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Pathogenicity of Septoria aquilina Isolated from Black Hills Bracken

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Septoria aquilina, a pathogenic fungus isolated from *Pteridium aquilinum* growing in the Black Hills was studied from 1991–1995. *S. aquilina* sprayed at 8,000 conidia/ml on transplanted fronds followed by high humidity caused severe necrosis seven days after inoculation and disease severity increased until fronds died. Severe necrosis also developed from inoculations at 4,000 and 2,000 conidia/ml. Less severe symptoms developed from inoculations at 1,000 conidia/ml. Inoculations at 4,000 conidia/ml not followed by high humidity caused less necrosis than inoculations at the same concentration with high humidity. Inoculations at 4,000, 2,000, and 1,000 conidia/ml on fronds grown from spores caused similar levels of necrosis as inoculations on transplanted fronds. Necrotic epidermal and mesophyll cells were observed in pinnules. In rachises, portions of cortex, endodermis, xylem, and phloem showed abnormal cells or cell destruction. Hyphae were observed infrequently and only in the epidermis and mesophyll of pinnules. *S. aquilina* is a candidate for field evaluation as a biocontrol agent.

INDEX DESCRIPTORS: biocontrol, *Pteridium aquilinum, Septoria aquilina.*

Pteridium aquilinum L., bracken fern is widely distributed in temperate areas of the world. Worldwide it is a weed (Taylor 1990) occupying acres of potential pasture and inhibiting reestablishment of conifers on disturbed sites in the northwestern United States (Stewart et al. 1979). Its toxicity and carcinogenicity to livestock have been established (Kingsbury 1964; Perring and Gardiner 1976; Fletcher and Kirkwood 1979).

The need to control bracken has generated interest in identifying fungi that may act as biocontrol agents. Killian (1918) described a "leaf roll" caused by *Cryptomyces pteridis* (Rebentisch) Rehm. and Bache-Wiig (1940) showed this fungus (=*Cryptomycina pteridis* [Rebent. ex Fr. Höhn.]) was systemic and overwintered in the rhizome. Studies by Gabel et al. (1996) determined that *C. pteridis* acted as an endophyte and did not significantly reduce populations to be an effective biocontrol agent.

Alcock and Braid (1928) described "Garelochead" disease in Scotland caused by a pycnidial fungus that rotted frond tips. Angus (1958) described "curl tip" disease, which produced dieback, uneven growth and death of fronds. Although he isolated *Stagonospora* sp.(=*Septoria* sp.?), *Ascochyta pteridis* Bres., and *Phoma aquilina* Sacc. and Penz., only *P. aquilina* caused disease when conidia of each fungus were inoculated to fronds. Burge and Irvine (1985) inoculated fronds with mycelia and spores of *P. aquilina, A. pteridis* and *Septoria* sp. and determined *P. aquilina* was the primary cause of "curl tip" disease. *Septoria* sp. produced no symptoms. Irvine et al. (1987) continued studies with *A. pteridis* and *P. aquilina,* and Irvine et al. (1991) concluded that *A. pteridis* was the primary disease organism. Webb and Lindow (1987) showed isolates of *A. pteridis* varied in virulence and age of fronds influenced susceptibility to the fungus.

In the Black Hills of South Dakota, *C. pteridis* was frequently identified on *Pteridium aquilinum* (Gabel 1993; Gabel, et al. 1996). In addition, in some areas after a wet spring, bracken exhibited a grey water-soaked appearance, became necrotic, wilted and died. *Septoria* was isolated from fronds infected with *C. pteridis,* from the wilted, necrotic fronds and from healthy appearing fronds. Two species of *Septoria, S. aquilina* Pass. (Saccardo 1884) described from populations of bracken in Europe and *S. pteridis* Peck (Saccardo 1892) described from populations of bracken in North America have been reported.

This study was initiated to identify the *Septoria* sp. that was isolated from bracken in South Dakota and to evaluate its pathogenicity as determined from inoculations with single-spore isolates under controlled conditions. This information is necessary prior to field evaluation of a potential biocontrol agent.

METHODS

Collections and Isolations

Pteridium aquilinum fronds were collected in June, July, and August of 1991–94, from Eleventh Hour Gulch site, section 12, T5N, R1E; Big Hill site, section 1, T5N, R1E and Deer Creek site, section 22, T5N, R1E in Lawrence County, South Dakota. Isolations were performed annually from 60 fronds per year (cut into 500 pieces from pinnae and rachises) which showed no apparent disease and from 40 fronds per year (cut into 500 pieces from pinnae and rachises) which were infected with *C. pteridis.* Isolations were performed in 1995 from 17 fronds (cut into 170 pieces from pinnae) showing necrosis and 10 fronds (cut into 100 pieces from pinnae) which showed no apparent disease. All isolations were performed on the day fronds were collected. Fronds were cut into 0.5–1.0 cm long pieces, dipped in 95% ethanol, surface sterilized for 2 min in 0.525% sodium hypochlorite, aseptically placed on potato dextrose agar acidified with .02% concentrated lactic acid (APDA), and incubated on the laboratory bench at 25° C (± 3° C) for 7–10 days. *Septoria* sp. colonies on isolation plates were washed with sterile water, and the suspension diluted and spread on APDA from which single spores were removed. Single spore cultures were subcultured, and conidial suspensions from selected subcultures were pooled for inoculum.

Spore Germination

Spore germination tests were conducted on the day of inoculation. Conidial suspensions were placed on APDA, incubated at 25° C (±
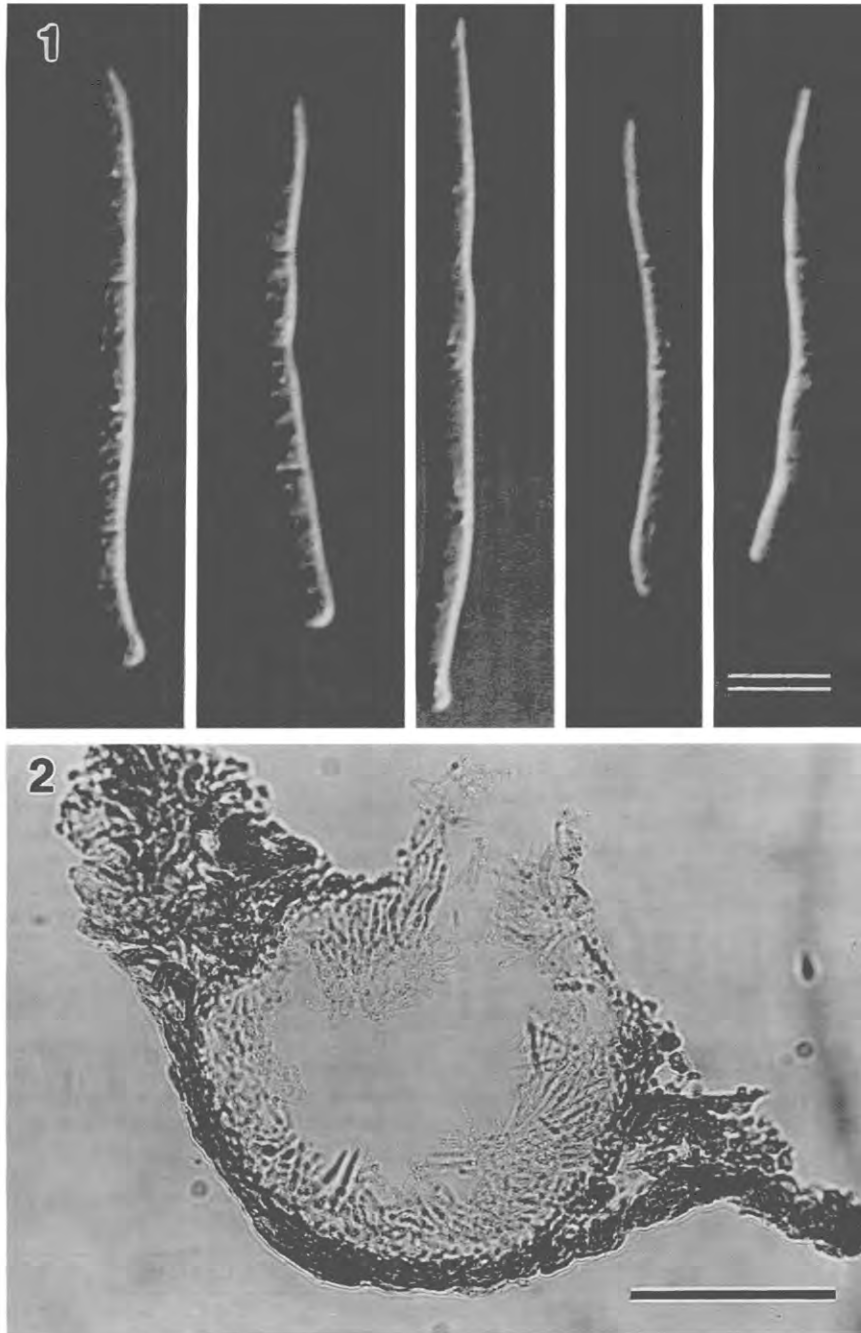
Fig. 1. Mature conidia of *Septoria aquilina* from host. Bar = 10 μm.

Fig. 2. Longitudinal section of pycnidium from host mounted in lactophenol/cotton blue showing wall cells and young attached conidia. Bar = 100 μm.

3° C), and percentage germination was determined from 10 random microscopic fields 24 h later. Percentage of germination ranged from 34%–100% and was adequate for infection.

Inoculations

In June fern rhizomes with young fronds were transplanted from the Schoolhouse site, section 9, T4N, R1E, and from Potato Creek site, section 20, T5N, R1E, in Lawrence County, South Dakota where plants had appeared disease-free for several years. Rhizomes with fronds were placed in well-drained 25 cm pots using soil from the collection sites. In the laboratory fronds were grown from *P. aquilinum* spores cultured in a liquid or solid mineral medium (Davis and Postlethwait 1966) and transplanted to clay pots when pinnae and rhizome development were adequate. Inoculum suspensions were

Table 1. Comparison of measurements (μm) of *Septoria aquilina* conidia from Black Hills bracken, from type material and from published descriptions.

	SIZE	SEPTA-TIONS
Black Hills (this study)	38.1 × 4.5[a]	0–4
	10–69 × 2.5–7.5	
Type (# 817 PAD)	37.0 × 4.8[a]	0–5
	30–52 × 3.8–5.0	
Diedicke (1915)	50–65 × 4–5	up to 4
Fisher & Punithalingam (1993)	50–65 × 4–4.5	3–7
Punithalingam (1993)	50–65 × 4–4.5	3–7
Saccardo (1884)	50–65 × 4–5	up to 4

[a]Average of randomly selected conidia

Table 2. Results of inoculation experiment 1: necrosis severity on transplanted fronds of *Pteridium aquilinum* inoculated with *Septoria aquilina* at 8,000 conidia/ml.

TREAT-MENTS	DAYS AFTER INOCULATION				
	2	7	20	43	55
Control	1.0[ab]	1.0	1.0	1.5	1.5
	1.0–1.0[c]	1.0–1.0	1.0–1.0	1.0–2.0	1.0–2.0
S. aquilina	2.5	3.8	5.0	5.0	6.0
	2.0–3.0	3.0–6.0	4.0–6.0	4.0–6.0	6.0–6.0

[a]Due to transplant shock control values are average of two replicate fronds and *S. aquilina* values are average of four replicate fronds
[b]Visual rating is based on a scale of 1–6 (see Methods)
[c]Values are the range of severity readings

prepared by washing conidia from subcultures of single spore cultures with distilled water, and concentrations were determined with a hemacytometer. Water was used for control treatments. All fronds were sprayed until runoff. Fronds were bagged for 48 h in the laboratory at 25° C (± 2° C) unless otherwise indicated.

Inoculation experiment 1

S. aquilina conidia were applied at a rate of 8,000 conidia/ml to four replicates of transplanted fronds. After bags were removed plants were randomized in a growth chamber set at 20° C during a 12 h day and 10° C during a 12 h night. Severity of necrosis was evaluated using the following rating system: 1 = no necrosis; 2 = necrosis < 25%; 3 = necrosis 25–50%; 4 = necrosis > 50%, but < 75%; 5 = necrosis > 75%, but < 100%, and 6 = necrosis at 100%.

Inoculation experiment 2

S. aquilina conidia were applied at rates of 1,000, 2,000, and 4,000 conidia/ml to four replicates of transplanted fronds for each treatment. After bags were removed, plants were randomized and maintained in the laboratory at 25° C (± 2° C). The severity rating system was the same as that used in Inoculation experiment 1. An unbagged control was included to determine if incidental inoculum occurred on field transplanted rhizomes. An unbagged 4,000 conidia/ml treatment was included to determine the effect of humidity on disease development.

Inoculation experiment 3

P. aquilinum fronds grown from spores were inoculated with *S. aquilina* at the same concentrations and under the same conditions as in experiment 2. Only one replicate was available for each treatment due to problems growing adequate sporophytes from spores. Evaluation was completed using the same severity rating system as in experiment 1.

Tissue Studies

Healthy fern tissue and tissue infected with *S. aquilina* were vacuum infiltrated with FAA immediately after collection. Samples were dehydrated in an ethanol series, infiltrated and embedded in LR White resin and cured for 20–24 h at 60° C. Staining with toluidine

Table 3. Results of inoculation experiment 2: necrosis severity on transplanted fronds of *Pteridium aquilinum* inoculated with *Septoria aquilina* conidia.

TREATMENT	DAYS AFTER INOCULATION					
	3	7	10	23	30	38
Control	1.0[ab]	1.0	1.0	1.0	1.0	1.0
	1.0–1.0[c]	1.0–1.0	1.0–1.0	1.0–1.0	1.0–1.0	1.0–1.0
1,000/ml	2.5	2.8	3.8	4.0	4.3	4.3
	0.9–4.1	1.2–4.3	1.0–6.5	1.8–6.3	2.2–6.3	2.2–6.3
2,000/ml	2.5	4.0	5.0	5.0	5.0	5.0
	1.6–3.4	1.8–6.3	2.8–7.3	2.8–7.3	2.8–7.3	2.8–7.3
4,000/ml	2.8	3.8	4.0	4.0	5.0	5.0
	1.2–4.3	2.2–5.3	2.2–5.9	2.2–5.8	3.7–6.3	3.7–6.3
4,000/ml unbagged	1.5	2.0	2.0	2.0	2.8	2.8
	0.6–2.4	2.0–2.0	2.0–2.0	2.0–2.0	2.0–3.5	2.0–3.5

[a]Values are average of four replicate fronds
[b]Visual rating is based on a scale of 1–6 (see Methods)
[c]Values are the range of 95% confidence intervals

Figs. 3–5. Control and inoculated fronds 10 days after inoculation (Inoculation experiment 2). 3. Control. 4. Inoculated with 1,000 conidia/ml. 5. Inoculated with 2,000 conidia/ml.

Table 4. Results of inoculation experiment 3: necrosis severity after inoculating *Pteridium aquilinum* sporophytes grown from spores with *Septoria aquilina* conidia.

TREATMENT	DAYS AFTER INOCULATION			
	2	4	8	15
Control # 1	1[a]	1	1	1
Control # 2	1	1	1	1
1,000 conidia/ml	2	3	4	4
2,000 conidia/ml	2	4	5	5
4,000 conidia/ml	2	3	4	4

[a]Visual rating is based on a scale of 1–6 (see Methods)

blue or methylene blue-azurII-safranin (Berlyn and Miksche 1976) followed.

RESULTS

Determination of Species

The Black Hills isolate was identified as *Septoria aquilina* Pass. Conidia, conidiogenous cells, and pycnidia were essentially similar to type material, to other exsiccatae examined and match descriptions of *S. aquilina* published by Diedicke (1915), Fisher and Punithalingam (1993), Punithalingam (1993) and Saccardo (1884). Measurements of *Septoria aquilina* conidia from Black Hills bracken are based on randomly selected conidia produced on the host and in culture. In culture, conidia were more variable in size and shape than those formed in the host (Table 1). Mature clavate conidia were straight to slightly curved, tapered at the apex, and rounded at the base (Fig. 1). Pale brown, partially immersed pycnidia were located on pinnules, costules, and costae of bracken. Pycnidia were globose, 180 μm mean diameter and had a central ostiole (Fig. 2). Conidiogenous cells were hyaline, short cylindrical to doliiform and not well differentiated from the hyaline, angular cells lining the inner wall. Bubák (1916), Diedicke (1915) and Saccardo (1884) described conidia as clavate with one end acute, the other rounded or blunt. Fisher and Punithalingam (1993) and Punithalingam (1993) described conidia as hyaline and clavate with subulate apical cells. Examination of the type specimen # 817 PAD (Holmgren et al. 1990) showed clavate conidia with subulate apical cells. The following exsiccatae from ISC and BPI (Holmgren et al. 1990) were also examined: *S. aquilina* # 1363 in Krieger Fungi Saxonici (ISC), # 2746 in Ellis and Everhart North American Fungi (ISC), # USO376818, # USO376819, and # USO376816 (BPI). A culture from one of the single spore isolates has been deposited in the ISC herbarium.

Frequency of Isolations

Septoria aquilina was isolated from pinnae, but not from rachises of field material. Frequency of isolations from July 1991–1993 collections ranged from 0–9% from healthy appearing bracken and 5–19% from bracken infected with *C. pteridis*. *S. aquilina* could not be isolated in 1994. Frequency of 1995 isolations from healthy appearing bracken was 15% and from the water-soaked, wilted and necrotic fronds was 48%. Although *S. aquilina* was isolated from all three groups of fronds, it was isolated more frequently from necrotic fronds.

Inoculations

In Inoculation experiment 1, *S. aquilina* infected pinnae and killed fronds at inoculum concentrations of 8,000 conidia/ml (Table 2). Two
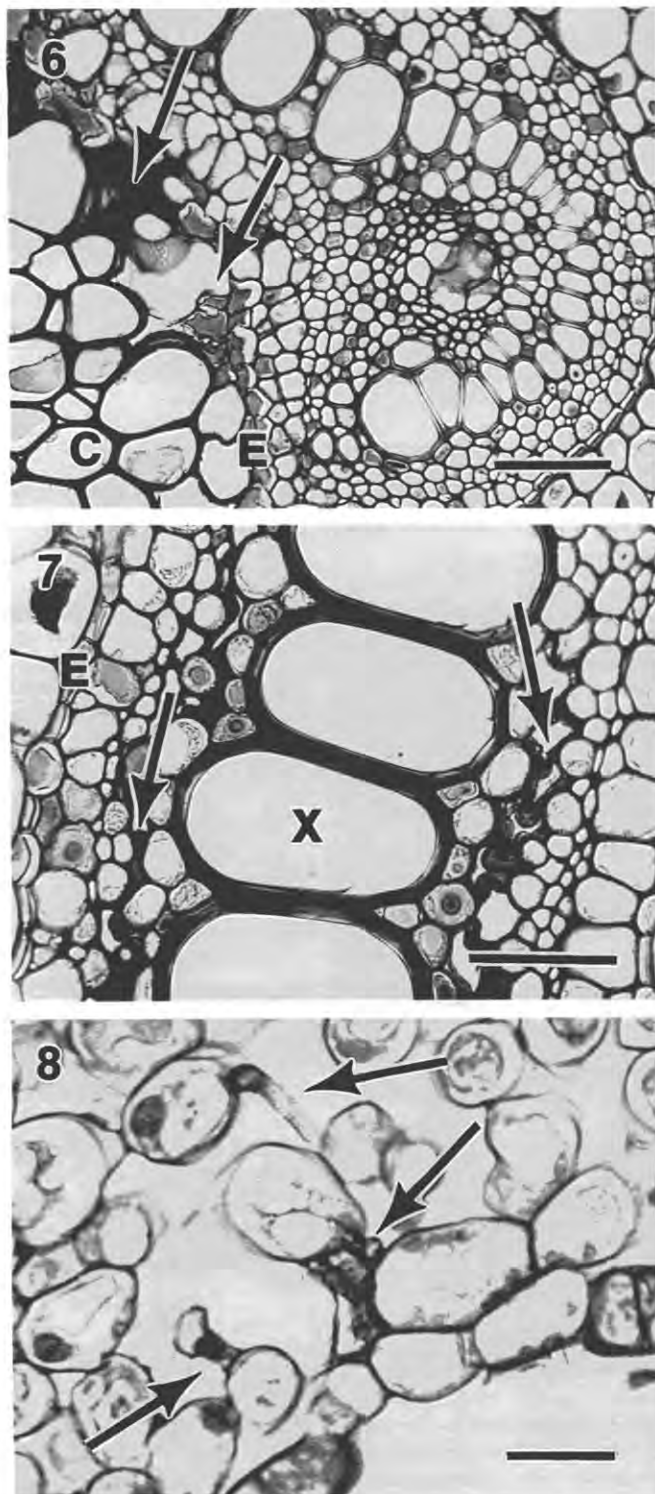
Figs. 6–8. Cross sections of *Pteridium aquilinum* tissues inoculated with *Septoria aquilina*. 6. Rachis 26 days after inoculation with 1,000 conidia/ml. Arrows point to abnormal endodermis and dead cortical cells. E = endodermis; C = cortex. Bar = 70 μm. 7. Rachis 26 days after inoculation with 1,000 conidia/ml. Arrows point to collapsing phloem. E = endodermis; X = xylem. Bar = 40 μm. 8. Abaxial section of pinnule 11 days after inoculation with 4,000 conidia/ml. Arrows point to hyphae. Bar = 20 μm.

days after inoculation, portions of pinnae had a grey water-soaked appearance and were wilted. Seven days after inoculation average necrosis severity was 3.8. Necrosis increased until 55 days after inoculation when all fronds appeared dead. Two fronds in the control treatment were lost because of transplant shock, resulting in low and unequal numbers of replicates. Consequently, the experiment was not statistically analyzed.

S. aquilina caused necrosis of fronds at lower inoculum concentrations in experiment 2 (Table 3). Three days after inoculation portions of pinnae appeared grey, water-soaked and wilted. Bagged treatments had a rating of 2.5 or higher. Ten days after inoculation the differences in necrosis severity between control, 1,000 conidia/ml and 2,000 conidia/ml treatments were conspicuous (Figs. 3–5). Necrosis severity at all inoculum levels followed by a high humidity period was significantly different (95% confidence intervals) from the control except for the 1,000 conidia/ml treatment at three days and 10 days after inoculation (Table 3). Variation in disease readings at 10 days increased the range of confidence intervals. Inoculations at 4,000 conidia/ml not followed by high humidity resulted in less necrosis than all levels of inoculation with *S. aquilina* followed by high humidity (Table 3). No necrosis occurred on bagged controls indicating any incidental inoculum was not a factor. Readings beyond 38 days after inoculation were considered invalid because fronds appeared to be senescing.

Results from Inoculation experiment 3, where fronds grown from spores were inoculated at the same concentrations as in Inoculation experiment 2 support earlier results in this study (Table 4). All inoculated treatments showed necrosis two days after inoculation and severity increased during the 15 day evaluation period. Readings beyond 15 days after inoculation were not considered valid because fiddleheads were appearing in all pots.

Tissue Studies

Tissue samples from controls and from fronds inoculated with *S. aquilina* at 1,000, 2,000, and 4,000 conidia/ml in Inoculation experiments 2 and 3 were examined. Tissues from controls had normal anatomy. Infected tissues of pinnae manifested a change in cell wall staining. Healthy cell walls when stained appeared blue-violet, but walls of dead cells appeared yellow-black. Three days after inoculation, epidermal cells of pinnae were dead but remained intact, and some cells of the spongy mesophyll had collapsed. Abnormal staining of cell walls was observed from adaxial to abaxial epidermis. The rachis endodermis was destroyed or appeared abnormal (Fig. 6), and xylem and phloem in some vascular bundles contained collapsed cells (Fig. 7) 26 days after inoculation. Hyphae were observed infrequently and only in the epidermis and mesophyll of pinnae (Fig. 8). No detectable differences in tissue damage between different concentrations of inoculum were observed.

DISCUSSION

Farr et al. (1989) reported *Septoria pteridis* on bracken in the United States. Conidial size and shape of the isolates in this study show the fungus on Black Hills bracken is *S. aquilina* and not *S. pteridis* Peck. The latter produces filiform conidia which are 70–100 μm long (Saccardo 1892) making it unmistakable from *S. aquilina*.

This is the first demonstration that *S. aquilina* is pathogenic on bracken. The fungus has been isolated, placed in single-spore culture, inoculated to healthy bracken resulting in necrosis and reisolated from inoculated fronds.

Obvious symptoms of disease occurred only in the early summers of 1993 and 1995, which were wetter than normal in the Black Hills. Differences between disease development in bagged and unbagged 4,000 conidia/ml treatments indicate high humidity for 48

h enhances disease development. All concentrations of inoculum followed by high humidity caused necrosis demonstrating the moisture need for disease development. The usual dry climate of western South Dakota may be a major factor for infrequency of disease.

In the field *S. aquilina* was not isolated until July, and not in every year of the study. Periods when the fungus survived as an endophyte, exhibiting no external symptoms may explain why the fungus was isolated from healthy appearing bracken and bracken infected with *C. pteridis*. It may also explain why pycnidia were observed only on isolation plates and on 1995 field material.

Since hyphae were rarely observed, and only in epidermis and mesophyll cells of pinnules, phytotoxins or enzymes produced by *S. aquilina* may be responsible for the widespread cell damage and death. This mode of action agrees with reports for *Septoria cirsii*, which produces a phytotoxin (Hershenhorn et al. 1993) or *Septoria nodorum*, which produces a variety of cell-wall degrading enzymes (Lehtinen 1993).

S. aquilina shows promise as a biocontrol agent. It grows well in culture, produces high numbers of conidia, which easily germinate and readily causes necrosis of bracken fronds. Further studies of inoculum levels, humidity regimes and host specificity are needed before *S. aquilina* can be recommended as a biocontrol agent.

ACKNOWLEDGEMENTS

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