroughly to ensure randomness before placement in treatment lots of 10 each. The surface-disinfestant employed was Clorox (5.25% NaOCl). Lesions were cut in half after treatment application, rinsed in sterilized, deionized water, and placed on potato-dextrose agar (Difco Laboratories, Detroit, MI 48232) amended with 100 μg streptomycin sulfate per milliliter of medium. Four pieces were placed on each of five plates and incubated for 5 days at 24–26 C with about 25 μE m^{-2} sec^{-1} fluorescent light (12 hr/day). Numbers of *Cylindrocladium* spp. colonies (four per plate possible) and saprophyte colonies (different genera were not distinguished) were recorded. The following factorial experiments were performed.

Experiment 1. The first experiment had a 4 × 3 × 2 design with four surface-disinfestant concentrations (0, 0.05, 0.52, and 2.6% NaOCl), three exposure times (1, 3, and 10 min), and two air pressures (ambient = 101.3 kPa and partially evacuated for infiltration = 81.3 kPa). Surface-disinfestant concentrations were chosen to cover a wide range of concentrations, including a standard concentration (0.52% NaOCl). This test was performed four times as described and once without air-pressure treatments.

Experiment 2. The second experiment was similar to the first, but a fourth factor was added—tissue condition. Three tissue conditions were tested that included fresh, precut and stored 1 wk, and uncut and stored 1 wk. Fresh tissue was prepared the day of collection. Precut and uncut tissue were stored in a paper bag (23–25 C) for 1 wk before plating. This experiment was performed twice as described and once with time of exposure fixed at 3 min.

Experiment 3. In the third experiment, the following factors were tested: storage temperatures of 5–6 and 23–25 C, storage in a paper bag or plastic bag with a wet paper towel and tissue ages of 0, 1, 2, 3, 4, 7, 10, and 14 days after excision (2 × 2 × 8 factorial). This experiment was performed three times as described and twice without the storage temperature factor. The amount of water loss was determined by weighing each treatment immediately after excision and before surface-disinfestation. In this experiment, all lesions were precut and stored under the appropriate conditions, then surface-disinfested using 0.52% NaOCl for 3 min without vacuum-infiltration.

RESULTS

Data were analyzed initially using an F test and regressions were performed using the entire data set for tests with significant F values ($P = 0.05$ or $P = 0.01$). Colonies of *C. heptaseptatum* and *C. pteridis* were grouped because initial analyses did not indicate differential effects of the treatments employed. Split lesions were treated as unrelated for the analyses. In cases where a factor was significant in only one of five tests, regression analysis was not included for that factor. Interactions between factors were rare and inconsistent among tests and therefore only main effects are discussed.

Experiment 1. Recovery of saprophytes and *Cylindrocladium* spp. were not affected by vacuum-infiltration (81.3 kPa air pressure). Recovery of saprophytes decreased exponentially as surface-disinfestant concentration increased from 0 to 2.6%, with as much as a 66% decrease (Table 1). Increased exposure from 1 to 10 min reduced recovery of saprophytes in one test (Table 1, test 4) and showed a similar trend in tests 1–3. Surface-disinfestant concentration was also usually important in recovery of *Cylindrocladium* spp., although exposure time did not affect recovery of the pathogens (Table 2). Increases in surface-disinfestant concentration resulted in increased pathogen recovery in three of the five tests. Even the lowest NaOCl concentration gave a higher recovery of *Cylindrocladium* spp. than the water

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control (Table 2) in these three tests.

Experiment 2. Results of these tests were similar to those noted earlier with respect to effects of surface-disinfestation procedure. Data on the tissue condition factor were similar among tests so only two of the three tests were included in Table 3. The highest recovery of pathogens occurred when tissue was cultured the day of excision (fresh). Significant reductions in recovery of *Cylindrocladium* spp. occurred when lesions were cut on the day of collection and stored in a paper bag for 1 wk before plating in two of three tests (Table 3). Tissue held for 1 wk, cut or uncut, appeared dry and gave lower recovery of the pathogens. Saprophyte recovery was unaffected in two of three tests with regard to storage conditions but was greater for tissue cut and stored 1 wk than for tissue uncut and stored 1 wk in the third test (Table 3).

Experiment 3. The role of tissue storage conditions in recovery of both pathogens and saprophytes was consistent in all five tests so data from only one test are presented. Recovery of pathogens from tissue stored at 5–6 C was greater up to 14 days when stored in a plastic bag (moist) than from tissue stored in a paper bag (dry), although recovery decreased slightly over the 14-day period (Fig. 1). Conversely, recovery of saprophytes was high under these conditions. Dry storage at 5–6 C had the lowest pathogen recovery and the highest saprophyte recovery. Tissue stored at 23–25 C gave

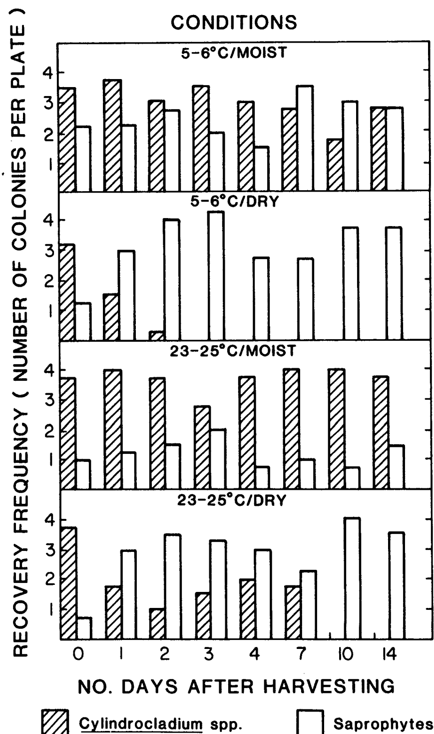

Fig. 1. Effect of storage conditions (temperature and moisture) and time after collection on recovery of *Cylindrocladium* spp. and saprophytes from leatherleaf fern fronds (experiment 3).

Table 1. Effect of surface-disinfestation procedures on recovery of saprophytic fungi from leatherleaf fern fronds (experiment 1)

Factor level	Frequency of recovery[a] Test 1	Test 2	Test 3	Test 4	Test 5
Surface-disinfectant concentration (%NaOCl)					
0	1.6	4.9	6.2	5.0	5.9
0.05	0.7	5.1	4.3	4.9	4.2
0.52	0.5	2.6	2.6	3.2	3.3
2.60	0.5	2.0	2.1	2.0	2.5
Exposure time (min)					
1	1.0	3.8	4.1	4.1	4.3
3	0.7	3.7	3.7	4.1	3.8
10	0.8	3.5	3.5	3.5	4.0
Air pressure (kPa)					
101.3	0.9	3.9	3.7	3.6	NT[b]
81.3	0.8	3.4	3.8	4.0	NT
Significant effects					
Surface-disinfestant concentration	*[c]	*	*	*	*
Exposure time	NS	NS	NS	*	NS
Air pressure	NS	NS	NS	NS	NT
Equation for surface-disinfestant	$y=1.1x^{-0.26}$ $R^2=0.68$NS	$y=5.3x^{-0.26}$ $R_2=0.94$**	$y=5.6x^{-0.27}$ $R_2=0.95$**	$y=5.4x^{-0.24}$ $R_2=0.97$**	$y=5.4x^{-0.2}$ $R_2=0.95$**

[a] Mean number of colonies per four pieces of tissue on each of 30, 40, or 60 plates, respectively.
[b] Not tested.
[c] * = Significant at $P = 0.01$, NS = not significant.

Table 2. Effect of surface-disinfestation procedures on recovery of *Cylindrocladium* spp. from leatherleaf fern fronds (experiment 1)

Factor level	Frequency of recovery[a] Test 1	Test 2	Test 3	Test 4	Test 5
Surface-disinfestation concentration (%NaOCl)					
0	3.9	3.2	3.8	0.6	1.2
0.05	4.0	3.8	3.7	1.3	1.9
0.52	4.0	3.9	3.9	1.5	2.5
2.6	3.8	3.9	3.8	1.5	2.6
Exposure time (min)					
1	3.9	3.8	3.9	1.3	2.1
3	4.0	3.7	3.7	1.1	2.1
10	3.9	3.7	3.8	1.3	2.0
Air pressure (kPa)					
101.3	4.0	3.7	3.8	1.2	NT[b]
81.3	3.9	3.7	3.8	1.3	NT
Significant effects					
Surface-disinfestant concentration	NS	*[c]	NS	*	*
Exposure time	NS	NS	NS	NS	NS
Air pressure	NS	NS	NS	NS	NT

[a] Mean number of colonies per four pieces of tissue on each of 30, 40, or 60 plates, respectively.
[b] Not tested.
[c] * = Significant at $P = 0.01$, NS = not significant. Regressions of significant data sets were not linear, quadratic, cubic, or exponential.

Table 3. Effect of storage condition on recovery of *Cylindrocladium* spp. and saprophytes from leatherleaf fern fronds (experiment 2)

Storage conditions	Saprophytes Test 1	Saprophytes Test 2	*Cylindrocladium* spp. Test 1	*Cylindrocladium* spp. Test 2
Freshly collected	5.5 ab[b]	3.4 a	3.5 b	2.4 b
Cut and stored 1 wk	6.3 b	3.9 a	1.9 a	1.2 a
Uncut and stored 1 wk	4.1 a	3.6 a	3.3 b	0.9 a

(All columns: Frequency of recovery[a])

[a] Mean number of colonies per four pieces of tissue on each of 120 plates (four possible for *Cylindrocladium* spp.).
[b] Means were separated by Duncan's new multiple range test ($P=0.05$).

the highest recovery of pathogens when maintained in a plastic bag and a low recovery of saprophytes. In this treatment, the effects of storage up to 14 days were negligible. Water loss was 16–18% for tissue stored in a plastic bag and 53–56% for tissue stored in a paper bag. Temperature did not appear to affect tissue weight loss in any of these tests. In most cases, weight loss occurred by the fourth day, with little or no weight lost after that time. The overall effect of storing tissue under any condition on recovery of *Cylindrocladium* spp. was consistent, characterized by the following exponential equation: $y = 5.42x^{-0.24}$, $R^2 = 0.77$ (significant at $P = 0.01$). A rapid decrease in recovery of the pathogen occurred within the first few days and leveled off as storage time increased.

DISCUSSION

Many important factors influence recovery of *Cylindrocladium* spp. from leatherleaf fern fronds. It seems opportune that the treatment giving the highest recovery of pathogens (2.6% NaOCl for 10 min) also gave lowest recovery of saprophytes. The high recovery of the pathogen in this treatment may have been affected by the relatively low populations of the saprophyte. *Cylindrocladium* spp. in leatherleaf fern fronds are apparently quite resistant to this level of exposure to surface-disinfestant. Allowing the tissue to dry out before plating results in loss of pathogens. If the pathogen dies in the lesion, saprophytes may invade the tissue and multiply. In treatments with high pathogen recovery, however, saprophytes cannot invade the lesions and their recovery remains low throughout the 14-day storage period. The cause and effect nature of this reaction is not clear, however, and the reverse could be true.

These results can be applied to other pathogen-suscept combinations only sparingly. Different combinations of pathogens and suscepts react differently to surface-disinfestation methods and storage conditions (A. R. Chase, *unpublished*). *Cylindrocladium* spp. fall into the group of pathogens that are resistant to relatively high concentrations of NaOCl (2.6%). These organisms are isolated more easily from host tissue under moist conditions because the high level of NaOCl allows a maximum recovery of pathogens and a minimum recovery of saprophytes. The response to storage temperature and time is probably dependent on the pathogen and suscept tissue because waxy or succulent leaves may dry more slowly than thin leaves like those of leatherleaf fern. Recovery of *Corynespora cassiicola* (Berk & Wei) Curt. from *Aphelandra squarrosa* Nees remained high, even when lesions were excised from tissue and stored in a paper bag for 1 wk (A. R. Chase, *unpublished*). Further research is under way to elucidate effects of tissue type on resistance to surface-disinfestation, tissue storage temperatures, and moisture loss on this and other pathogen-suscept combinations.

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