Fungal endophytes of bracken *(Pteridium aquilinum)*, with some reflections on their use in biological control

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Endophytes were isolated from the pinnules, leaf vein, rachis, and rhizome of healthy spring and autumn plants of *Pteridium aquilinum* growing on two moorland and two woodland sites. Colonization of bracken by endophytic fungi appeared to be quite uniform in both types of habitats. Endophytes were isolated on average from approximately 25% of the pieces of each tissue investigated. Differences between the autumn and the spring samples were statistically significant, with the exception of both rhizome samples, in which the colonization frequencies at the two sampling dates were nearly equal. Within each sampling period, all aerial tissue types generally had homogeneous colonization frequencies. Three species (*Aureobasidium pullulans, Cylindrocarpon destructans,* and *Phoma* sp.) dominated the endophyte community in the spring, four (*A. pullulans, Ramichloridium schulzeri, Stagonospora* sp., and *Sordaria fimicola*) in the autumn. Ordination analysis by nonmetric multidimensional scaling indicated that the endophyte communities were not very organ–specific. The analysis also did not show pronounced patterns of seasonal succession or any clear differences in endophyte community composition between moorland or woodland sites. The potential role of *Aureobasidium pullulans* and *Stagonospora* sp. as candidates for use in the biological control of *Pteridium aquilinum* is briefly discussed.

Keywords: Coelomycetes, Hyphomycetes, ecology, fungi.

Bracken [*Pteridium aquilinum* (L.) Kuhn] is a dominant weedy species in moorlands and pastures of Great Britain (Macleod, 1982; Scragg, 1982). Much information exists on the mycorrhizal symbionts of ferns (e.g., Boullard, 1979) but the mycoflora of their aerial organs has been poorly investigated. Godfrey (1974) has briefly characterized the phylloplane mycoflora of *P. aquilinum* and more extensive investigations have been carried out by Frankland (1966, 1969, 1976) on the succession of fungi on decaying petioles of bracken and on its litter. Hutchinson (1976) has described possible fungal pathogens of bracken colonies in the field, finding only a limited number of fungal species associated with *P. aquilinum*. Its prothalli are not

axenic, but disease symptoms are infrequent (Hutchinson, 1976). Braid (1940) has reported that diseases of bracken sporophytes are rare and Angus (1958) has described only *Ascochyta pteridis, Phoma aquilina,* and a species of *Stagonospora* associated with the curled tip disease of the plant. Frankland (1966) has isolated species of *Phoma* and *Stagonospora* from apparently healthy petioles. This suggests that at least some of the fungi so far recorded from ferns may co-exist within the tissues of their host as symptomless endophytes.

Various attempts have been made at biological control of bracken (e.g., Burge & Irvine, 1985; Lawton, 1988; Lawton & al., 1988; Webb & Lindow, 1981). Epiphytic and endophytic fungi, specific to bracken, could be used either in first-order or second-order biocontrol (Burge & Irvine, 1985) although claims have also been made for the use of insects as control agents (Lawton & al., 1988). However, successful use of fungi in biocontrol requires a thorough knowledge of the microbial communities present in or on the target species, not only for selection of possible biocontrol agents but also to subsequently monitor any changes or interactions that may occur during or after the application of the mycoherbicide. Following this idea, the present investigation has been undertaken to describe the endophytic fungal communities present in the tissues of bracken sporophytes collected at four different sites and two seasons and to identify the dominant species of each community.

Materials and methods

Isolations

Endophytes were isolated from healthy spring and autumn plants of *Pteridium aquilinum* growing on two moorland sites at Scorr Hill (grid reference SY 657873) and South Tawton Common (grid ref. SY 655925), as well as on two woodland sites at Steps Bridge (grid ref. SY 804883) and Gidleigh (grid ref. SY 677885), all situated in the Dartmoor National Park in Devon, U.K. Mature plants were collected at the end of September 1990, before the first frost, and the spring collection was made at the end of May 1991 from very young plants, when the pinnules had just completed unrolling. At each sampling date, ten plants were collected from each site, placed in polyethylene bags, taken to the laboratory and processed within 24 hours after collection. Five pieces each of rhizome, taken from close to the emerging rachis, rachis, principal vein of the compound pinnule, and pinnule were sampled. All pieces (approx. 10 x 15 mm) appeared free of disease and blemishes.

The plant material was thoroughly washed in running water before surface sterilization of the pieces was performed by the immersion sequence 75% ethanol for 1 min, 20% NaClO for 3 min and 75% ethanol for 30 sec. The pieces were then placed in groups of five onto Petri dishes containing 1.5% Oxoid malt extract agar (MEA) supplemented with 250 mg l^{-1} Terramycin to suppress bacterial growth. All plates were incubated at 20 ± 2 C for $5 - 14$ d depending upon the growth rates of the emerging fungi. Isolation was by transfer of mycelium or conidia to 2% MEA plates without antibiotics. Near UV–light (Philips TL 40W/05) was used to induce sporulation.

Statistical Methods

Colonization frequency of the plants by a fungal species was defined as the total number of pieces of a given tissue (pinnules, leaf vein, rachis, or rhizome) colonized by a given fungus. Analysis of variance was used to detect differences in colonization frequencies among the different tissues studied and among sampling periods.

Community ordination was performed on the matrix of the raw data of the colonization frequencies. The ordination analysis was carried out on a reduced matrix containing only those taxa with a relative importance (dominance) index (Ludwig & Reynolds, 1988) of at least 10% in either of the two sampling periods. The resulting matrix was analyzed by monotonic multidimensional scaling (MDS), since non-linearity in the dataset did not allow the use of principal components analysis. The statistical package SYSTAT, release 5.1 (Wilkinson, 1989), was used for all computations and graphical displays of the data.

Results

Colonization of bracken by endophytic fungi is quite similar in both types of habitats. Endophytes have been isolated on average from approximately 25% of the pieces of each tissue investigated, with homogeneous frequencies of colonization among the different tissue types (Fig. 1). Differences in colonization frequencies between the autumn and the spring samples are statistically significant (P Aureobasidium pullulans, *Cylindrocarpon destructans,* and *Phoma* sp.) have relative importance values of more than 50%. Four species *(A. pullulans, Ramichloridium schulzeri, Stagonospora* sp., and *Sordaria fimicola)* dominate the endophyte community in the autumn sampling. Both distributions are lognormal.

Of the 66 fungal taxa recorded (Tab. 1), only a limited number were isolated with frequencies large enough to detect patterns of variation among samples. Inspection of Fig. 3a reveals some patterns of tissue specificity. *Cylindrocarpon destructans* and *Mortierella* sp.
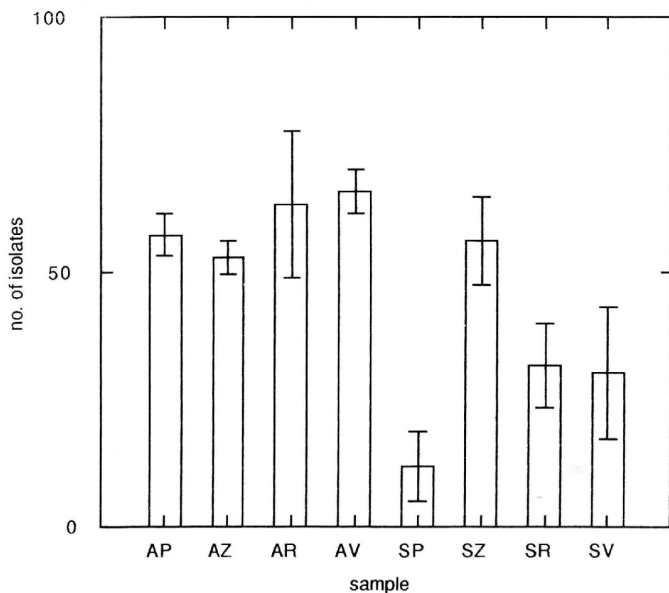
Fig. 1.– Mean values and standard deviation bars of overall colonization frequencies for the two sampling dates. The total possible number of isolations for each tissue is 200.– A: autumn; S: spring; P: pinnule; R: rachis; V: leaf vein; Z: rhizome.

1 preferentially colonize the bracken rhizome, whereas *Stagonospora* sp. can be found mainly in the aerial organs of the fern. *Aureobasidium pullulans* and *Cladosporium tenuissimum,* on the other hand, are most frequent in the rachis and leaf vein of bracken (Tab. 1). Most of the frequently occurring species have been isolated at both sampling dates; on the other hand, some taxa (e.g., *Sesquicillium buxi)* are present only in the young plants and are replaced by other fungi at a later stage, whereas others (*Phomopsis aquilina, Ramichloridium schultzeri, Virgariella* sp.) apparently infect the young plants during the summer and can thus be detected only in mature plants (Fig. 2, Fig. 3b). This may indicate a succession within the endophyte communities of bracken.

Ordination analysis does not show the endophyte communities to be good discriminators of the bracken tissues, nor does it detect pronounced patterns of seasonal succession. With a low stress of the final configuration (0.0834) and an almost linear Shepard diagram,

285

Tab. 1.– Fungal isolates from *Pteridium aquilinum*. Only those fungi that accounted for a relative importance (RI) of at least 10% have been included in the detailed list. Figures represent the mean values of the overall frequencies of isolation [number of pieces (out of 50) infected for each tissue type and site] for the two habitats and seasons. A: autumn; B: spring; M: moorland; W: woodland; P: pinnule; R: rachis; V: leaf vein; Z: rhizome.

Taxon	Code	APM	APW	ARM	ARW	AVM	AVW	AZM	AZW	SPM	SPW	SRM	SRW	SVM	SVW	SZM	SZW
Absidia cylindrospora Hagem	AC	0	1	1	1	0	0	9	6	0	0	0	0	0	0	2	0
Aureobasidium pullulans (De Bary) Arnaud	AP	0	0	6	14	0.5	17	0	0	0	0	5	5	1	15.5	3	7
Cladosporium tenuissimum Cooke	CT	0	4	7	4	5.5	4.5	0	0.5	0.5	0.5	1.5	0.5	7	3	0	2.5
Cylindrocarpon destructans (Zins.) Scholten	CD	0	0	0	0	0	0	3	4.5	0	0.5	0.5	5.5	0.5	0.5	5.5	11.5
Endoconospora sp.	EA	0	0.5	0	6.5	0	7.5	0	0	0	0	0	0	0	0	0	0
Exophiala sp.	EX	0	0	0	0	0	0	0	0	1	1	3.5	0	6.5	1	1	0.5
Fusarium avenaceum (Corda: Fr.) Sacc.	FA	0	0	0	0	0	0	0	0	0	0	0.5	3.5	0.5	0	1	0.5
Mortierella sp. 1	MA	0	0	0	0.5	0	0	7.5	7.5	0.5	0	0.5	2.5	0	0.5	4.5	7
Mortierella sp. 2	M2	0	0.5	0	1	0	1	5.5	5	0	0	0	0	0	0	0	0
Phoma sp.	PO	0.5	0.5	0	1	0.5	1.5	0.5	1.5	3	0	4.5	2.5	3	1	6.5	0.5
Phomopsis aquilina Petr.	PA	0	10.5	1	0	0.5	7.5	0	0	0	0	0	0	0	0	0	0
Ramichloridium schulzeri (Sacc.) de Hoog	RS	5	1.5	25.5	2	11.5	1	7	1.5	0	0	0	0	0	0	0	0
Seimatosporium sp.	SE	0	0	0	0	0	0	0	0	0.5	0.5	1.5	0	1	0.5	1.5	0
Sesquicillium buxi (Link: Fr.) W. Gams	SB	0	0	0	0	0	0	0	0	0	0	1	0.5	0.5	0	0	1

Sordaria fimicola (Rob.) Ces & De Not.	SF	15.5	1	1.5	0	14	0.5	3.5	0.5	1.5	0	0	0	0.5	0	3	0
Stagonospora sp.	SG	20	3.5	11	4.5	23.5	0.5	0	4.5	4	0.5	0.5	1	1.5	1	0.5	0.5
Sterile Basidiomycete	SS	0	0	0	0	0	0	0	0	0	0	0.5	1	0	0	7.5	3.5
Trichoderma pseu-dokoningii Rifai	TR	0	0	0	0	0	0	8	6	0	0	0	0	0	0.5	2.5	1
Trichosporiella cere-briformis (de Vries & Kleine Natrop) W. Gams	TC	0	2.5	0.5	3.5	0	1	1.5	6	0	1	1.5	2	0.5	0	0.5	4
Virgariella sp.	VA	0	13	0	2.5	0.5	11	0	0	0	0	0	0	0	0	0	0
Xylaria sp.	XY	0.5	3	0	0.5	0	2.5	0.5	0	0	0	0	0	0	0	0	0
Sterile		9	1.5	8.5	3	2.5	0.5	0	2	5	1	2.5	4.5	2	2	12.5	8.5

Rare isolates (less than 10% RI): *Acremonium* spp., *Aporothielavia leptoderma* (Booth) Malloch & Cain, *Botrytis cinerea* Pers., *Ceuthospora* cf., *Chaetomium indicum* Corda, *C. ochraceum* Tschudy, *C. spinosum* Chivers, *Cladosporium herbarum* (Pers.: Link) Gray, *Colletogloeum* sp., *Colletotrichum* sp., *Cryptocline* sp., *Cryptomycella pteridis* (Kalkbr.) Höhn., *Cryptosporiopsis* sp., *Exophiala* sp., *Fusarium graminearum* Schwabe, *F. lateritium* Nees, *F. oxysporum* Schlecht., *F. solani* (Mart.) Sacc., *Geomyces pannorum* (Link) Siegler & Carmichael, *Gilmaniella humicola* Barron, *Gnomonia* sp., *Humicola fuscoatra* Traaen, *Leptostroma filicinum* Fr., *Melanconium* sp., *Melanospora brevirostris* (Fuck.) von Höhnel, *Monostichella* cf. *indica* Sutton, *Mortierella ramanniana* (Möller) Linnem. var. *ramanniana* Linnem., *Mucor circinelloides* van Tieghem, *M. indicus* Lendner, *M. tuberculisporum* Schipper, *Nigrospora oryzae* (Berk. & Br.) Petch, *Penicillium* spp., *Phialophora* spp., *Phythium* sp., *Pleospora herbarum* (Pers.: Fr.) Rabenhorst, *Scytalidium* sp., *Sphaeriothyrium filicinum* Bub., *Sporormiella intermedia* (Auersw.) Ahmed & Cain, *Thysanophora penicillioides* (Roum.) Kendrick, *Trichoderma polysporum* (Link: Pers.) Rifai.

multidimensional scaling appears to describe the ecological model very well. The separation of the samples by collecting dates, origins or plant tissues is not clear-cut, although some patterns emerge from the analysis. The first two dimensions (Fig. 4a) are determined mainly by *C. destructans, Stagonospora* sp., and *Absidia cylindrospora*. The spring root samples are characterized by high colonization frequencies of *C. destructans* and low frequencies of *Stagonospora* sp., whereas in the autumn samples *Stagonospora* sp. is the dominant endophyte and *C. destructans* is rare. The plot of dimensions 1 and 3 (Fig. 4b) highlights the importance of *C. destructans* in distinguishing the rhizome tissues from all other samples. Only the spring samples of rachis tissues from the woodland sites, heavily colonized by *C. destructans,* are included in the rhizome clusters in both Fig. 4a and 4b.
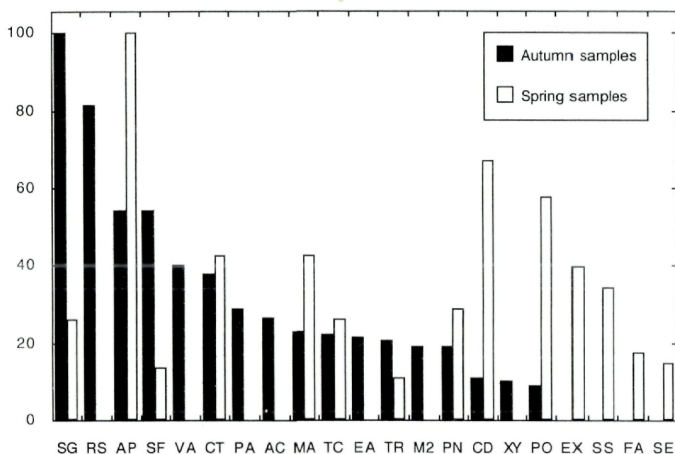
Fig. 2.– Relative importance (%) of each individual fungal taxon isolated. Only those taxa that account for a relative importance of at least 5% have been included in the graph. The most frequent species has been assigned the value of 100% after the method described by Ludwig and Reynolds (1988) and the importance of all other taxa has been computed accordingly. Both curves are typical for a lognormal distribution of the species. AC: *Absidia cylindrospora*; AP: *Aureobasidium pullulans*; CT: *Cladosporium tenuissimum*; CD: *Cylindrocarpon destructans*; EA: *Endoconospora* sp.; EX: *Exophiala* sp.; FA: *Fusarium avenaceum*; MA: *Mortierella* sp. 1; M2: *Mortierella* sp. 2; PO: *Phoma* sp.; PA: *Phomopsis aquilina*; PN: *Penicillium* spp.; RS: *Ramichloridium schulzeri*; SE: *Seimatosporium* sp.; SF: *Sordaria fimicola*; SG: *Stagonospora* sp.; SS: Sterile Basidiomycete; TR: *Trichoderma pseudokoningii*; TC: *Trichosporiella cerebriformis*; VA: *Virgariella* sp.; XY: *Xylaria* sp.
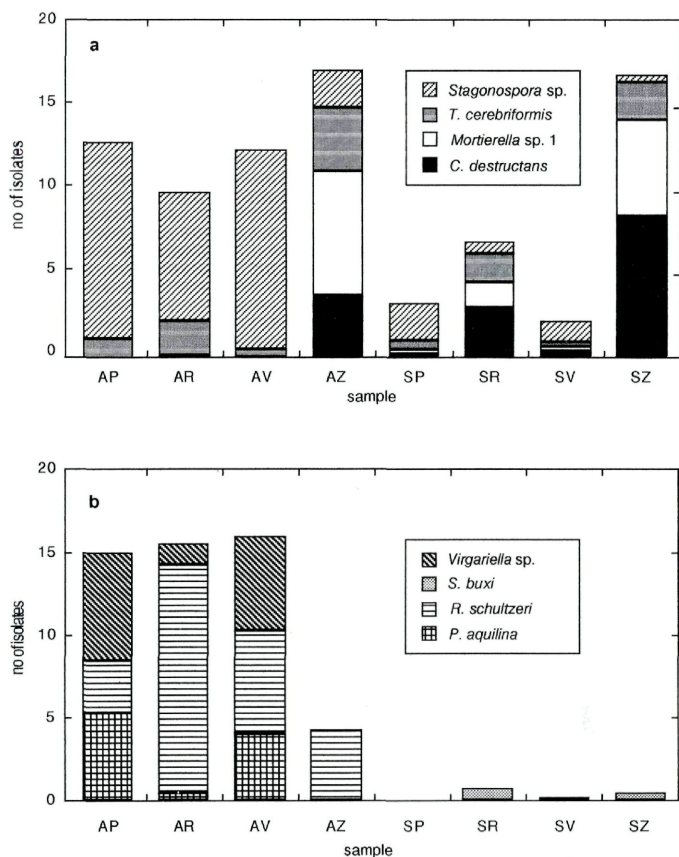
Fig. 3.– Bar chart of the colonization frequencies (raw data) of some endophytes isolated.– a: taxa showing patterns of tissue specificity. b: taxa showing patterns of seasonal variation.– A: autumn; S: spring. P: pinnule; R: rachis; V: leaf vein; Z: rhizome.

The first two dimensions are also useful to distinguish seasonal variation. The rather peculiar position of the two autumn samples ARW and AVW is determined by low levels of colonization by both *Stagonospora* sp. and *C. destructans*. Accordingly, SPM is the only spring sample with low *C. destructans* values. The separate position
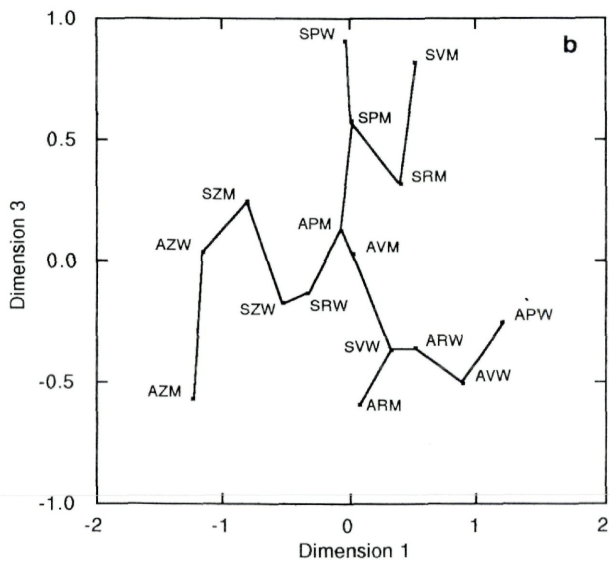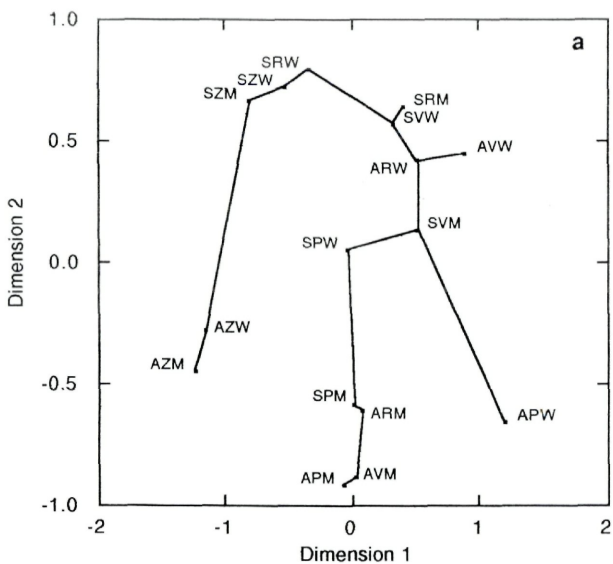
of APW is determined by high colonization levels by *Phomopsis aquilina* and *Virgariella* sp.

The ordination analysis does not detect any clear differences in endophyte community composition between moorland or woodland sites.

Discussion

Host tissue and organ specificity have been demonstrated for wheat endophytes (Sieber, 1985) and for endophytes of Norway spruce (Sieber, 1988, 1989). Fisher & al. (1991) have shown that aquatic and soil root samples of *Alnus glutinosa* (L.) Gaertn. are colonized by two different endophyte communities and have postulated that organ specificity may result from the adaptation by some endophytes to the particular microecological conditions present in a given organ. In bracken, the endophyte flora of the rhizome, the only perennial organ of this plant, is distinct from that of the aerial parts, as already shown for other hosts (Fisher & Petrini, 1992; Oberholzer, 1982). On the other hand, the composition of the endophytic communities of the pinnules, rachis, and leaf veins of bracken appear to be quite similar, with only selected species being more abundant in any given organ. For instance, *Stagonospora* sp. is the most common colonizer of the pinnules, and *Aureobasidium pullulans* and *Cladosporium tenuissimum* are prevalent in the rachis and leaf veins.

Surprisingly, no site-specific patterns appeared in this investigation. Edaphic components were shown by Hunter (1953) to affect only marginally the chemical composition of bracken tissues. It can be assumed, therefore, that the nutritional requirements for most fungal specialists will be adequately covered in all sites where bracken can grow. However, it can also be expected that microclimatic differences would account for among-sites variation in colonization frequencies. Although no statistically significant clustering of the samples by geographic origin was detected, the consistent tendency for samples originating from the same sites to group quite closely on the ordination plots leaves the possibility open that more extensive sampling could confirm this trend.

Striking differences in colonization frequencies, and partly also in endophyte community composition, can be seen between samples

Fig. 4.– Results of the ordination analysis by MDS. For the analysis only those fungi with a relative importance of at least 10% have been used. The samples units are linked by a minimum spanning tree to help detecting similarities and preventing misinterpretations. The path of the tree corresponds to the level of similarity among sample units.– a: plot of Dimension 2 vs Dimension 1; b: Plot of Dimension 3 vs. Dimension 1. A: autumn; S: spring; M: moorland; W: woodland; P: pinnule; R: rachis; V: leaf vein; Z: rhizome.

collected at different times of the year. The marked increase in colonization in the mature plants suggests that airborne and rain-dispersed inoculum lands on the plants and infects them, as demonstrated by Kinkel & al. (1987, 1989) for apple leaf epiphytes. Infection by airborne and rain dispersed spores can thus be postulated for endophytic species isolated exclusively from samples collected in autumn, such as *Cryptocline* sp., *Endoconospora* sp., *Gilmaniella humicola, Mortierella* sp. 2, *Phomopsis aquilina, Ramichloridium schulzeri, Virgariella* sp., and *Xylaria* sp.

Frankland (1966) has reported a number of fungi from decaying bracken petioles, including *Acremonium murorum, A. pullulans, Mucor* spp., and an unidentified *Stagonospora* sp. Decaying bracken material on the ground may provide inoculum for subsequent spring and summer infection, although Frankland (1976) observed a drastic decrease of *A. pullulans* in first-year bracken litter.

The use of endophytes as mycoherbicides has been proposed by Sieber & al. (1991), who also discussed the related advantages and disadvantages. In the case of bracken, *A. pullulans* and *Stagonospora* sp., both known pathogens of *Pteridium,* are potential candidates. *A. pullulans* causes superficial lesions on the petiole of bracken (Frankland, 1966) and a species of *Stagonospora* is always associated with the curled tip disease of bracken (Angus, 1958; Hutchinson, 1976). Both fungi are dominant components of the endophyte flora in all sites studied and have already been isolated by Frankland (1976) from non–diseased bracken plants. If appropriate infection conditions can be determined for *A. pullulans* and *Stagonospora* sp. or if virulence genes could be introduced into the genome of these two species (Petrini, 1991), they may prove effective in the biological control of *Pteridium aquilinum.*

Acknowledgments

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