Influence of Environment and Variation in Host Susceptibility on a Disease of Bracken Fern Caused by *Ascochyta pteridis*

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ABSTRACT

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Virulence of isolates of *Ascochyta pteridis* from diseased bracken fern in various geographical areas differed significantly in greenhouse inoculations. Isolates also differed significantly in desiccation survival and spore production in vitro. Infection of bracken fern occurred at temperatures as low as 10 C, but a leaf wetness duration of at least 18 hr was required for infection at this temperature. Infection frequency increased with increasing incubation temperatures up to 20 C. Decreasing durations of leaf wetness required for infection were observed as temperature during leaf wetness periods increased from 10 to 20 C. The susceptibility of bracken fern to infection by *A. pteridis* decreased greatly with increasing frond maturity after 2 wk. Infection of mature bracken fern fronds by *A. pteridis* was less than about 12% of that of immature fronds. Frond tissue showed two distinct classes of susceptibility to infection. Immature frond tissues and some parts of fronds of intermediate maturity had an ED_{50} for *A. pteridis* infection of about 10^3 spores per milliliter. Mature fronds and portions of fronds of intermediate maturity had an ED_{50} of 2×10^4 spores per milliliter.

Bracken fern (*Pteridium aquilinum* (L.) Kuhn) is a weed in pasturelands and timberlands throughout tropical and temperate regions of the world (3). Attempts to control this weed conventionally (i.e., chemicals and repeated cultivations or burnings) have not been cost-effective except in areas under intensive cultivation (5). The primary means of bracken fern reproduction is vegetative via dichotomous rhizomes that may grow as much as 2.5 m within one season (7). As the rhizome grows and branches, the older portions die, disintegrate, and give rise to apparently distinct, unrelated colonies that actually originated from one parental rhizome. The genetic uniformity of bracken fern and the high costs of effective conventional control methods make it a promising candidate for biological control.

Bracken ferns infected with *Ascochyta pteridis* (Bres.) Sacc. were observed in various areas in northern California during the spring of 1980. Control of bracken fern, using *A. pteridis* as a biological control agent in preliminary field trials, was inconsistent and highly variable (8). It was suspected that much of the variability was due to variations in fungal virulence, variable environmental parameters, and host phenology at the time of inoculation.

The objectives of this study were to examine variance in survival of physical stresses, sporulation capabilities, and virulence of *A. pteridis* isolates collected from different areas of California; to determine the influence of free moisture, temperature, and their interactions on infection and symptom development; and to determine the relative susceptibility of bracken fern fronds of different phenological stages to infection by *A. pteridis*.

MATERIALS AND METHODS

Isolation, culture, and harvesting of conidia. Initial isolations were made from extruded conidia removed aseptically with a needle from pycnidia in diseased tissue. All *A. pteridis* isolates were cultured in petri dishes containing a modified minimal medium (MM) of the following composition: KH_2PO_4, 2.0 g; $K_2HPO_4 \cdot 3H_2O$, 1.7 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.2 g; asparagine, 1.9 g; sucrose, 10.0 g; malt extract, 7.5 g; yeast extract, 2.5 g; agar, 15.0 g; and distilled water, 1,000 ml. This medium produced prolific growth and sporulation. Cultures were maintained under 40W fluorescent lights at a height of 55 cm (12-hr light period per day) at 21 C. Conidia were harvested after 2 wk of growth by washing hyphal mats in distilled water, using the ultrasonic vibration method described by Haefele and Webb (2). Spore suspensions were adjusted to appropriate concentrations with distilled water with a hemacytometer.

Determination of in vitro desiccation tolerance of conidia. An aqueous suspension containing 10^5 conidia per milliliter from each of six *A. pteridis* isolates was atomized onto six glass coverslips and allowed to air-dry for 3 min at 21 C. The coverslips were then suspended over $CaSO_4$ in a sealed glass jar and placed in an incubator maintained at 21 C (21 ± 1% relative humidity). After 0, 5, 10, 20, 30, and 60 days, one of the six coverslips was inverted on water agar and the percentage of spores that had germinated after 12 hr was determined by counting total spores and germinated spores observed in 10 random microscope fields with a minimum of 200 spores per field. This trial was conducted twice for all isolates except V780.

Determination of in vitro sporulation capabilities. Individual colonies originating from single conidia from each of six *A. pteridis* isolates were grown at 21 C on 25 ml of MM agar in four covered 125-ml Erlenmeyer flasks for each isolate. After 2 wk of growth, 50 ml of sterile distilled water was added to each flask and flasks were placed in an ultrasonic bath for 5 min. The number of conidia recovered per colony was estimated with a hemacytometer. This trial was conducted twice for all isolates except V780.

Determination of relative virulence of *A. pteridis*. Conidia from each of six *A. pteridis* isolates were harvested from cultures grown on MM agar, and their concentrations were adjusted to 10^5 spores per milliliter with sterile distilled water. Four replicates of these spore suspensions were applied in aerosol sprays to individual greenhouse-grown ferns. Inoculated ferns were placed in a dew chamber maintained at 21 C for 24 hr, then returned to greenhouse benches at 24 C. Disease evaluations were made 1, 2, 3, and 4 wk after inoculation. Symptom development was quantified with a disease assessment key containing 10 categories constructed for visual estimation of infection of bracken fern with *A. pteridis* that had different simulated disease severities (available from S. E. Lindow).

Determination of the effects of free moisture and temperature on infection. To meet the requirements of large numbers of inoculum

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concentrations needed in infectivity titrations as well as in determinations of free moisture and temperature requirements for infection, it was necessary to develop a rapid leaf-disk assay of frond infections that was representative of an in situ response. Six replicate leaflets were excised from each of four fronds and mist-inoculated with 10^5 conidia of isolate 0779 per milliliter. The leaflets were maintained in darkened moist chambers kept at 6, 10, 12, 15, and 20 C. They were removed from the water-saturated atmosphere 0, 4, 8, 10, 18, 24, 36, and 48 hr after incubation at each of the temperatures, blotted dry, and kept in a sealed container held at a relative humidity of 80% and the temperature corresponding to the original moist incubation until all samples were removed from moist incubation (up to 48 hr). The samples were then surface-sterilized for 3 min in a 0.57% sodium hypochlorite solution. Ten 3-mm disks were excised from each of six leaflets incubated under each combination of temperature and free moisture duration and placed aseptically on 2% water agar. The plates were incubated for 5 days at 21 C under fluorescent lights (12-hr light period per 24 hr). Pycnidia embedded in infected tissue and in the agar surrounding infected disks could be readily observed. The proportion of all disks that contained at least one infection was determined. The average number of infections (n) per leaf disk was calculated as $n = \ln(1/1 - x)$, where x is the fraction of leaf disks infected. This experiment was conducted twice in its entirety. In this same experiment, a comparative study was made of the relationship between this leaf-disk infection assay and disease symptoms resulting from whole-plant inoculations. Leaflets from greenhouse-grown ferns inoculated with 10^5 conidia per milliliter were excised, surface-sterilized immediately after drying, and incubated for 24 hr at 21 C in a mist chamber. Leaf disks were examined for infections as described. Estimations of percent symptomatic tissue were made with the visual disease assessment key 2 wk after treatment of whole plants.

Determination of susceptibility of different-aged fronds to infection by *A. pteridis*. Frond phenology was determined by variations in morphology observed as fronds matured. A scale of 0–III was used based on morphological characteristics easily distinguished both in the field and greenhouse: 0 = fiddlehead stage (0–1 wk old), I = partially unfolded and succulent (1–2 wk old), II = fully unfolded, still succulent (2–3 wk old), and III = mature, hardened frond (more than 3 wk old).

Excised leaflets from fronds of various phenotypic stages were inoculated with atomized spore suspensions containing 10, 30, 10^2, 3×10^2, 10^3, 3×10^3, 10^4, 3×10^4, 10^5, 3×10^5, and 10^6 conidia per milliliter to uniform wetness. The leaflets were incubated in a moist chamber at 21 C for 24 hr. Infections of individual leaf disks were determined after removal from the moist chamber as described earlier. The proportion of leaf disks infected at each spore concentration were transformed by the Weibull equation $[\log_{10}(-\log_e(1 - x))]$, where x = the fraction of leaf disks infected (4), and plotted against the logarithm of inoculation concentration. Spore concentration necessary to obtain infection of 50% of the leaf disks (ED_{50}) was also estimated from these curves.

TABLE 1. Relative spore survival, sporulation capabilities, and virulence of isolates of Ascochyta pteridis collected in different areas of California

Isolate	Source[w]	Desiccation survival[x] (% spores viable)[y]	Spore production[x] ($\log_{10}$ spores per colony)	Disease severity[x,z] (% diseased tissue)
BL680	Blodgett	33.20 a	10.49 a	40.0 ab
FT680	Berkeley	1.11 b	10.38 a	32.5 b
PR580	Point Reyes	0.00 b	9.34 b	20.0 bc
MT779	Talmalpias	0.00 b	9.30 b	20.0 bc
V780	Usal	0.00 b	9.04 bc	17.5 c
0779	Bolenas	0.00 b	8.47 c	52.5 a

[w] All isolates collected within California.
[x] Values in a single column followed by a common letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.
[y] Spore viability measured after 20 days at a RH of ca. 21% over $CaSO_4$.
[z] Results for isolates 0779 represent the mean of three trials containing four replications each. Results of the other isolates represent the mean of one trial with four replications.

RESULTS

Description of pathogen. Pycnidia observed in necrotic leaf tissue and in agar culture were globose, brown to olive, immersed, later becoming erumpent, opening by apical ostioles, and 100–150 μm in diameter. Conidia were hyaline, cylindrical with rounded ends and with a single septum, and $10–15 \times 3–5$ μm in diameter. Colonies on MM agar produced abundant pycnidia when incubated 10 days under fluorescent lamps (12-hr light period per 24 hr).

In vitro tolerance of conidia to desiccation. Isolates of *A. pteridis* collected at different locations in California differed greatly in conidial tolerance to desiccation (Table 1). Isolate BL680 consistently displayed the highest germination of all isolates after desiccation for up to 30 days. Nearly 100 and 30% of the conidia of this isolate were viable after drying for 5 and 20 days, respectively. Fewer than 60% of the conidia from the other five isolates were viable after 5 days and fewer than 1% were viable after 20 days of desiccation (Table 1).

In vitro sporulation capabilities. Isolates of *A. pteridis* differed greatly in conidial production in vitro. The number of conidia produced per colony after 2 wk of growth ranged from about 3×10^8 for isolate 0779 to more than 3×10^{10} for isolate BL680. Isolates BL680 and FT680 consistently produced 10–100 times more conidia per colony than the other four isolates (Table 1).

Relative virulence of isolates of *A. pteridis*. *A. pteridis* isolates differed significantly in the severity of foliar necrosis that they incited. Under greenhouse conditions, maximum disease severity was observed 2 wk after inoculation. Isolates 0779 and BL680 were most virulent, causing necrosis of 52.5 and 40% of the total surface area of treated fronds, respectively (Table 1). The amount of foliar necrosis caused by the remaining isolates under identical conditions in this comparison was only about 30–50% of that caused by isolates 0779 and BL680 (Table 1).

Effects of free moisture and temperature on infection. Infection of bracken fern increased with increasing periods of free moisture and with increasing temperature during wet periods. No infections were observed at 6 C regardless of the duration of free moisture. A minimum duration of free moisture of 18 hr was required for measurable frequencies of infection of fronds held at 10 C (0.63 infections per disk) (Fig. 1). Maximum infection frequencies were

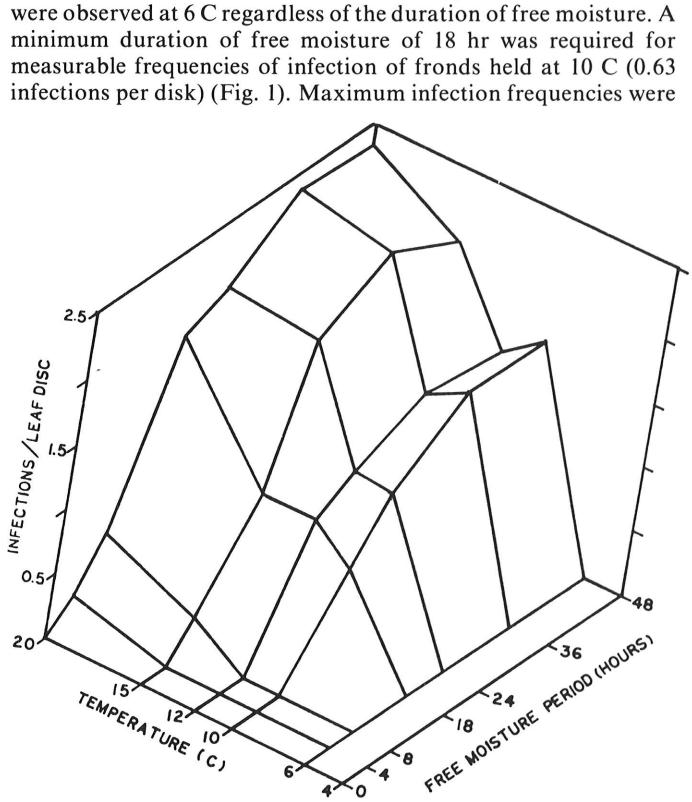

Fig. 1. Influence of temperature and free moisture on the number of *Ascochyta pteridis* infections in 3 mm disks of *Pteridium aquilinum* leaflets after spray inoculation of foliage.

achieved after 36 hr of free moisture at all temperatures above 6 C (1.5–2.3 infections per disk). The optimum temperature was 20 C under all free-moisture durations.

Susceptibility of fronds of different phenological stages to infection and symptom development. Fronds of differing phenological stages treated with *A. pteridis* under identical environmental conditions differed significantly in subsequent disease severity (Fig. 2). Fronds of stage III showed less than 30% foliar necrosis, whereas those in stages II and I showed between 30 and 75% and 60 and 90% necrosis, respectively. Similar phenological differences in symptom expression were observed under other combinations of free moisture and temperature. Fronds in the fiddlehead stage did not show disease symptoms under the conditions used in this and other experiments. Fronds of different phenological stages also differed significantly in their frequency of infection by *A. pteridis*. Fewer than 0.2 infections per leaf disk occurred in tissue excised from stage III fronds. However, up to 1.3 and 0.85 infections per disk were observed on tissue removed from fronds in phenological stages I and II, respectively (Fig. 2). No infections were observed in tissue taken from stage 0 fronds.

The amount of foliar necrosis that developed in fronds was correlated highly ($R = 0.931$) with the number of infections present in disks of tissue excised previously from the same fronds (Fig. 2). Fronds of phenological stage III showed both a very low severity of frond necrosis and low frequencies of infection. Fronds from stage I, however, were frequently infected by *A. pteridis* and showed a greater degree of foliar necrosis.

Three distinct disease-inoculum relationships were observed when the Weibull transformation of percent infected leaf disks from fronds of phenological stages I, II, and III was plotted against log spore concentration applied to plants in infectivity titrations (Fig. 3). Fronds of phenological stage I showed a linear response-probability curve indicative of a homologous distribution of susceptible tissue. The ED_{50} (ED_{50}I) for infection of stage I fronds was estimated to be about 10^3 spores per milliliter (Fig. 3). Fronds of phenological stage II displayed a bimodal infection response to applied inoculum, indicating that tissues of two susceptibilities occurred within stage II fronds. Distinct ED_{50} values of about 10^3 and 2×10^4 spores per milliliter ($ED_{50}II_a$ and $ED_{50}II_b$, respectively) were observed for fronds of this stage. Stage III fronds, like stage I fronds, displayed a homogeneous, linear response-probability curve, demonstrating a simple-exponential relationship of infection to inoculum dosage. The ED_{50}III value of about 5×10^4 spores per milliliter is similar to the $ED_{50}II_b$ value.

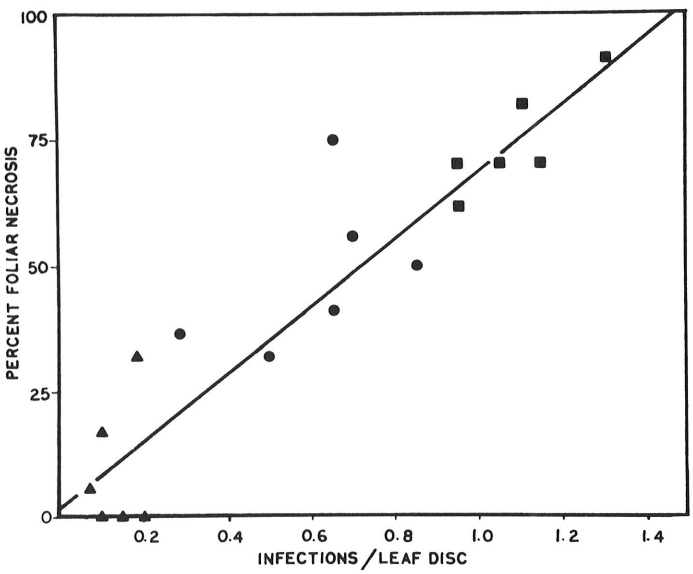

Fig. 2. Comparison of infection of *Pteridium aquilinum* leaf disks with severity of whole-plant necrosis observed after inoculation of fronds of phenology stage I (■), stage II (●), or stage III (▲) with *Ascochyta pteridis*. The line drawn represents the linear relationship $y = 68.7x + 1.6$ ($r^2 = 0.865$), $P < 0.01$ between severity of foliar necrosis of intact fronds and number of infections per 3-mm leaf disk detached from these same fronds. All fronds were treated with a conidial suspension of 10^5 spores of *A. pteridis* per milliliter and incubated at 21 C for 24 hr before excision of leaf disks or transfer of plants to a greenhouse for symptom development.

DISCUSSION

Evaluations of stands of ferns from diverse locations in California made from 1978 to 1984 revealed that <1% of the plants displayed infections by *A. pteridis*. Where the disease was observed, however, disease severity occasionally was 100% of frond area within small geographical areas (about 1–100 m^2). Diseased plants and plant tissues did not show a uniform spatial distribution but occurred in distinct foci.

An important factor apparently limiting the development of natural field epidemics caused by *A. pteridis* is the decrease in frond susceptibility to infection with age. This variation in host susceptibility could be identified and quantified with a Weibull transformation (Fig. 3) in conjunction with infectivity titrations of inoculum (1). Host heterogeneity to susceptibility to infection is not as obvious in untransformed dose-response relationships. Shortley and Wilkins (4) demonstrated that detection of heterogeneity of host susceptibility to infection could be enhanced by plotting the transformed percentage (x) of hosts infected, i.e., $[\log_{10}(-\log_e(1-x))]$, on a Weibull scale against log inoculum dose. This response-probability transformation reduces the simple-exponential curve generated by tissues of uniform susceptibility, when challenged with increasing inoculum dosage, to a straight line. This response is approached by fronds of phenological stages I and III, where the mean effective dosage (ED_{50}I and ED_{50}III, respectively) is the same for all tissues within a given stage of development. However, if the challenged tissues vary in susceptibility such that a proportion of the tissues are more susceptible to infection than the rest, as fronds in phenological stage II apparently do, a bimodal curve with two asymptotic projections identifying two different ED_{50} values is generated. In the case of stage II fronds, the two ED_{50} values observed (ED_{50}IIa and ED_{50}IIb) are very similar to the ED_{50} values for the tissue in fronds of phenological stages I and III, respectively. This indicates that fronds of phenological stage II contain tissue with two distinct disease susceptibilities similar to categories I and III. Therefore, a distinct transition in susceptibility apparently occurs upon maturation of bracken fronds at an age of about 2 wk.

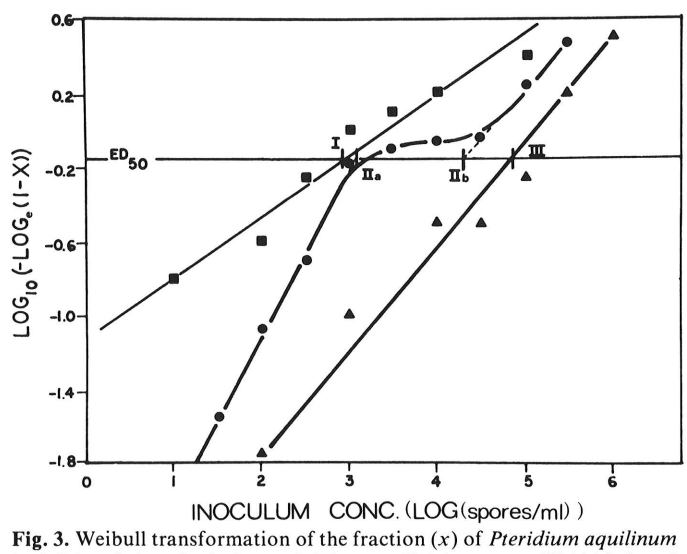

Fig. 3. Weibull transformation of the fraction (x) of *Pteridium aquilinum* leaf disks of phenological stage I (■), stage II (●), or stage III (▲) infected by *Ascochyta pteridis* after spray inoculation of foliage with the different inoculum concentrations shown on the abscissa. The estimated ED_{50} for each phenological stage of fern is shown as the interception of the dose-response curve, with the horizontal line representing 50% infection (■ and ▲) or the asymptotic projections of two linear proportions of the dose-response curve (●).

The leaf disk assay allowed us to maintain a large number of samples in a closely controlled physical and chemical environment and minimized variations in leaf phenology that occurred within whole-plant samples (Figs. 2 and 3). The good correlation between infection frequency as determined by the leaf disk assay and subsequent disease severity (Fig. 2) indicates this in vitro method of estimating infection closely approximated whole-plant responses. Some underestimation of the true infection frequency could, however, have occurred due to death of very recent infections by the surface-sterilization procedure used to eliminate nongerminated spores and ineffective germ tubes.

Temperature and free-moisture conditions conducive for infection and symptom development of this disease fell within a narrow range (Fig. 1). Such definitive free-moisture and temperature requirements probably limit disease progress from sporadic initial infections under field conditions. Weather records during 1979–1984 from target field areas in California and in other areas of the United States and the world where bracken fern is a weed indicate that free-moisture durations and temperatures during early growth periods are within the thresholds established for infection, especially early in the season (April and May). However, as the growing season in California progresses (June and July), environmental conditions favorable to infection decrease in frequency and tissue susceptibility decreases with age. Therefore, natural epidemics of this disease are self-limiting and of short duration.

A low level of primary inoculum and poor secondary inoculum dispersal probably also limit disease severity and rate of increase within a season. In field trials, these factors could be overcome by the application of primary inocula to susceptible tissues under environmental conditions shown to be conducive to disease development (8).

Results of this study indicate that a high degree of diversity exists among isolates of *A. pteridis* with regard to sporulation, tolerance to desiccation, and virulence. The "mycoherbicide" approach to biological weed control as identified by Templeton and Tebeest (6) is most applicable when factors limiting disease development can be identified and manipulated, and the degree of diversity among isolates of the pathogen exploited to optimize survival, pathogenicity under diverse environments, and inoculum production. *A. pteridis* is a highly virulent pathogen of bracken fern that can cause severe defoliation when applied under favorable environmental conditions. Because natural epidemics of this pathogen frequently occur but are of short duration and localized, the biological control of bracken fern should be successful if additional primary inoculum is applied at an early stage of bracken fern development.

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