The northern flying squirrel *(Glaucomys sabrinus)* as a vector for inoculation of red spruce *(Picea rubens)* seedlings with ectomycorrhizal fungi

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Caldwell I.R., Vernes K. & Bärlocher F. (2005) The northern flying squirrel (*Glaucomys sabrinus*) as a vector for inoculation of red spruce (*Picea rubens*) seedlings with ectomycorrhizal fungi. – Sydowia 57 (2): 166–178.

Mycophagous mammals excavate and ingest fruiting bodies (ascomata) of hypogeous ectomycorrhizal fungi and produce faeces containing numerous spores. To evaluate the significance of mycophagy to plant hosts we compared inoculation rate and degree of fungal development on red spruce (*Picea rubens*) seedlings treated with (1) faeces of the northern flying squirrel (*Glaucomys sabrinus*) against seedlings treated with (2) ascospores of *Elaphomyces granulatus*, and (3) those grown in natural forest soil or (4) forest soil that had been rendered sterile. No seedlings grown in sterilised soil showed fungal colonization. Significantly more seedlings were colonized in natural forest soil (97.5 %) than in sterile soil treated with squirrel faeces (69.2 %) or fruiting body spores (27.5 %). Treatment with squirrel faeces produced significantly more colonization than treatment with fruiting body spores. Fungal development was significantly greater on seedlings grown in forest soil compared with other treatments, but did not differ significantly between squirrel faeces and fruiting body treatments. These results demonstrate that passage through the digestive tract of flying squirrels may enhance germination and inoculation potential of fruiting body spores, although actively growing mycelium in forest soil may be the primary and most effective means by which seedlings develop mycorrhizae under natural conditions.

Keywords: mycophagy, hypogeous fungi, spore germination, fruiting body.

Hypogeous ectomycorrhizal fungi form important symbiotic associations with woody plants of forested ecosystems (Smith & Read 1997). In North America, several tree species, including those in the genus *Picea* (spruces), require such fungal symbionts for growth and survival (Castellano 1994). In epigeous fungi, spores are liberated into the air and subsequently dispersed by wind and water; however, in hypogeous species, spores are contained within the peridium of an

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ascoma or basidioma that develops beneath the soil surface (Kendrick 2000). Hypogeous fungi therefore require excavation, either by soil microfauna or a mammalian mycophagist, before spore dispersal can occur (Allen 1991).

Mature hypogeous fungal fruiting bodies often emit strong odours (Maser *et al.* 1978) that attract mycophagous mammals (Donaldson & Stoddart 1994, Pyare & Longland 2001) and ingested spores remain viable after passage through the mammalian gut (Kotter & Farentinos 1984, Claridge *et al.* 1992, Reddell *et al.* 1997, Terwilliger & Pastor 1999, Colgan & Claridge 2002). In North America, several mammal species regularly consume hypogeous fungi (e.g. Fogel & Trappe 1978, Ovaska & Herman 1986, Trappe 1988, North *et al.* 1997, Cazares *et al.* 1999). The northern flying squirrel (*Glaucomys sabrinus* Shaw) may be a particularly important mycophagist because it is common throughout much of northern North America (Nowak 1999) and consumes fruiting bodies of many hypogeous species on a year-round basis (e.g. Maser *et al.* 1985, Hall 1991, Rosentreter *et al.* 1997, Lehmkuhl *et al.* 2004, Vernes *et al.* 2004). In southern New Brunswick, northern flying squirrels consume at least 16 hypogeous taxa (Vernes *et al.* 2004), and they have great potential to disperse ingested fungal spores far from the host tree by way of their gliding locomotion (Vernes 2001) and relatively large home range of up to 12.5 hectares (Gerrow 1996).

Only seven experimental studies have examined inoculation of plants with ectomycorrhizal fungi through the application of mammal faeces. In North America, Kotter and Farentinos (1984) inoculated ponderosa pine (*Pinus ponderosa* Douglas) seedlings with faeces from tassel-eared squirrels (*Sciurus aberti* Woodhouse); Miller (1985) inoculated seedlings with faeces from captive white-footed mice (*Peromyscus leucopus* Rafinesque) that had been fed *Tuber* spp. fruiting bodies; Terwilliger & Pastor (1999) inoculated black spruce (*Picea mariana* Miller) with faeces collected from the red-backed vole (*Clethrionomys gapperi* Vigors); and Colgan & Claridge (2002) inoculated seedlings of Douglas-fir (*Pseudotsuga menziesii* Mirbel) and ponderosa pine with faeces from the Californian red-backed vole (*Clethrionomys californicus* Merriam), Townsend's chipmunk (*Tamias townsendii* Bachman), and northern flying squirrel. In the Terwilliger & Pastor (1999) study, inoculation success was greatest with forest soil (93–100 %) versus 0 % for sterilized soil, but was also greatly enhanced with *C. gapperi* faeces (11–83 %). Success varied in response to the time of year that faeces were collected, with August-collected faeces having greater success than faeces collected in May (Terwilliger & Pastor 1999). In Australia, Lamont *et al.* (1985) and Claridge *et al.* (1992) experimentally inoculated eucalyptus seedlings with faeces from mycophagous marsupials (woylie, *Bettongia penicillata* Gray and long-nosed potoroo, *Potorous tridactylus* Kerr respectively); and Reddell *et al.* (1997) inoculated eucalyptus seedlings with

faeces of a further five Australian mammal species. In most instances, mammalian inoculum proved effective in producing mycorrhizae.

The objective of our study was to determine whether northern flying squirrels could be effective vectors for the spores of hypogeous ectomycorrhizal fungi. In particular, we were interested in whether spores from northern flying squirrel faeces had enhanced inoculation potential on red spruce seedlings (*Picea rubens* Sargent) compared with spores that had not passed through the squirrel digestive system, and how these inoculation rates differed from soil containing active fungal mycelia. We hypothesized that mycophagy by the flying squirrel would enhance fungal inoculation and, therefore, seedlings inoculated with spore-containing faeces would exhibit greater fungal colonization than those inoculated with spores from a fruiting body, but less than those grown in forest soil containing mycelium. The importance of hypogeous ectomycorrhizal fungi to plant growth is well documented (Kendrick 2000, Allen 1991, Ingham & Molina 1991, Amaranthus 1998). This paper contributes to the less well-understood relationship among mammals, plants and fungi – a relationship vital to maintaining the long term viability of forested ecosystems.

Materials and methods

Faecal and Cleistothecium Samples

Northern flying squirrel faecal samples were collected from animals live-trapped at Fundy National Park (centre: 45°37'N, 65°02'W) between May and August 1999. Samples were frozen at time of collection. Squirrel faecal pellets which were dominated by spores from the genus *Elaphomyces* (Ascomycota) were preferentially selected for the study, since fruiting bodies of this genus were commonly collected in the study area (K. Vernes, unpublished data). Although faecal spore quantities differed over the collection period (Vernes *et al.* 2004), pellets were chosen solely based on the relative abundance of *Elaphomyces* within the sample, not overall quantity. Each faecal pellet was crushed using a mortar and pestle in 20 mL of distilled water to create a faecal-spore suspension. Spores in faeces were then counted in a haemocytometer and the total number of spores in the pellet was calculated. Inoculation on the roots of a developing seedling has been successful with as few as 100 spores (Theodorou & Bowen 1973). To ensure number of spores was not limiting, we created a faecal-spore suspension in distilled water that contained 2 500 spores per mL.

Elaphomyces granulatus Fries fruiting bodies (cleistothecia) were also collected at Fundy National Park between May and August 1999 and frozen until use. Cleistothecia were sliced open with a sterile scalpel and a sub-sample of the spore material was removed with a

sterile spatula. This sub-sample was weighed and mixed with 20 mL of methyl cellulose to reduce surface tension and allow spores to be counted using the haemocytometer. We then created a spore suspension in distilled water that contained 2 500 spores per mL.

Seedling Preparation

Red spruce seeds (J.D. Irving Ltd, Sussex, NB) were allowed to germinate in the dark on moist, sterile filter paper prior to planting. After germination, seeds showing the healthiest and most vigorous radicle growth were selected for planting. Soil was collected from Fundy National Park in the same habitat where squirrels were trapped and fungi collected. Although fungal strands were observed within the forest soil, fungal content was not quantified within these samples. Most of the soil to be used in the experiment was autoclaved to kill any living material, but a portion was left unaltered for use in Treatment 1 (below). Each 30 mm-diameter cell of seedling propagation trays was randomly assigned to one of four treatments, resulting in 60 cells per treatment. Cells in Treatment 1 were filled with untreated forest soil while remaining cells were filled with autoclaved forest soil. One seed was planted per cell and allowed to grow for five weeks before fungal inoculum was applied. Our experiment was undertaken in a heated greenhouse between November 2000 and April 2001, with a combination of natural and artificial light to mimic typical mid-summer day-lengths for this latitude (16 hrs light, 8 hrs dark). Average greenhouse temperatures were 15 °C during the light phase and 10 °C during the dark phase. Seedlings were watered approximately every 48 hours, or when the soil was dry.

Experimental inoculation

Any seedling that had not emerged after five weeks of growth was removed from the experiment, leaving at least 40 seedlings per treatment. Treatments for the experiment consisted of (1) untreated forest soil; (2) autoclaved forest soil (control); (3) autoclaved forest soil with squirrel faecal inoculum added as a 20 mL spore suspension (50 000 spores); and (4) autoclaved soil with a 20 mL suspension of *Elaphomyces granulatus* spores (50 000 spores) added. At ten weeks of growth a second dose of treatments 3 and 4 was applied to ensure that sufficient numbers of spores were present at a time when mycorrhizal formation can occur.

Analysis

Between 12–18 weeks growth, five seedlings were removed from each treatment per week and their roots examined for fungal hyphae.

Coarse soil particles were removed from roots with fine forceps, and finer particles by soaking seedlings in a 70 % ethanol solution for one hour. Cleaned seedling roots were placed in vials of 0.01 % Chlorazol Black E (CBE) for 48 h. CBE is a fungal specific stain that has been used both in ectomycorrhizal (Brundrett *et al.* 1994) and endomycorrhizal studies (Brundrett *et al.* 1996). We applied the stain to spruce roots with ectomycorrhizal fungi and found it to be equally successful for staining their hyphae.

Following staining, seedlings were washed in 70 % ethanol for one hour to remove remaining detritus from roots before observation under a dissecting microscope. If fungal hyphae (stained black) were found associated with the roots of a seedling, the percentage of the root containing hyphae was classified according to the scheme proposed by Kormanik & McGraw (1982) where 1 = 0–5 %, 2 = 6–25 %, 3 = 26–50 %, 4 = 51–75 % and 5 = 76–100 % coverage. The intensity of fungal colonization was also estimated with a scheme proposed by Kormanik *et al.* (1980) where 1 = roots with small colonization sites widely scattered; 2 = larger colonization sites more uniformly distributed but rarely coalescing; and 3 = feeder roots that are almost completely colonized.

Fluorescent staining

We used florescent staining to gather more information about the types of fungal hyphae associated with seedlings. After 15 weeks of growth two seedlings from each of the untreated soil and untreated faeces treatments were removed. Several whole root sections were sliced and combinations of stains applied in an attempt to identify morphological structures. CBE and trypan blue were used along with two fluorescent nuclear stains, DAPI (4',6-diamidino-2-phenylindole) and Hoechst 33342 dye (Sigma), and cotton blue (a general stain for fungi). Those sections stained with fluorescent nuclear stains were microwaved for five seconds on high to facilitate movement of the stain into the root. Sections were mounted on slides and viewed at magnifications of up to 800X. Qualitative observations of the appearance of the hyphae were recorded.

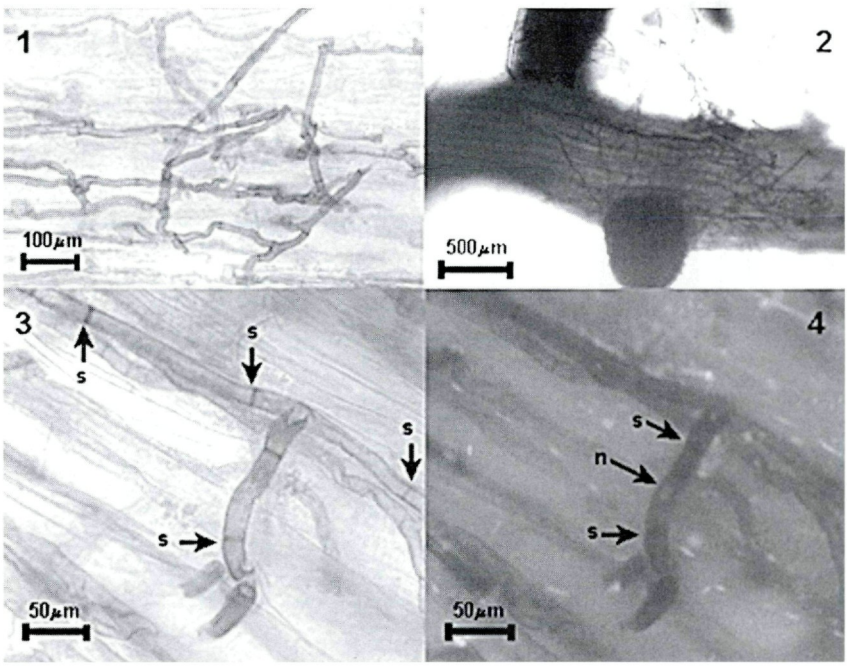
Statistical analysis

Differences in fungal growth over the five treatments were assessed with the Kruskal-Wallis test. The dependent variable was the measurement of fungal growth (percentage coverage classification or intensity) and the factor was treatment. Dunn's multiple comparisons test was used to analyse differences between each pair of treatments.

Results

Fungal Development

After twelve weeks of growth hyphae were found associated with the roots of seedlings grown in unsterilised soil, squirrel faeces and *Elaphomyces* spore treated seedlings. Hyphae turned a distinct black colour that contrasted with the translucent root tissue (Figs. 1–2). Hyphae in the three treatments appeared similar. Hyphae were approximately 10 µm wide; no mycelia were seen to have invaded plant cells and hyphae were clearly septate (Fig. 3; septa evenly spaced at 65–100 µm). Although a fully formed Hartig net was not found, there was evidence of early stages in its formation. These observations suggest that the fungus was ectomycorrhizal. Fluorescent staining revealed additional details about hyphae; only one nucleus was seen between septa (Fig. 4), and dolipores (a septal pore-type found in Basidiomycetes but not Ascomycetes) were not seen. These observations point toward the fungi belonging to the Ascomycetes.



Figs. 1–4. Root whole-mounts of red spruce *(Picea rubens)* seedlings grown in natural forest soil after 12 weeks **(1.)** and 15 weeks **(2.)**, stained with Chlorazol Black E (CBE); after 15 weeks **(3.)** and stained with CBE and Hoechst dye. **4.** Represents the same sample as **3.**, but examined under ultraviolet light. Dark stained hyphae are clearly visible against the translucent root tissue at **1.** and **2.**; septa (s) between hyphae are seen in **3.**; and the nucleus (n) of the hypha is evident at **4.**

Inoculation Success

No seedlings grown in sterilised soil developed fungal growth and significant differences in inoculation success were observed among the three treatments ($F_{2,21} = 53.4$; $P < 0.0001$). In the unsterilised forest soil treatment, 97.5 % of seedlings were colonized by fungi, a significantly greater percentage than values from both squirrel faeces (69.2 %; $P = 0.0002$) and *Elaphomyces* treatment (27.5 %; $P = 0.0001$). The squirrel faeces treatment, in turn, had significantly greater success than the *Elaphomyces* treatment ($P = 0.001$).

From 12 weeks of growth onward, neither fungal coverage nor intensity of coverage was correlated with week of growth, so we pooled fungal growth data across weeks within treatments. Kruskal-Wallis tests revealed significant differences between treatments for both coverage of fungi ($H = 32.8$; $P < 0.0001$) and intensity of coverage ($H = 21.0$; $P < 0.0001$; Fig. 5). Dunn's multiple comparison test revealed significant differences between forest soil and faeces treatments ($P < 0.001$ for both coverage and intensity) and forest soil and *Elaphomyces* treatments ($P < 0.001$ for coverage and $P < 0.01$ for intensity). Fungal coverage and intensity did not differ significantly between faeces and *Elaphomyces* treatments.

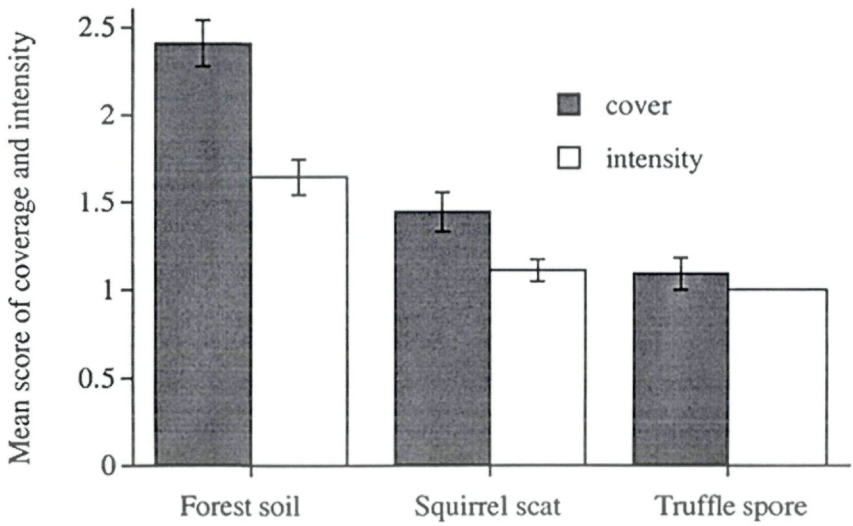


Fig. 5. Mean coverage (rank scale of 0–5) and intensity (rank scale 0–3; see text) ± S.E. of fungal hyphae on the roots of seedlings examined between weeks 12–20 of growth. Seedlings were grown in three treatments: (1) natural forest soil; (2) sterile forest soil treated with squirrel faecal inoculum, and (3) sterile forest soil treated with fruiting body spore inoculum. No seedlings grown in sterile soil (control) developed hyphae. $N = 40$ for each treatment.

Discussion

Observations of the mycelia that developed during our experiment provide some evidence that seedlings formed symbiotic associations, probably with ectomycorrhizal ascomycetes. At high magnifications, hyphae were observed growing around cortical cells but not invading them, as would occur if fungi were endomycorrhizal (Kendrick 2000). We did not observe any dead roots cells near the fungal hyphae, making it unlikely that pathogenic or saprobic fungi were involved. Furthermore, evidence of early formation of the Hartig net, which is only found in ectomycorrhizae (Smith & Read 1997), was apparent in several of our samples. Clamp-less septa were seen at regular intervals within hyphae in samples from all collection dates, suggesting the fungi belonged to asco- or basidiomycetes rather than to the Zygomycetes (Kendrick 2000).

Combinations of stains with both UV and visible light at high magnifications revealed more detailed information about the hyphae. A single nucleus present between each pair of septa indicated that the fungus was in a uninucleate stage, which precludes the presence or absence of clamp connections as a diagnostic character (Moore-Landecker 1990). However, Vernes *et al.* (2004) identified only one ascomycete genus, the genus *Elaphomyces,* in northern flying squirrels faecal samples at Fundy National Park. We used the same faecal samples in our experiment that Vernes *et al.* (2004) had examined, and chose samples with high proportions of *Elaphomyces* spores. Furthermore, *Elaphomyces* was the most common fungus collected at squirrel foraging locations at Fundy National Park, (K. Vernes, unpublished data), and was therefore used in our fruiting body spore treatment. Unfortunately, many of the morphological characteristics used to identify ectomycorrhizae are based on fully developed mycorrhizae (Goodman *et al.* 1996), making it difficult to identify the fungus any further using morphological criteria. However, all of the above factors lead us to believe that the hyphae observed on roots of our seedlings belonged to *Elaphomyces.*

Our study is one of only a small number that have examined the role small mammal mycophagists play in plant-fungal dynamics. In our study, untreated forest soil proved to be the most effective inoculum, with 97.5 % of seedlings developing associations with fungal hyphae. The squirrel faeces treatment was the next most effective inoculum (69.2 %), and was significantly more effective than spore inoculum from the *Elaphomyces* cleistothecium (27.5 %). Previous studies comparing soil and mammal inoculum reported similar results (Table 1), suggesting that the primary source of inoculum may be forest soil. In the same studies, mammal inoculum was 30–70 % effective, although Reddell *et al.* (1997) reported very high rates of inoculation using faeces from native Australian mycophagists (Tab. 1).

Tab. 1. – Percentage of seedlings infected with fungi in the present study, and in previous studies with mammalian and non-mammalian inocula (n.d. = no data).

Treatment	This Study	Kotter & Farentinos (1984)	Terwilliger & Pastor (1999)	Claridge *et al.* (1992)	Reddell & Spain (1997)
Sterilised soil	0.0	0.0	0.0[a]	0.0	n.d.
Non-sterilised soil	97.5	n.d.	96.5[b]	80	100
Mammal faeces	69.2	33.3	37.3[b]	45[c]	97.2
Fungal spores	27.5	20.0	n.d.	0.0	n.d.

[a] Terwilliger & Pastor (1999) used soil from an abandoned beaver pond that contained no mycorrhizae

[b] Represents the mean of several trials

[c] Results of the treatment where Claridge et al. (1992) deposited inoculum on the soil surface. Greater success (80 %) was achieved in that study when inoculum was mixed into the soil

Measurements of fungal growth were positively correlated suggesting that they were similarly effective indicators of fungal development. However, neither fungal coverage nor intensity of colonization increased over the seven-week sampling period, and colonised roots generally had only 6–50 % coverage or intensity. Mycorrhizae sometimes develop slowly (Smith & Read 1997) and the short duration of our experiment (20 weeks) may account for low levels of colonization and lack of measurable increase over time.

Untreated forest soil resulted in significantly greater coverage and intensity of fungi than other treatments at all time periods. The forest soil treatment likely contained a suite of living organisms (e.g. mycorrhizal helper bacteria) not found in the other treatments, which may stimulate rapid fungal colonization of the root. Furthermore, active hyphae were likely present in the soil at the start of the experiment, which could initiate immediate invasion of the plant root without having to first germinate. Even if only spores (rather than hyphae) were present in the soil treatment, germination may have begun during the first 12 weeks of growth, before inoculum was introduced in the other treatments. Several micro-organisms, some of which may have been present in the untreated soil, are also known to stimulate germination of ectomycorrhizal spores (Smith & Read 1997).

Hyphal growth from plant to plant may be the primary means by which new seedlings become inoculated with fungi in a functioning forest ecosystem. While efficient over short distances, this dispersal mechanism is slow (Allen 1991), and not well suited to rapid recolonization in areas where fungal biota may have been lost or interrupted. For example, ectomycorrhizal fungi have been found at the forefront of a receding glacier (Cazares & Trappe 1994) where soil

had been rendered bare of mycorrhizal fungi following thousands of years of glaciation. In all likelihood, mycophagous mammals transported spores to the glacial forefront in faeces, leading to a more rapid colonization of the area by forest trees (Cazares & Trappe 1994). Terwilliger & Pastor (1999) suggest that mammal mycophagists might also provide impetus that allows abandoned beaver ponds to return to forest, and other authors have suggested a similar role for mycophagous mammals in areas altered by forest fire (Claridge *et al.* 2001).

The squirrel faeces treatment in our study resulted in greater colonization of seedlings with discernible fungal hyphae than the *Elaphomyces* spore treatments, although coverage and intensity of fungi on roots that developed mycorrhiza were similar in the two treatments. After feeding *Rhizopogon* spores to three mammalian mycophagists, Colgan & Claridge (2002) found that colonization of seedlings was not significantly greater after application with northern flying squirrel feaces than after application with basidiospores. This result differs from our findings, but this may be due to their use of *Rhizopogon* while we used *Elaphomyces* or their use of Douglas-fir and ponderosa pine while we used red spruce seedlings.

Our study provides experimental support to suggestions that northern flying squirrels and other mycophagous mammals are important and efficient dispersal vectors for ectomycorrhizal fungi and that ingestion of spores may be required for optimal spore germination. Cork & Kenagy (1989) demonstrated that after passage through the gut of a golden-mantled ground squirrel (*Spermophilus lateralis* Say), the surfaces of ectomycorrhizal spores were visibly altered and Colgan & Claridge (2002) found that spores eaten by Californian red-backed voles and Townsend's chipmunks had greater metabolic activity than uneaten spores. Fogel & Trappe (1978) suggested that ingested fungal spores would be subjected to body heat, enzymatic action and co-culture with microorganisms during digestion and in faeces, all of which might increase the spore's propensity to germinate. Mycorrhizae grow faster when in contact with ammonium and phosphorus (Smith & Read 1997), and both are present in substantial amounts in mammal faeces. Furthermore, some mycorrhizal fungi germinate more readily when exposed to yeast extracts (Fries 1987) and Li *et al.* (1986) and Maser (1988) suggested that yeasts in the faeces of mammals might contribute to enhanced germination. Although there has still been no unequivocal demonstration that passage through the gut of a mammalian mycophagist enhances germination of fungal spores (Claridge *et al.* 1996), our data and previous research (e.g. Kotter & Farentinos 1984, Lamont *et al.* 1985, Claridge *et al.* 1992) support such a mechanism.

Acknowledgements

We thank Krista Cox and staff at the Nova Scotia Agricultural College (NSAC) for use of the greenhouse facility. Anna Fitzgerald, Dr Randy Olson (NSAC) and Dr David Garbary (St Francis Xavier University) gave expert technical assistance in preparation, staining and photographing hyphae. Parks Canada gave permission to collect samples in Fundy National Park and generously supported the project with equipment and personnel. We thank Dr Douglas Campbell (Mount Allison University) for reading and commenting on an earlier draft of the manuscript. Funding for the project came from Mount Allison University internal research funds; an NSERC Discovery Grant awarded to F. Bärlocher; and a McCain Postdoctoral Fellowship awarded to K. Vernes.

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(Manuscript accepted 3 October 2005)