The effects of selective cutting and liming on mushroom formation and mycorrhizal colonization of mature yellow birch (*Betula alleghaniensis* Britt.) in a deciduous forest within the Réserve faunique de Portneuf, south central Québec

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Abstract

The effects of selective cutting and liming on mushroom formation and mycorrhizal colonization of mature yellow birch (*Betula alleghaniensis* Britt.) in a deciduous forest within the Réserve faunique de Portneuf, south central Québec

To test the effects of liming (~500 kg/ hectare) and the cutting of ~1000 m^2 gaps on the formation of mushrooms in a yellow birch/sugar maple (*Acer saccharum* Marsh.) forest, all mushrooms were collected and quantified from twelve 49 m^2 plots. The liming had a neutral or positive affect on the fruiting of most species, for example, certain *Hygrophorus* species. Ectomycorrhizal taxa (eg. *Entoloma salmoneus*, *Lactarius rufus*) generally had decreased fruiting in the gaps whereas many non-mycorrhizal taxa were unaffected by the cuts. Species of *Hebeloma* and *Laccaria* however did not show any response to the treatments. The ectomycorrhizae on mature yellow birch were quantified using morphological and molecular analyses. Ectomycorrhizal abundance was compared to mushroom abundance and confirmed the lack of correlation between fruiting and mycorrhizal colonisation of yellow birch roots. The proportion of ectomycorrhizal ITS RFLPs matching mushroom RFLPs (34%) agreed with other studies. The matches included species of *Cortinarius*, *Hebeloma*, *Laccaria* and *Russula*. The data from seedlings from the same site showed that fungal taxa colonizing mature yellow birch had a higher diversity, due to a more even distribution. Some species of *Hebeloma* and *Laccaria* appeared to be more abundant on seedlings while species in the *Russulaceae* appeared to be more abundant on mature trees. These differences may reflect the importance of the fungal partners for the establishment and survival of yellow birch.

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Chapter 1

Introduction

Ectomycorrhizae are symbiotic mycorrhizal associations between the fine roots of plants and fungi. Ectomycorrhizae have been estimated to occur between 8000 plant species (Smith & Read 1997) and at least 6000 fungal species (Dahlberg 2001), mostly from the phylum Dikaryomycota. Many of the fungi are from the subphylum Basidiomycotina and the subphylum Ascomycotina, but one ectomycorrhizal genus in the Zygomycotina has also been documented (Smith & Read 1997). While this particular mycorrhizal association includes only about 3% of known plant species (Smith & Read 1997), they are mainly large woody plants which perform a large portion of the world's carbon cycling and provide much of our timber.

The fungi colonize these plants by forming a mantle of hyphae around the root tips. Hyphae also penetrate between the epidermal and/or cortical cells of the roots to form an intercellular structure called the Hartig net. The Hartig net is thought to be the point of nutrient and carbon exchange, where up to 30% of photoassimilate may be transferred to the fungus (Smith & Read 1997). Recent molecular and biochemical data using *Amanita muscaria*, have shown an increase in expression of the one sugar transporter protein found to date, bolstering the evidence that the fungus is creating a carbon sink in the roots of the plant (Wright et al. 2000, reviewed in Nehls & Hampp 2000, Nehls 2001). Labelled uptake experiments strongly suggest that the nitrogen, phosphorus and other trace elements, taken up by ectomycorrhizae, are transferred to the plants (Rygiewicz et al. 1984, Andersson et al. 1997, Colpaert et al. 1999, Perez-Moreno

& Read 2000, Bucking & Heyser 2000, Bucking & Heyser 2000b, Jentschke et al. 2001) via the Hartig net. Numerous recent studies also show other benefits to the host plant, including increased water uptake and stomatal conductance (Boyd et al. 1986, Mason et al. 2000, Muhsin & Zwiazek 2002) and increased resistance to pathogens (Duchesne et al. 1987a, Duchesne et al. 1989a, Duchesne et al. 1989b, Marx 1969), either due to a production of antibiotics by the fungus or an accumulation of antimicrobial compounds in the roots, induced by the fungus (Duchesne et al. 1987b).

The fungus associated with the plant is an infinitesimally small part of the fungal thallus or genet living beneath the soil. The genet of an ectomycorrhizal fungus consists of a hyphal network growing outward from the root tips in search of nutrients. For inorganic nutrient uptake, the small size of the hyphae allows for the exploration of soil pores which roots can not access (Simard et al. 1997). The absorption surface is much larger than plants can build, due to the comparatively large diameter of their root tips (Smith & Read 1997). In addition, the fungi can better harvest nutrients, either by producing enzymes to break down organic matter (Trojanowski et al. 1984, Durall et al. 1994, Perez-Moreno & Read 2000) and/or by colonizing saprophytic fungi (Lindahl et al. 1999). When the genet gains enough energy and the correct abiotic conditions are attained for a particular species (Kues & Liu 2000) it produces a reproductive/dispersal structure, the mushroom.

The soil fungal community is diverse and, like the mycorrhizal fungi, parasitic and saprophytic fungi are growing throughout the soil, absorbing nutrients and carbon compounds from live organisms or dead organic matter (Kendrick 1992, Colpaert & Van Laere 1996). The evidence suggests that ectomycorrhizal trophic status is variable

between species, some fungi grow very poorly without the host (Smith & Read 1997) while others can even fruit without the host present (Trojanowski et al. 1984, Durall et al. 1994). The data presented by Bruns et al. (1998) has suggested that ectomycorrhizal fungi have evolved several times from saprotrophic or parasitic lineages. Thus, some of these fungi may gain part of their carbon by saprophytic (Trojanowski et al. 1984) or parasitic (Lindahl et al. 1999) means.

The community of ectomycorrhizal fungi exists as a below ground, interwoven set of mycelial networks consisting of different species and different individual genets. The evidence to date suggests that the natural communities are dominated by a few species which have high colonization rates in conjunction with high species richness (Visser1995, Goodman & Trofymow 1998, Gehring et al. 1998). Genets vary in size depending on species (Gherbi et al. 1999, Redecker et al. 2001) and, most probably, abiotic factors and/or microsite size. Additionally, the evidence suggests that genets can move in space (Last et al. 1984, Dahlberg & Stenlid 1995) and time (Kranabetter 1999, Taylor & Bruns 1999), with differences between species (Taylor & Bruns 1999) and conditions (Kranabetter 1999). Comparative studies of undisturbed and disturbed sites generally show a shift in the dominant colonizers and a reduction in species richness in the disturbed habitats (Visser 1995, Lilleskov et al. 2002). The functional result of these shifts is difficult to determine, due to a lack of knowledge of the functional differences between species (reviewed in Cairney 1999). However Durall et al. (1999) have suggested that the diversity at a site is functionally important in allowing the plants to associate with a fungus which is best adapted to exploit specific soil microsites.

Differences in the growth parameters of species and in their respective abilities to assist the plant in its growth support this theory.

Gap creation is one of the main disturbances in the forest and is important for the regeneration of yellow birch (Ricard et al. 2002, Godman & Krefting, 1960). Due to increased light and increased temperature, gaps have low soil water contents and thus different nutrient availabilities. There are also changes in plant species composition which can affect the ectomycorrhizal community. Ectomycorrhizal fruiting is affected by these factors and also by the decrease in the carbon source and disruption of the mycelial network. The below ground ectomycorrhizal community changes in composition within 8-10 m from the forest edge (Durall et al. 1999, Hagerman et al. 1999) in conjunction with a significant decrease in mushroom production in gaps over 629 m^2 (Durall et al. 1999).

Ectomycorrhizal species are quantified in two ways, by counting the different species of mushrooms and by counting the number of root tips colonized by different species. Mushrooms are an important structure for the quantification of ectomycorrhizal species richness, since the species fruiting are often not abundant colonizers on a forest scale (Dahlberg et al. 1997, Kernaghan 2001, Kernaghan & Harper 2001, Peter et al. 2001). However, a species may exist in the soil without fruiting. Thus, when restriction fragment length polymorphisms (RFLPs) from mushrooms and from ectomycorrhizal tips are compared, there is generally only a small overlap in the RFLP types found (Jonsson et al. 1999, Peter et al. 2001, Lilleskov et al. 2002). The changes in ectomycorrhizal fruiting may signal future changes in the below ground colonization patterns (Fellner & Peskova 1995) and decreases in the fruiting of certain species seem to

be followed by changes in the below ground colonization structure within two years (Peter et al. 2001).

Quantification of ectomycorrhizal fungi on root tips does allow for a direct assessment of one aspect of the below ground ectomycorrhizal community but it is limited by the number of samples which can be processed in a given amount of time (Goodman et al. 1996). To separate species morphologically one must look at 50 or more characters under dissecting and compound microscopes (Goodman et al. 1996) and the characters can still vary to some extent with substrate and external environmental conditions (Yamada et al. 2001, Lilleskov et al. 2002, Farmer & Sylvia 1998, Stocchi et al. 1994). In addition to morphological assessment, the fungal symbionts can also be determined using their DNA (Gardes & Bruns 1993, Kernaghan 2000). To date most DNA-based ectomycorrhizal studies have relied on the internal transcribed spacer (ITS) region of the ribosomal DNA for several reasons, 1) it is a tandemly repeated region, the length of which is usually under 1kb, thus making it easier to amplify from small samples such as individual root tips, 2) it has been shown to have high interspecific variability and low intraspecific variability for many species, 3) the conserved ribosomal genes on either side are adequate for group specific primer design (Gardes et al. 1991, Gardes & Bruns 1993). Molecular methods are making the identification process easier and it will become more so with increases in knowledge, but there are still complicating factors. There are cases where intraspecific ITS variability has been shown to be higher than interspecific variability such as with *Cenococcum geophilum* (LoBuglio et al.1991). *Pisolithus tinctorius* was thought to have a very high intraspecific variability but recent evidence suggests that what was identified as one species is a species complex composed

of several species with very similar morphological characters (Junghans et al. 1998, Martin et al. 2002). Cases of low interspecific variability in the ITS region have also been seen, such as for certain *Cortinarius* species (Dahlberg et al. 1997, Peter et al. 2001), where restriction digests with 3 enzymes do not always separate all species. And there are even *Russula* isolates which seem to have been separated into species groups by an obvious morphological character which has since been attributed to one gene when there was no variation found in their ITS sequences and little in their AFLP (amplified fragment length polymorphism) data (Redecker et al. 2001). Still, to get a picture of what is happening below ground one must identify the symbiont on tens of thousands of root tips and, even then, most studies find ectomycorrhizal mushrooms for species which are not identified on the roots (Peter et al. 2001).

As useful as it is to get a direct measure of the ectomycorrhizae fungal species on the roots, there could be inherent problems associated with this scale of measurement when calculating diversity. Diversity is based on the frequency and distribution of individuals (or their respective bio-masses) of a population within a community (Begon et al. 1998). As of yet there is no evidence suggesting a relationship between the number of tips colonised and the volume of soil being explored, or the rate of nutrient transfer to the host, but there does seem to be a large variation in functional differences between fungal species, (Boyd et al. 1986, Duchesne et al. 1987a, Bucking & Heyser 2000, Bucking & Heyser 2000b, Mason et al. 2000, Perez-Moreno & Read 2000, Muhsin & Zwiazek 2002) probably resulting in differences in the growth of these species. We also know that the colonization of many root tips within a soil core by a single RFLP type is likely to be from one individual, thus the evenness of ectomycorrhizal fungal distribution

is generally better gauged from presence or absence of fruiting in a plot, colonisation within a core, or on the root system of a small seedling.

Understanding the below ground community is important because this community provides nutrition for a variety of soil organisms (Zhu & Ehrenfeld 1996) as well as for small mammals (Pyare & Longland 2001). Ectomycorrhizal fungi may play an important role as bioindicators (Peter et al. 2001) and they are of direct economic importance, as mushroom consumption by humans has increased by magnitudes over the last two decades (Kues & Liu 2000). Mushroom production can be worth as much as the harvested timber (Pilz & Molina 2002) in some areas. The nutrition of trees is very important, not only for increased timber production, but, probably more importantly, in counteracting the rise in atmospheric carbon. Ectomycorrhizal fungi are probably irreplaceable in the nutrition of ligneous plants (Lindahl et al. 1999, Perez-Moreno & Read 2000) and the diversity of the communities has an impact on seedling productivity (Baxter & Dighton 2001, Jonsson et al. 2001). To make sure that we do not decrease productivity over time, the effects of forest harvesting on the ectomycorrhizal community structure need to be taken into account when designing forestry management practices. To date only 5 studies have examined the fruiting of fungal communities in Quebec (Villeneuve 1985, Bussieres 1986, Lessard 1986, Villeneuve et al. 1989, Nantel & Neumann 1992) and there have been no studies to determine the fungal partners of mature *Betula alleghaniensis*.

This study was, therefore, undertaken to morphologically and molecularly characterise mushroom taxa. The abundance of fruiting of some fungal taxa, particularly the ectomycorrhizal fungi, was expected to change between experimental liming and gap

treatments. The DNA of both mushrooms and mycorrhizal root tips was sampled to create an RFLP database for the identification of fungal taxa occurring as mycorrhizae on both trees and seedlings, in order to test the hypothesis that the mycorrhizae of mature trees and seedlings would differ.

Chapter 2

Mushroom formation in response to selective partial cuts and liming in a deciduous, south central Québec forest

Abstract

In order to test the effects of cutting and liming on fruitbody production, all visible mushrooms were collected from twelve 49 sq m plots. Treatments were either limed (~500 kg/ hectare) or not, within ~1000 m^2 cut gaps and the forest during 2 seasons in a mixed deciduous forest. The level of lime used in this study had a neutral or positive affect on the fruiting of most species, particularly on the fruiting of certain *Hygrophorus* species, which were more abundant in the limed plots. Ectomycorrhizal taxa (eg. *Entoloma salmoneus*, *Lactarius rufus*) generally had decreased fruiting in the gaps whereas many non-mycorrhizal taxa were unaffected by the cuts. Species of *Hebeloma* and *Laccaria* however did not show any response to the treatments.

Introduction

Mushrooms are the reproductive and dispersal structures of certain fungi. The fungal body, the genet or thallus, grows as a mycelial mat below ground. Fungi are heterotrophic organisms, each species gaining their energy by parasitic means, by saprophytic means, by forming mutualistic relationships or by a combination of these trophic strategies. Mushroom formation is dependent on the environmental conditions and the level of nutrition of the below ground genet in conjunction with the need to encounter a compatible mating type (Kues & Liu 2000).

Comparative studies of undisturbed and disturbed sites generally show a shift in the dominant species (Senn-Irlet & Bieri 1999) and a reduction in species richness in disturbed habitats (Visser 1995, Lilleskov et al. 2002). The functional result of these shifts is difficult to determine, due to a lack of knowledge of the functional differences between species (reviewed in Cairney 1999).

Gap creation is one of the main natural disturbances in the forest (Godman & Krefting 1960) and has become an important tool in selective sylviculture practices. Due to increased light and increased temperature, gaps have lower soil water contents and thus differences in nutrient availability (Begon et al. 1996, Zhang & Zak, 1998). There are also changes in plant species composition which can affect the fungal community. The disruption of the mycelial network will affect fruiting as well. Gaps probably change the competitive ability of wood decomposer fungi (Senn-Irlet & Bieri 1999), by changing the proportion of substrate available, and parasitic fungi by increasing the number of

potential points of infection (Rizzo et al. 2000). The below ground fungal community changes in composition within 8-10m from the forest edge and, in conifer forests, there is a significant decrease in ectomycorrhizal mushroom production in gaps over 629m^2 (Durall et al. 1999, Hagerman et al. 1999).

Another sylvicultural practice which may negatively affect mushroom fruiting is the addition of lime. Liming has been shown to increase soil pH and nutrient availability (Began et al. 1996). Some studies have shown that the effects of liming on mushroom formation is similar to mushroom formation in response to other disturbances

Understanding the fungal community is important because it provides nutrition for a variety of soil organisms (Zhu & Ehrenfeld 1996) as well as for small mammals (Pyare & Longland 2001). Fungi can also play a role as bio-indicators (Garcia et al. 1998, Peter et al. 2001, Alonso et al. 2003), dependent on our understanding of their physiology and responses to the exterior environment. Mushrooms are of direct economic importance, as their consumption by humans has increased by magnitudes over the last two decades (Kues & Liu 2000) and mushroom production can be worth as much as the harvested timber (Pilz & Molina 2002) from some forest ecosystems.

To date only 5 studies have examined the fruiting of fungal communities in Quebec (Villeneuve 1985, Bussieres 1986, Lessard 1986, Villeneuve et al. 1989, Nantel & Neumann 1992) and no studies have tested the effects of cutting or liming on mushroom fruiting in these forests. In order to test the hypothesis that the community of fungi forming mushrooms would change with treatment, mushrooms were collected from anc counted from gaps and from the control forest. In order to test for the responses of

individual taxa to changes in the soil parameters, these parameters were measured for each treatment and correlations were performed.

Materials and Methods

Site Description

This study took place in a mixed deciduous forest of the Réserve faunique de Portneuf located near Rivière à Pierre in south central Québec (47° E 04' N and 72° E 15' W). The forest was dominated by sugar maple (*Acer saccharum* Marsh.) and yellow birch (*Betula alleghaniensis* Britt.), which made up 75% of the vegetation cover. Other tree species present were American beech (*Fagus grandifolia* Ehrh.), red spruce (*Picea rubens* Sarg.), red maple (*Acer rubrum* L.) and balsam fir (*Abies balsamea* (L.). Of the trees, the maples are not ectomycorrhizal. The under story ligneous vegetation consisted of mountain maple (*Acer spicatum* Lamb.), striped maple (*Acer pensylvanicum* L.), Canadian yew (*Taxus canadensis* Marsh.), Nannyberry (*Viburnum alnifolium* Marsh.), and beaked hazelnut (*Corylus cornuta* Marsh.) which was the only ectomycorrhizal species in the understory. The soil was made up of an undifferentiated till with a depth of about 1 m covered with an orthic humo-ferric podzol with a mean organic horizon pH of 5.57 (Ricard, 1999). The altitude at the site ranged from 320 to 400m (Ricard 1999). The forest had last been disturbed ~25 years ago when approximately 30% of the timber had been removed through partial selective cutting (Ricard, 1999).

In November of 1996 fifty gaps of about 1000 m^2 were created using heavy machinery, 12 of which were chosen for treatment in the spring of 1997. In each gap, two, 7 x 7 m plots were used from which all the remaining live trees had been cut after the plots had been established. Another 24 plots were set up in the undisturbed forest or in single downed tree gaps (control forest plots). Twelve randomly chosen plots in both

the gaps and in the control forest received a 3.2 kg crushed dolomite treatment, containing $CaCO_3$ 92% (Ca: 36%), $MgCO_3$ 0.76% (Mg: 0.35%) and 0.16kg of KCl, once per summer season. The treatment extended 0.5 m outside the plots to reduce edge effects. If there was a slope, the lower plot received the treatment. In late July to early August vegetation surveys determining percent cover of each species present at 4 height levels was performed in 4, 1 m^2 subplots.

Soil samples were collected from the first 5 cm of the organic horizons, in 3 places in each plot, placed in Ziplock© bags and stored at 4°C. pH was determined using a Piccolo ATC pH meter (Hanna instruments) after creating a paste, from a sub-sample of the 3 pooled samples, using distilled water. The water content was determined by removing all live roots and stones from a sub-sample, then drying it overnight at 90°C. Combustible organic matter was determined at 500°C for 4 hours.

Mushroom Sampling

For the month of September in 1998 mushrooms were surveyed and identified in an unorganised fashion to gain an initial idea of species richness in the area. In 1999 all visible (>1 mm) mushrooms were sampled from every plot each month from May through August and twice in September. Mushrooms were photographed, spore prints were taken and macroscopic features were described. The fruiting abundance for each mushroom taxona was determined. Each mushroom was then removed from the plot so as to not count it again in the next survey.

Data Analyses

Statistical Tests Between Treatments

Total and individual mushroom data in the form of abundances and presence/absence data from plots as well as variable measurements were tested for conformation to the requirements for parametric tests. The only variable with a normal distribution, percent cover of maple, was tested for significant differences between treatments using a t-test. The rest of the variables (including mushroom taxa) were tested for differences using the Kruskal Wallis test for multiple samples. All statistical tests were performed using the SPSS statistical package. (Norusis 1997)

Ordination Analysis

Canonical correspondence analysis (CCA) was performed to find the factors having the greatest effect on mushroom fruiting abundance. Initially included in the analysis were 53 fungal taxa, which had a fruiting abundance of greater than 40 mushrooms and occurred in more than one plot. The mushrooms were initially tested in conjunction with 13 variables, the mean percent cover of 9 ligneous plants and 4 abiotic factors. Outlier Analysis was performed using PCORD Multivariate Analysis of Ecological Data version 3.0 for windows (McCune and Mefford, 1997) resulting in the removal of 3 fungal taxa, and 7 plots from the analysis. Through analysis, all the variables except percent soil water content, and percent cover of birch, maple, american yew and raspberry were removed.

CCAs were performed using PCORD (McCune and Mefford, 1997) with their axis scores centred and standardized to unit variance and with the scores for graphing

plots being linear combinations of the variables, the difference between the two graphic representations is that one has axes scaled to optimize representation of the variables (variable scores are weighted mean scores for plots) while the other was scaled to optimize representation of the plots (plots scores are weighted mean scores for variables). The resulting scores were plotted on a Biplot using the same program.

Diverity measures

Diversity was calculated using the Shannon's diversity index (H) (Begon et al 1996):

$$H = -\sum_{i=1}^{s} P_i \ln P_i$$

Individual taxa were defined using morphological descriptions. The morphological data were analysed based on abundance of fruiting and on the presence or absence of the fungus in a plot.

Results

Soil Nutrients

Using the Kruskal Wallace test it was shown that the gaps had a significantly higher pH ($p<0.05$) than the forest plots and that the soil organic matter content as well as magnesium levels were significantly higher ($p<0.05$) in limed compared to unlimed plots.

Woody Plant Cover

Among the woody plants, only the cover of sugar maple conformed to the

requirements of parametric tests. T-tests showed a significant decrease ($p<0.000$) in the cover of sugar maple in the gap openings, but liming had no significant effect on the cover of sugar maple. In the forest, beech and American yew had significantly higher cover while yellow birch showed a tendency towards a higher percent cover ($p<0.056$). In the gaps black cherry, striped maple, redberry elder and raspberry showed a significantly higher percent cover. Liming had no significant effects on the percent cover of ligneous plants.

Mushroom Fruiting

There were over 9100 mushrooms collected belonging to 376 morphologically distinct fungal taxa over the two year period of collection. Sixty three percent of the taxa were observed in the 1999 survey and 14% of the species found in 1999 were found in both sampling seasons. The total mushroom fruiting was not normally distributed but there was a trend for overall fruiting in the limed forest plots to be higher (Fig. 1).

Using the Kruskal Wallis test, 29 fungal species showed significant differences ($p < 0.05$) in fruiting between treatments (Fig. 2). Some species fruited more in gaps (ie unknown sp. 151 and unknown sp. 191) or in gaps which had been limed, such as the wood decomposers, *Polyporus* sp. 24, *Polyporus* sp. 26, *Polyporus* sp. 28. *Lactarius rufus* (Fr.) Fr., unknown sp. 28, *Entoloma salmoneus* (Pk.) Sacc., *Clavaria vermicularis* Micheli: Fr., *Clavulinopsis helvola* , *Geoglossum* sp., unknown sp. 178, unknown sp. 202 fruited more under forest conditions. The three species of *Lycoperdon* (*L. perlatum* Pers., *L. pyriforme* Schaeff. Ex Pers., *L. echinatum* Pers.) increased in abundance as a result of liming, while only *Collybia dryophyla* (Bull. Ex Fr.) Kummer decreased.

Figure 1. Mean number of mushrooms fruiting for each treatment (+F = +lime, -F = -lime)

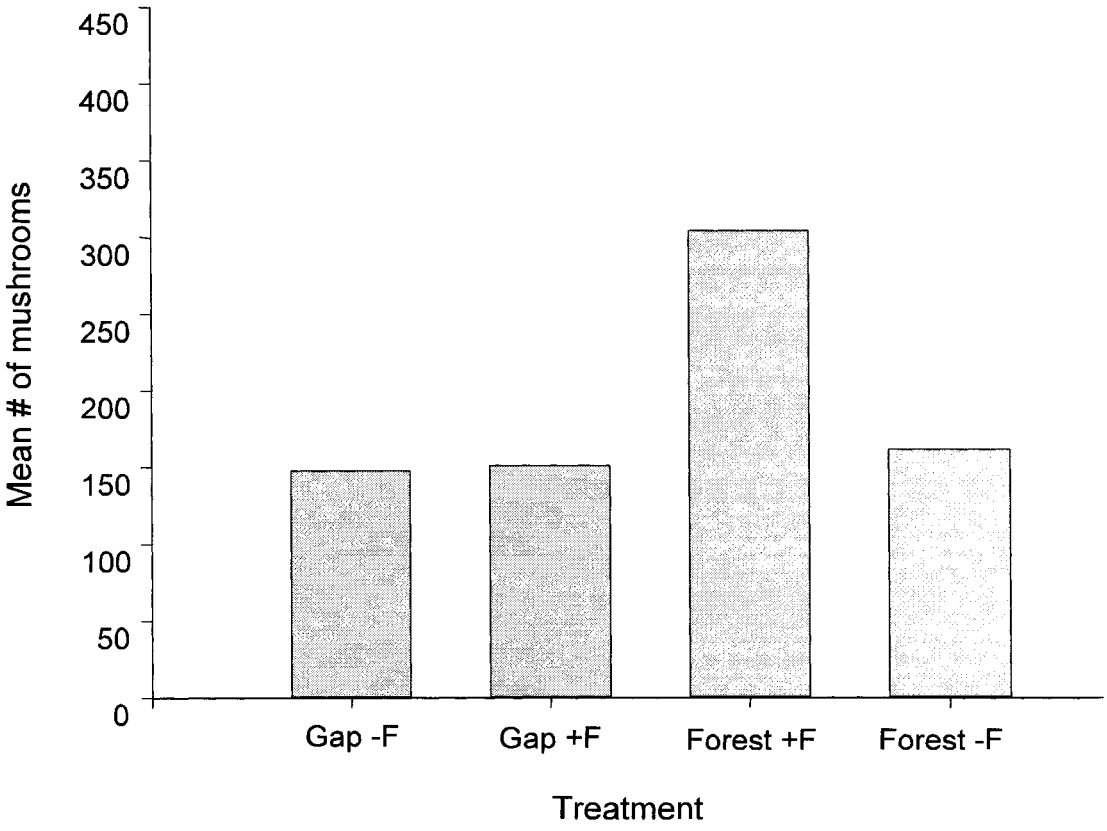
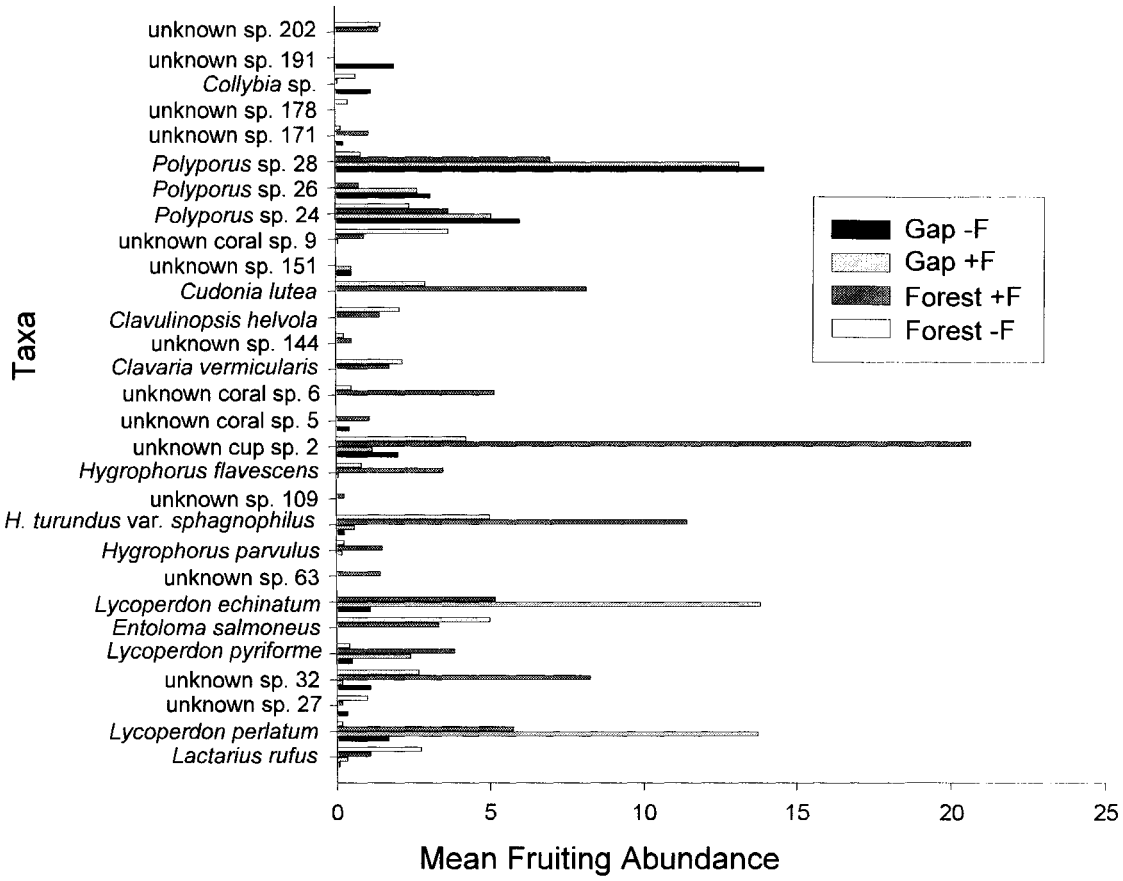

Mean # of mushrooms
450
400
350
300
250
200
150
100
50
0
Gap -F
Gap +F
Forest +F
Forest -F
Treatment

Figure 2. Individual taxa's fruiting abundance for each treatment (+F = +lime, -F = -lime)


unknown sp. 202
unknown sp. 191
Collybia sp.
unknown sp. 178
unknown sp. 171
Polyporus sp. 28
Polyporus sp. 26
Polyporus sp. 24
unknown coral sp. 9
unknown sp. 151
Cudonia lutea
Clavulinopsis helvola
unknown sp. 144
Clavaria vermicularis
unknown coral sp. 6
unknown coral sp. 5
unknown cup sp. 2
Hygrophorus flavescens
unknown sp. 109
H. turundus var. sphagnophilus
Hygrophorus parvulus
unknown sp. 63
Lycoperdon echinatum
Entoloma salmoneus
Lycoperdon pyriforme
unknown sp. 32
unknown sp. 27
Lycoperdon perlatum
Lactarius rufus
Taxa
Gap -F
Gap +F
Forest +F
Forest -F
0
5
10
15
20
25
Mean Fruiting Abundance

Regardless of liming, the forested plots seemed to have increased fruiting of several species, such as unknown sp. 32 , unknown sp. 63 , *Hygrophorus parvulus* Pk., *Hygrophorus turundus* var. *sphagnophilus* (Pk.) Hesler and A. H. Sm., unknown sp. 109 , *Hygrophorus flavescens* (Kauffman) Smith & Hesler syn. , cup fungus sp. 2 , coral fungus sp. 5 , *Ramaria* sp. 6 , unknown sp. 144 , *Cudonia lutea* (Pk.) Sacc. and unknown sp. 171 .

Some of the differences revealed by the Kruskal Wallis tests were also supported by the Canonical correspondance analyses in conjunction with the differences we know existed between plots due to the changes in plant cover. The percent cover of birch and maple as well as raspberry seemed to control axis 1 (λ_1=0.486 with a Kendall Corr., Spp-Envt 0.707) and by looking at the bi-plot of the quadrats, the gaps (G) are on the left and the forest plots (P) are on the right (Fig. 3). Fig. 4 shows the mushroom taxa in the same orientation. Axis 2 (λ_2=0.355 with a Kendall Corr., Spp-Envt 0.563) is mostly determined by the soil water content. While Axis 3 (λ_3=0.288 with a Kendall Corr., Spp-Envt 0.610) seems to be dictated almost equally by sugar maple and yellow birch, in fact creating a level of separation between the two, in respect to the fungi fruiting. The ectomycorrhizal species whose fruiting abundance were most constrained by yellow birch cover included *Cudonia lutea* , *Lactarius rufus* , *Clavaria vermicularis* Micheli Fr. and coral fungus sp. 8 (Figs. 4 & 5). There are other fungal species which were constrained by birch and maple together, some of which are probably ectomycorrhizal, including *Entoloma salmoneus* , *Hygrophorus turundus var. sphagnophilus* , unknown coral sp. 6 , and *Lactarius* sp. 7 . Then there are the species which were more constrained on the ordination by soil water content such as *Hebeloma*

Figure 3. Canonical correspondence analysis scaled to optimize representation of the plots

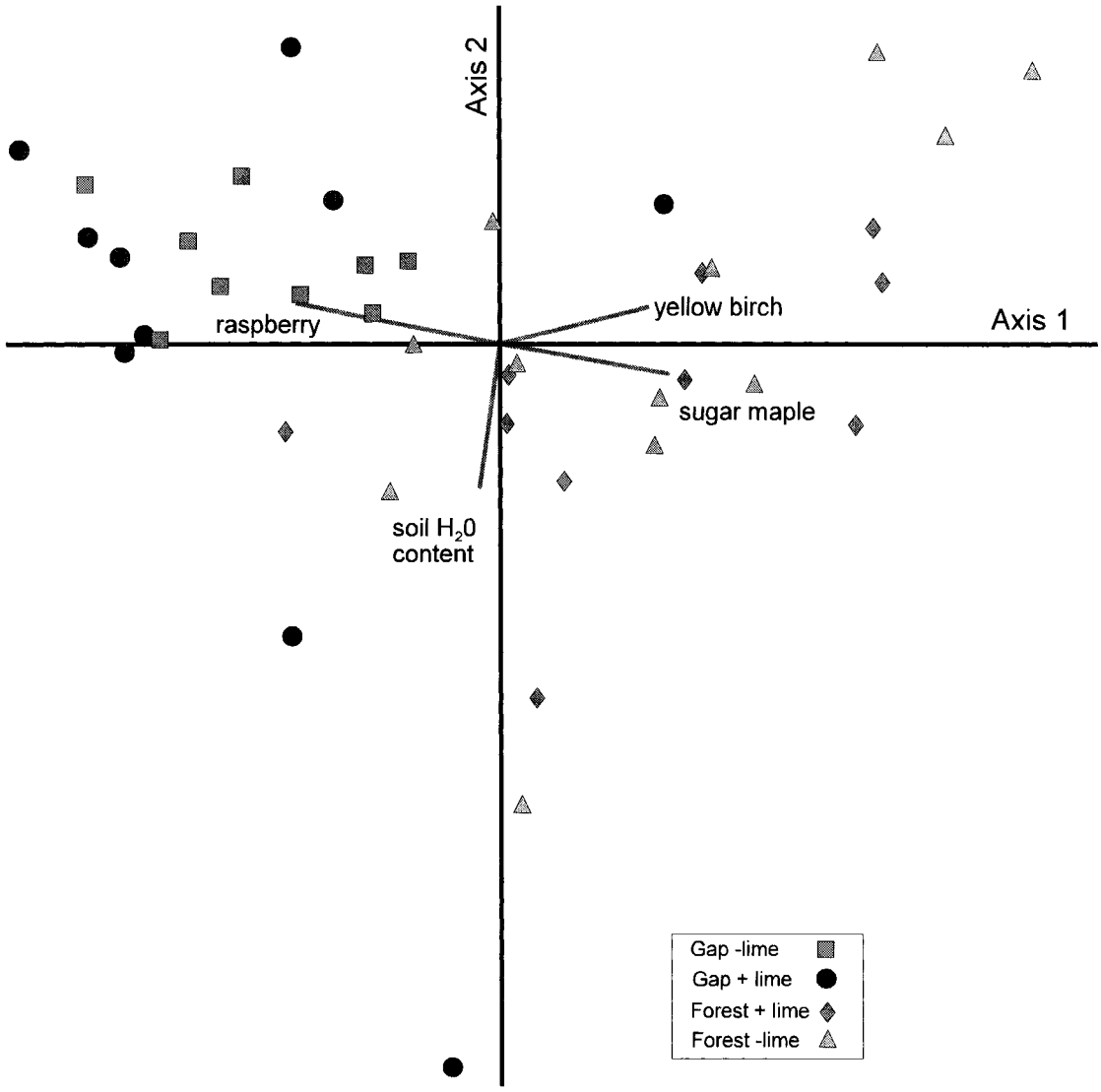

Axis 2
Axis 1
raspberry
yellow birch
sugar maple
soil H_20 content
Gap -lime
Gap + lime
Forest + lime
Forest -lime

Figure 4. Canonical correspondence analysis scaled to optimize representation of the variables, axes 1 and 2

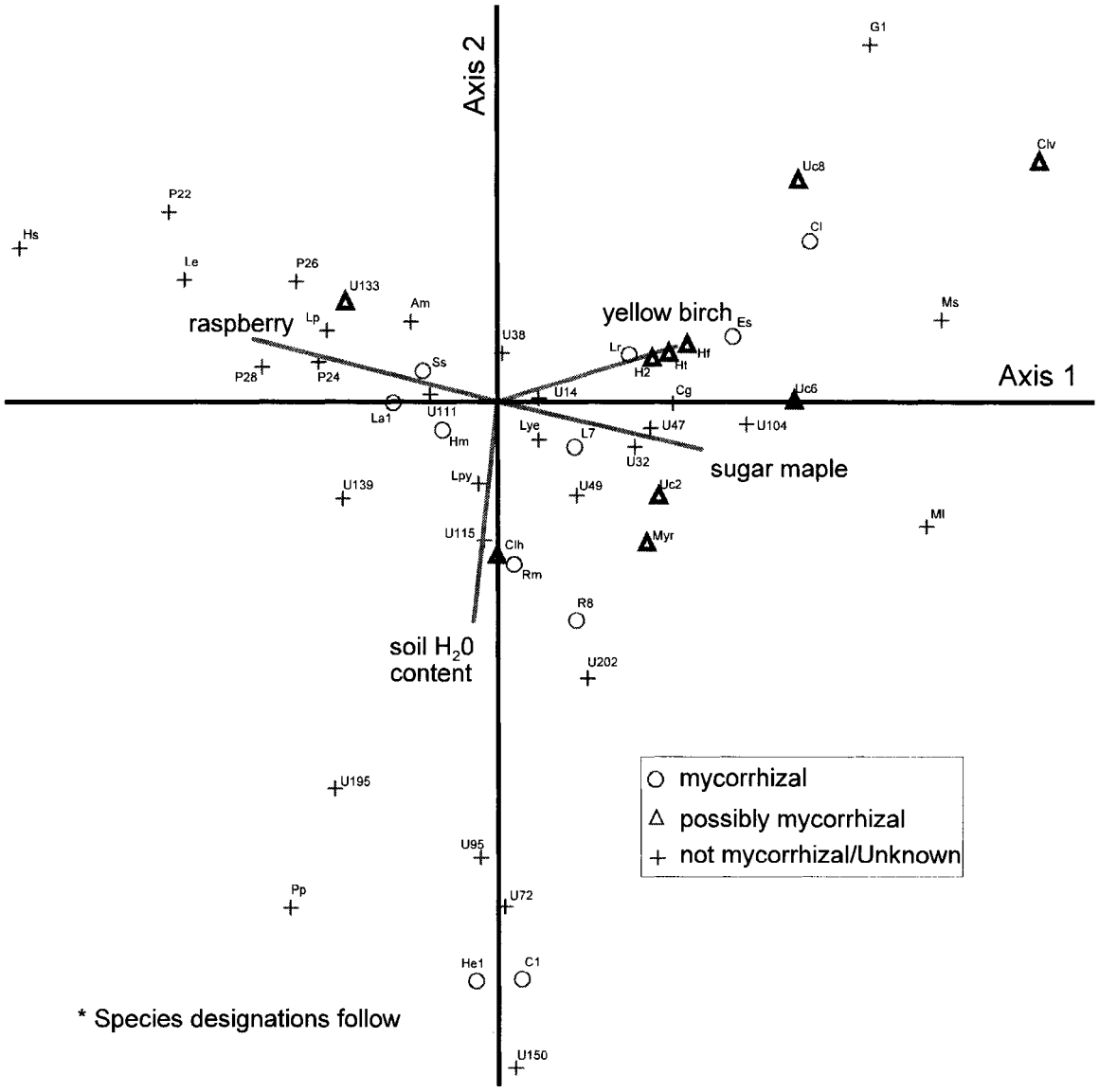
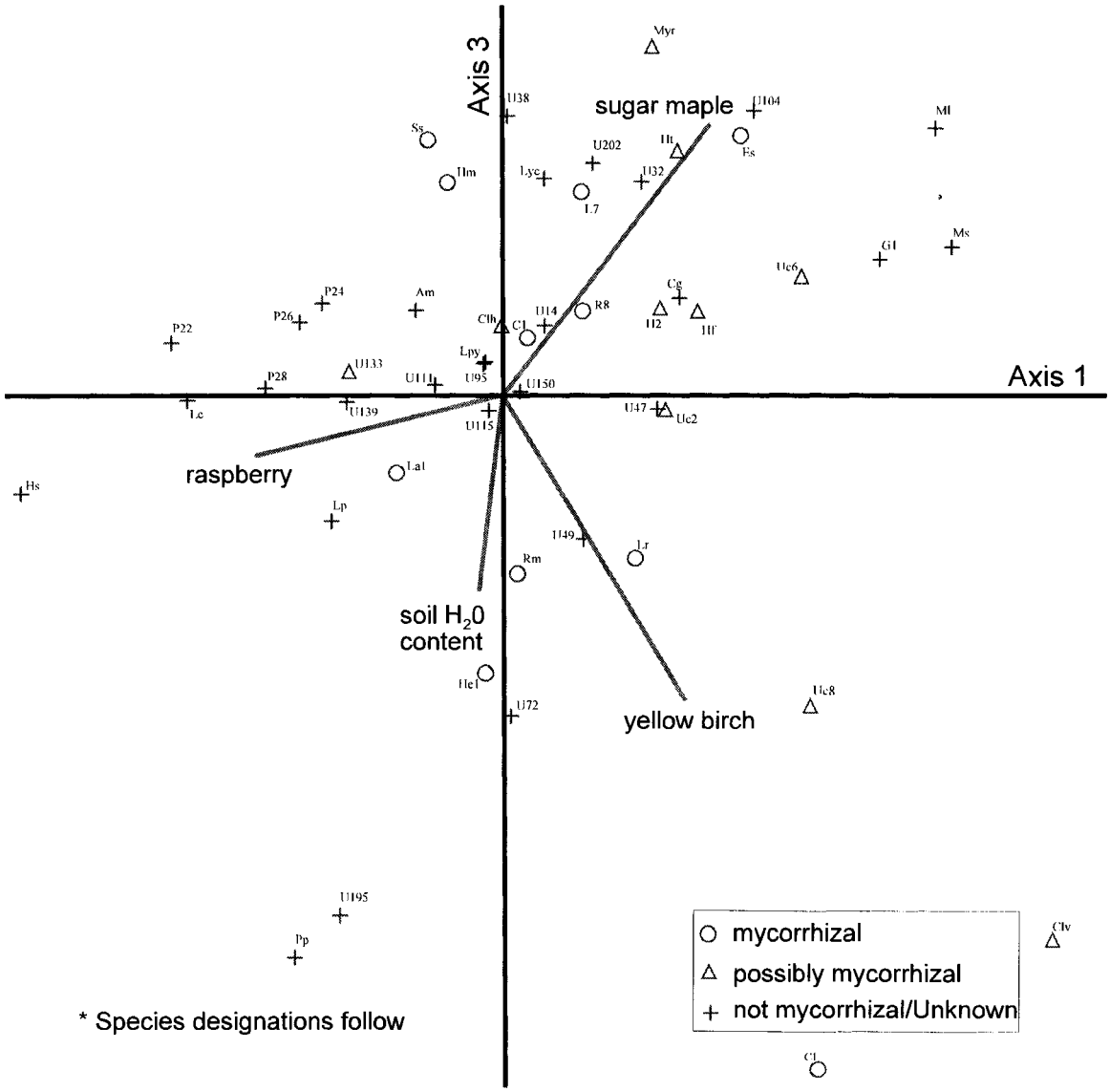

Axis 2
Axis 1
G1
Clv
Uc8
Cl
P22
Hs
Le
P26
U133
Am
Lp
raspberry
yellow birch
Es
Ms
U38
Lr
Hf
Ht
H2
P28
P24
Ss
U14
Cg
Uc6
La1
U111
Hm
Lye
L7
U47
U104
U32
sugar maple
Lpy
U139
U49
Uc2
MI
U115
Clh
Myr
Rm
R8
soil H_2O content
U202
U195
U95
Pp
U72
He1
C1
U150
mycorrhizal
possibly mycorrhizal
not mycorrhizal/Unknown
* Species designations follow

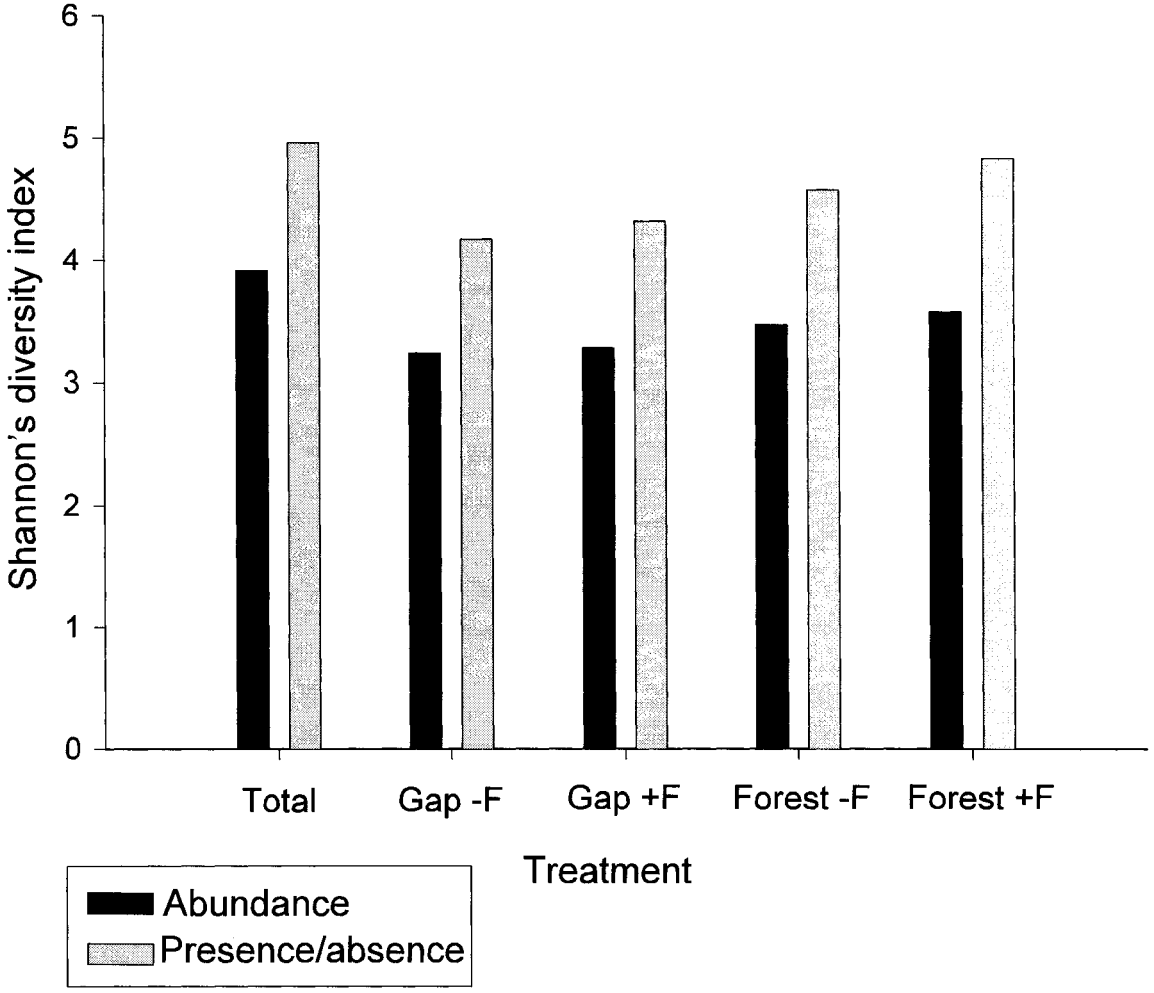
Figure 5. Canonical correspondence analysis scaled to optimize representation of the variables, axes 1 and 3


Axis 3
Axis 1
sugar maple
raspberry
soil H_2O content
yellow birch
Myr
U138
U104
Ss
Es
Ml
U202
Ht
Hm
Lyc
U32
L7
Ms
Gl
Uc6
P24
Am
Cg
P26
Clh
Cl
U14
R8
U2
Hf
P22
Lpy
U133
P28
U111
U95
U150
Le
U139
U115
U47
Uc2
Hs
Lal
Lp
U49
Lr
Rm
Hel
U72
Uc8
U195
Pp
Clv
Cl
○ mycorrhizal
△ possibly mycorrhizal
+ not mycorrhizal/Unknown
* Species designations follow

Taxonomic designations for figures 4 and 5

Am	*Armillariella mellea*	P24	*Polyporus* sp. 24
C1	*Cortinariaceae* sp.	P26	*Polyporus* sp. 26
Cg	*Clitocybe gibba*	P28	*Polyporus* sp. 28
Cl	*Cudonia lutea*	R8	*Russula* sp. 8
Clh	*Clavulinopsis helvola*	Rm	*Russula mariae*
Clv	*Clavaria vermicularis*	Ss	*Scutellinia scutellata*
Es	*Entoloma salmoneus*	U14	unknown sp. 14
G1	*Geoglossum* sp.	U32	unknown sp32
He1	*Hebeloma* sp	U38	unknown sp38
Hm	*Helvella macropus*	U47	unknown sp. 47
H2	*Hygrophorus* sp. 2	U72	unknown sp72
Hf	*H. flavescens*	U91	unknown sp. 91
Ht	*H. turundus* var. *sphagnophilus*	U95	unknown sp. 95
Hs	*Hypholoma sublateritium*	U104	unknown sp.104
La1	*Laccaria* sp. 1	U111	unknown sp. 111
Lr	*Lactarius rufus*	U114	unknown sp. 114
L7	*Lactarius* sp. 7	U115	unknown sp. 115
Le	*Lycoperdon echinatum*	U133	unknown sp. 133
Lp	*Lycoperdon perlatum*	U139	unknown sp139
Lpy	*Lycoperdon pyriforme*	U150	unknown sp. 150
Lye	*Lycogala epidendrum*	U183	unknown sp. 183
Mr	*Marasmius rotula*	U195	unknown sp. 195
Ml	*Mycena leaiana*	U202	unknown sp. 202
Ms	*Mycena stylobates*	Uc2	unknown cup sp. 2
Myr	*Mycroglossum rufum*	Uc6	unknown coral sp. 6
Pp	*Pleurocybella porrigens*	Uc8	unknown coral sp8
P22	*Polyporus* sp. 22		

Figure 6. Mushroom fruiting diversity as determined by abundance and presence/absence


6
5
4
3
2
1
0
Shannon's diversity index
Total
Gap -F
Gap +F
Forest -F
Forest +F
Treatment
Abundance
Presence/absence

sp., *Russula* sp. 8, *Clavulinopsis helvola*, and Cortinariaceae sp. 1. The species more constrained by the percent cover of raspberry seem to be the saprophytes that are more drought tolerant, including the polypores , two of the *Lycoperdon* species and a couple of ectomycorrhizal species including *Laccaria* sp. 1 and *Helvella macropus*. As with these ectomycorrhizal species, there should be saprophytic and parasitic species which are less drought-tolerant and may be more constrained by the soil water content than raspberry which indicates an open gap.

Mushroom Diversity

The diversity of mushrooms was lower in the cuts (Fig. 6) but the control (forest - F) treatment had almost the same diversity as the all the treatments grouped together. When diversity was calculated with respect to the presence or absence of species per plot (treatment) the diversity increased and the increase was even across all treatments.

Discussion

The total of 377 epigeous fungal taxa found fruiting in this region is higher than has been previously reported. Villeneuve et al. (1989) found 195 species fruiting with a much larger sampling area during a 2 year sampling period, located north of our study site while Nantel and Neumann (1992) found about 240 basidiomycete taxa in a yellow birch-maple forest north of Montreal ~80 km. In Virginia forests with similar tree species Bills et al. (1986) found only 54 species of fruiting epigeous fungi. The list of mushrooms Villeneuve, *et al.* (1989) found to be associated with yellow birch-maple stands correspond with some of the abundantly fruiting species found in this study.

Lactarius rufus and *Amanita fulva* (Schaeff.) Per Pers. as fruiting ectomycorrhizal species, *Hygrophorus flavescens*, with unknown trophic status in addition to the saprophytes *Crepidotus* sp. and *Marasmius rotula* (Scop. Ex Fr.) Fr. which were abundant in this study. Despite the fact that the number of taxa found in this study was higher than that found in many other studies, it is likely to be an underestimate of the fungal species richness in this forest. Even if the two years sampled had optimum fruiting conditions for most fungi, there are still almost certainly fungal species requiring different fruiting conditions which were not observed in this study (Bills et al. 1986, Villeneuve et al. 1989, Nantel & Neumann 1992, Kues & Liu 2000).

Twenty nine fungal taxa, including some ectomycorrhizal species, showed differences in fruiting between treatments. Many of these fungi associate with various biotic and abiotic factors (Fig. 4 &5). While this suggests changes to the below ground community structure, the ectomycorrhizal taxa colonizing seedlings did not seem to change due to the creation of gaps (De Bellis, 2000). These changes in fruiting are most likely due to differences in the growth and function of the different species (Cairney 1999, Kues & Liu 2000, Leake 2001).

Most studies have shown that the fruiting of members of the *Hygrophoraceae* are very sensitive to disturbances. Cutting or thinning of the forest and nitrogen addition have all been shown to reduce the fruiting of members of the *Hygrophoraceae* (Visser 1995, Kropp & Albee 1996, Jonsson et al. 2000). In this study 500kg/hectare of dolomite (Ca 36% & Mg: 0.35%) was used whereas in most other studies of the effects of liming, approximately 4000kg/hectare of dolomite (Agerer 1989, Nowotny et al. 1998) has been used. The increase in fruiting of members of the *Hygrophoraceae* (Fig. 2) could be a

response to a slight increase in nutrients or pH (although not significant), as generally occurs with the addition of lime (Begon et al. 1996), whereas a larger increase might be detrimental to the fungus.

Some ectomycorrhizal taxa, such as members of the *Russulaceae* and some *Cortinarius* species fruited less in the gaps. Durall et al. (1999) and Visser (1995) have observed similar fruiting responses in larger gaps. The decrease in fruiting could be detrimental to the survival of these fungi due to a decrease in spore propagation and dispersal. Redecker et al. (2001) have shown the importance of fruiting for the propagation of species in the *Russulaceae*.

In Tasmania, Packham et al. (2002) found that multivariate analysis of presence/absence data differentiated between the fungal communities in old versus re-growth forest sites. In this study the canonical correspondence analysis of fruiting abundance data separated forest plots from gap plots showing that different species fruit in the gaps than fruit in the forest (Fig. 3). Upon the creation of gaps ectomycorrhizal fruiting diversity decreases in conjunction with a decrease in the fruiting of *Russula*, *Cortinarius* and *Hygrophorus* species (Durall et al. 1999, Stocchi et al. 1994). The CCAs (Fig. 4 & 5) associated some fungal species with yellow birch cover. They include one of the most common ectomycorrhizal mushrooms, *Lactarius rufus*, two species from the Clavariaceae, and *Cudonia lutea*, which is closely related to an ascomycete that has the same RFLP type as one of the ectomycorrhizal RFLP types. One species in the *Clavariaceae* has been shown to associate with a *Betula* species in addition to the numerous members of the *Clavariaceae* which associate with other ectomycorrhizal trees including *Pinus*, *Cedrus*, *Quercus*, *Picea*, *Abies*, *Fagus* and *Larix* trees species (Trappe

1969). Only two *Cudonia* species have been observed colonizing a *Picea* species but they have not been observed on *Betula* species to date (Maia et al. 1996). One of the more common species found fruiting on decomposing wood was *Lycoperdon perlatum* which forms an ectomycorrhizal association with several tree hosts, though again it has not yet been observed on birch (Trappe, 1969).

This study, as in most others, found changes in fruiting of certain species. These changes have been attributed to forest type, changes in soil organic matter levels or soil humidity (Villeneuve et al. 1989, Nantel & Neumann 1992, Claridge et al. 2000a&b). Nantel and Neumann (1992) found that abiotic gradients have a more linear effect on the community of fruiting ectomycorrhizal fungi than they do on the host tree community. Many factors are implicated in the changes in fruiting but, overall, the responses to abiotic gradients will be different for each fungal species. This means that measures of community structure will only show differences for those that are affected and for which we measured the biotic and abiotic factors at the correct scale (Zak and Visser 1996).

The diversity of fruiting fungi was quite high in this region and was much higher than the fungal diversity colonizing mature birch roots. This would be expected as many of the mushrooms collected were saprophytic and parasitic taxa. This does not explain the total difference in diversity as the mushrooms from 77 potentially ectomycorrhizal taxa were collected and only 11 matched ectomycorrhizal RFLPs. Other factors included the five ectomycorrhizal plant species living within this forest, while the expectation is that they will share many of their fungal partners, there is most likely some level of host specificity (Massicotte et al. 1999). Thus the lack of match of all the ectomycorrhizal mushroom RFLPs with fungal RFLPs from yellow birch roots suggests some of the

ectomycorrhizal fungal taxa fruiting in this forest were not necessarily associated with mature yellow birch roots.

Conclusions:

The creation of 1000 m^2 gaps in the forest increased the pH of the soil while liming increased the soil organic mater content and the availability of magnesium. This was reflected in the changes in fruiting that took place for certain species. Ectomycorrhizal taxa (eg. *Entoloma salmoneus*, *Lactarius rufus*) generally had decreased fruiting in the gaps whereas many non-mycorrhizal taxa were unaffected by the cuts. The level of lime used in this study generally had a neutral or positive affect on the fruiting of most species, particularly on the fruiting of certain *Hygrophorus* species, which were more abundant in the limed plots. Species of *Hebeloma* and *Laccaria* however did not show any response to the treatments.

Chapter 3

The ectomycorrhizal community on the roots of mature yellow birch in a Québec forest

Abstract

The ectomycorrhizae on mature yellow birch were quantified using both morphological and molecular analyses. To test whether the fungi forming mycorrhizae were correlated with fruibody formation, the ectomycorrhizal abundance data was compared to data on mushroom abundance. The correlation between fungi present as friutbodies and those found as mycorrhizae was weak while 34% of the ectomycorrhizal RFLP types matched mushrooms that had been collected from this forest. The matches included species of *Cortinarius*, *Hebeloma*, *Laccaria* and *Russula*. The data was then compared with existing data from seedlings, collected from the same study site. The ectomycorrhizal fungal taxa colonizing mature yellow birch were more evenly distributed among individual samples than the distribution on seedlings and they, therefore had a higher diversity. Some species of *Hebeloma* and *Laccaria* appeared to be more abundant on seedlings while species in the *Russulaceae* appeared to be more abundant on mature trees. These differences between trees and seedling may reflect the importance of the fungal partners for the establishment and survival of yellow birch.

Introduction

Ectomycorrhizae are symbiotic mycorrhizal associations between the fine roots of plants and fungi. Nutrients are transferred to the plant by the fungus (Rygiewicz et al. 1984, Andersson et al. 1997, Colpaert et al. 1999, Perez-Moreno & Read 2000, Bucking & Heyser 2000, Bucking & Heyser 2000b, Jentschke et al. 2001) and up to 30% of the plant's photo-assimilate may be transferred to the fungus (Smith & Read 1997, Wright et al. 2000, reviewed in Nehls & Hampp 2000, Nehls 2001). Numerous studies also show other benefits to the host plant, including increased water uptake, increased stomatal conductance (Boyd et al. 1986, Mason et al. 2000, Muhsin & Zwiazek 2002) and increased resistance to pathogens (Duchesne et al. 1987a, Duchesne et al. 1989a, Duchesne et al. 1989b, Marx 1969).

The ectomycorrhizal community is diverse and evidence regarding the trophic status of ectomycorrhizal fungi suggests a high degree of variation between species. Some "ectomycorhizal" fungi gain carbon and nutrients by additional means, including saprophytism (Trojanowski et al. 1984, Durall et al. 1994) and/or parasitism (Bruns et al. 1998, Lindahl et al. 1999). The fungi exist as a below ground, interwoven set of mycelial networks consisting of different species and different individual genets. The evidence to date suggests that the natural ectomycorrhizal communities are dominated by a few species which have high colonization rates but they have a high species richness (Visser1995, Goodman & Trofymow 1998, Gehring et al. 1998). This implies the existence of a large number of rare species.

The functional effect of community shifts in species composition is difficult to

determine, due to a lack of knowledge of the differences between species (reviewed in Cairney 1999). However Durall et al. (1999) have suggested that the diversity at a site is functionally important in allowing the plants to associate with a fungus which is best adapted to exploit specific soil microsites. Differences in the growth parameters of species (Bonello et al. 1998, Redecker et al. 2001) and in their respective abilities to assist the plant in its growth (Duchesne et al. 1989a, Duchesne et al. 1989b, Colpaert et al. 1999, Bucking & Heyser 2000, Mason et al. 2000, Perez-Moreno & Read 2000, Muhsin & Zwiazek 2002) support this theory.

Disturbances in the mixed forests of southern Québec create gaps which are important for the establishment of yellow birch (Ricard et al. 2002, Godman & Krefting 1960). Due to increased light and increased temperature, gaps have lower soil water contents and thus different nutrient availabilities. The ectomycorrhizal commmunity is may be affected by these factors, by the decrease in the carbon source, and by the disruption of the mycelial network.

Understanding the below ground community is important because it provides nutrition for a variety of soil organisms (Zhu & Ehrenfeld 1996) as well as for small mammals (Pyare & Longland 2001). The nutrition of trees is very important, not only for increased timber production, but, probably more importantly, in counteracting the rise in atmospheric carbon. Ectomycorrhizal fungi are probably irreplaceable in the nutrition of woody plants (Lindahl et al. 1999, Perez-Moreno & Read 2000) and the diversity of ectomycorrhizal communities has an impact on the productivity of seedlings (Baxter & Dighton 2001, Jonsson et al. 2001). If high forest productivity over time is the objective, the effects of harvesting on the ectomycorrhizal community structure needs to be taken

into account when designing forestry management practices. To date there have been no studies to determine the mycorrhizal fungal partners of mature *Betula alleghaniensis*.

This study was, therefore, undertaken to quantify the fungal species forming ectomycorrhizae with mature yellow birch. To do this an RFLP database of the fungal taxa forming mushrooms was created and compared with ectomycorrhizal taxa on mature yellow birch, classified molecularly and morphologically. To test whether the mycorrhizal community on mature trees differed from that of seedlings, and whether this was affected by the creation of gaps, the ectomycorrhizal fungi on mature trees were compared with those on seedlings growing in the forest (this study) and in the gaps (DeBellis 2000).

Materials and Methods

Site Description

This study took place in a mixed deciduous forest of the Réserve faunique de Portneuf located near Rivière à Pierre in south central Québec (47° E 04' N and 72° E 15' W). The forest was dominated by sugar maple (*Acer saccharum* Marsh.) and yellow birch (*Betula alleghaniensis* Britt.), which made up 75% of the vegetation cover. Other tree species present were American beech (*Fagus grandifolia* Ehrh.), red spruce (*Picea rubens* Sarg.), red maple (*Acer rubrum* L.) and balsam fir (*Abies balsamea* (L.)). Of the trees, the maples are not ectomycorrhizal. The under story woody vegetation consisted of mountain maple (*Acer spicatum* Lamb.), striped maple (*Acer pensylvanicum* L.), Canadian yew (*Taxus canadensis* Marsh.), Nannyberry (*Viburnum alnifolium* Marsh.), and beaked hazelnut (*Corylus cornuta* Marsh.) which was the only under story ectomycorrhizal species. The soil was made up of an undifferentiated till with a depth of about 1 m covered with an orthic humo-ferric podzol with a mean organic horizon pH of 5.57 (Ricard, 1999). The altitude at the site was between 320 and 400 m (Ricard 1999). The forest had last been disturbed ~25 years ago when approximately 30% of the timber had been removed through partial selective cutting (Ricard, 1999).

Ectomycorrhizal Sampling

In the spring of 1999, 200 mature yellow birch (~35-80 years old) trees, in the undisturbed forest, were numbered. One hundred and seventy five trees were chosen at random to be sampled. At a random compass direction from each tree a soil core with a diameter of 5.5 cm and a depth of 11 cm (tulip bulb corer) below the un-decomposed

litter surface, was taken 2-3 m from the mature tree. Thus all of the tips within one core came from a space with a maximum distance of 10 cm between them. If the core location was within range of the root system of another ectomycorrhizal plant, determined by canopy size, other than American beech, then another location was chosen. Samples were placed in a Ziplock© bag and were stored at 4°C until processing could take place, a maximum of 8 weeks from the sampling date.

Soil cores were carefully washed over 850 micron and 500 micron screens (Combustion Engineering Canada Inc., St. Catherines, Ont.) to separate all the roots. The yellow birch roots were separated from the others under a dissecting microscope (Brundrett et al. 1990). Of the 175 cores, 46 contained roots which could be identified as those of yellow birch and were ectomycorrhizal. All ectomycorrhizal root tips were then separated and counted based on morphological characters as discussed in Agerer (1991), Goodman et al. (1996) and Agerer (1995), under a Wild Heerburg dissecting microscope (128-800 X) using a fibre optic light source. Tips were also sub-sampled to obtain and analyse mantle peels, which were observed under a Nikon Optiphot differential interference contrast (DIC) microscope (200X, 400X, or 1000X). Samples of each morphotype were collected for DNA analysis from each soil core if there was enough material in addition to that used for morphological analysis. One to three root tips per sample were placed in a 1.5 ml ependorf tube and the tubes were frozen in liquid nitrogen or placed on ice until the samples could be lyophilized. Samples of each morphotype were randomly chosen for DNA analysis until 10 successful amplifications were achieved or until all samples had been tested.

Mushroom DNA sampling

As mushroom taxa were collected and described in 1998 and 1999 (See Chapter 1), two samples from the interior of the cap and/or stipe tissue were taken from each species and placed separately into 1.5 ml ependorf tubes. Samples were either stored in 2% CTAB solution at 4°C or directly in liquid nitrogen until they could be lyophilized.

DNA Extraction

The extraction procedure used was a modification of that described by Henrion et al. (1992). A small piece of mushroom (5-20 mg) or 1-3 root tips were suspended in 300 µl of β-mercaptoethanol (0.02%)/CTAB (100 mM Tris HCl pH 9.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB) solution. The sample was frozen in liquid nitrogen and crushed several times, using a plastic pestle, until the solution was homogeneous. Five micrograms of proteinase K/ TE buffer solution with 0.5 µl lauryl sulfate sodium salt (SDS) were added to the sample which was then incubated for 1 h at 64°C. An equal volume of a 24:24:2 solution of phenol:chloroform:isoamyl alcohol was added, mixed gently and then centrifuged at 13000 x g for 15 min. The upper phase was removed to a clean ependorf tube and the step was repeated with chloroform:isoamyl alcohol (24:2). One hundred micrograms of RNAse/TE buffer solution were added to the sample and the mixture was incubated for 1 h at 37°C. The phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol separation steps were repeated. The upper phase was then mixed with either 80% of its volume of cold isopropanol or 2X its volume of 95% cold ethanol, and then with 10% of the final volume of 3 M sodium acetate (pH 5.2) and left

for a minimum of 1 h at -20°C. The sample was then centrifuged at 13000g for 15 minutes and the supernatant was discarded. Eight hundred microlitres of cold 70% ethanol were then added to wash the pellet and centrifuged for 15 min before discarding the supernatant. The pellet was dried at room temperature for storage until re-suspension at 37°C for 1 h in 50 µl of ultra-pure water. Upon re-suspension the samples were stored at 4°C.

DNA Amplification

To amplify the ITS region of the ribosomal DNA a nested approach was used. The PCR cycling protocol followed that of Gardes and Bruns (1993) with the exception that the annealing temperature for the primary PCR with ITS1-F (Gardes & Bruns 1993) and NL6C (Kernaghan et al. 2003) was 57°C and the internal PCR using ITS1 and ITS4 (Gardes & Bruns1993) had an annealing temperature of 55°C. The primary PCR was run on a gel of 1% low melt agarose, DNA was excised using a sterile pipette tip and re-suspended in 300 µl of ultra pure water. PCR reactions contained 20 mM Tris (pH 9.5), 25 mM KCl, 0.5% Tween 20, 0.1 mg/ml BSA, 0.5 mM or 0.6 mM of each dNTP, equal amounts of either 0.5 pmol/µl or 0.6 pmol/µl for each oligonucleotide, 3 mM $MgCl_2$ and 0.04 u/µl to 0.2 u/µl of Taq plus 10 µl of undiluted, 1/10, 1/100 or 1/1000 dilution of genomic DNA in a 25 µl primary PCR or 5-25 µl of dissolved excised DNA in a 50 µl nested PCR. The PCRs were performed using either a MiniCycler PTC-150-16 or PTC-100 Programmable Thermal Cycler (MJ Research Inc., Watertown, Massachusetts).

Restriction Digestion

The PCR product was digested with Hinf I, Taq I and Nde II, an isoschizomer of Mbo I, provided by Promega Corporation (Madison, WI). Digestions were performed on 10 µl of PCR product from the nested PCR using 1.5 µl of their respective buffers (B, E & D, provided by the manufacturer), 2 µg of BSA and 5 units of enzyme diluted in a 21.7 µl reaction. The digestions were done at 37°C, 65°C, and 37°C for ~2 h each, respectively.

Gel Examination

In all cases DNA visualization was done using 1-2% ethidium bromide on a 2% agarose gel. Excision of the primary product from the gel was done using UV light and a protective shield. All other gels were examined on a GeneGenius Bio-imaging system (Synoptics, Cambridge, England) with the GeneSnap 4.00.00 software (©Synoptics Ltd.), lengths of products and restriction digests were measured using GeneTools 3.00.22 software (©Synoptics Ltd.) in comparison to a 100kb plus molecular marker (MBI-Fermentis Corp.).

For sequencing, PCR products from nested PCR reactions were quantified, by comparison with a Lamda molecular marker (Promega Corp., Madison, WI.), on a 2% agarose gel using the GeneTools software. The PCR product was then cleaned (QIA Quick PCR purification kit, Quiagen, Missisauga, ON) and requantified. If samples were not concentrated enough, two were pooled and then sent via courier to the Core

Molecular Biology Facility at York University, Toronto. Sequencing was done using primers ITS1 and/or ITS4.

Data Analyses

Restriction Fragment Analysis

All restriction fragment data was entered into a Quatro Pro (Corel software) spreadsheet. RFLP patterns were considered similar when the first 2 bands of each digest were within a +/-10% bp range of each other. After this analysis usually fewer than 10 samples remained. Similar types were separated based on presence or absence of other prominent fragments and finally based on proportion of the distance between fragments, between individual samples. Similar ectomycorrhizal RFLP types which did not occur on the same gel originally were run together on the same gel in conjunction with matched mushroom RFLP types to verify the match.

Sequence Analysis

The DNA sequences were aligned using ClustalX 1.81 (Hall 2001). Pairwise alignment parameters were set to slow-accurate, with gap opening penalties between 5 and 10, gap extension penalties between 0.2 and 0.8 and with the IUB DNA weight matrix. Multiple alignment parameters had gap opening penalties of between 5 and 15, gap extension penalties between 0.5 and 3.0 and with the delay of divergent sequences set to 25%. The varying penalties were used on different parts of the ITS, dependent upon which gave the better alignment. The alignment was then imported into PAUP v. 4.0b10 (Swofford 2000) to perform a bootstrapped (1000 replicates) heuristic search with

parsimony as the optimum search criterion to create a cladogram file. In this study "cladogram" is used to describe an unrooted tree showing only the branching order of nodes with no distance information. The numbers at the branches are the bootstrap values. TreeView (Page 1996) was used to produce an image of the cladogram.

Diverity Measures

Diversity was calculated using the Shannon's diversity index (H) (Begon et al. 1996):

$$H = -\sum_{i=1}^{s} P_i \ln P_i$$

Individual taxa were defined using morphological descriptions as well as being reclassified using RFLP types to compare between diversity of morphotypes and diversity of RFLP types. The morphological data were analysed based on abundance of ectomycorrhizal colonization, i.e. % tips colonized, and on the presence or absence of an ectomycorrhizal morphotype in a core.

Results

Ectomycorrhizal Colonization

There were 12247 ectomycorrhizal root tips observed in this study. Of all the tips observed, 99.3% were colonized and 2.6% had immature mycorrhizae which could not be morphotyped. From these root tips, 29 morphotypes were described (Table 1). Of the 29 morphotypes observed, 6 occurred in at least 10% of the cores while 12 were present in more than 20% of the cores. Two morphotypes (Morphotypes 7 and 9) were much

Table 1. Brief descriptions of ectomycorrhizal morphotypes on mature yellow birch

Morpho-type	Mantle Color, Lustre & Texture	Mantle Type	Tip Shape	Branching Pattern	R*	Septa/CC† Anastomoses	Eminating Hyphae	Defining Features	Relative abundance (%)
1	light to dark beige, slightly grainy	H	straight to slightly bulbous at base	none	none	none	few		1.53
2	white, reflective, patchy texture	A	straight to slightly bulbous at base	none	none	none	few		2.1
3	brownish-beige shiny	H	slightly bent	none	none	none	few		0.05
4	redish brown, slight sheen, slightly grainy	H	straight		none	none	few		0.07

5	dark brown, slight sheen, smooth to grainy	E	straight club shaped	mono-podial pinnate	none	Septa/CC	few	0.6
6	thin translucent white over redish brown, slight sheen, smooth	A	straight, slightly twisted	none	none	CC&in H-bridges	few	4.6
7	light yellowish orange to redish orange, slight sheen, smooth	C	straight	irregular/ none	none	Septa/CC& H-bridges	few	19.1
8	white/grey, dirty-matt, rough	C	slightly bent	none	none	Septa/CC& H-bridges	few	0.55
9	black, matt, spiny-long	G	straight to slightly bulbous	none	none	Septa	common	27.3

	10	beige white, slight sheen, smooth to grainy	N	straight	none	none	none	no		2.78
	11	brown, slight sheen, lightly wooly	C	straight to slightly bulbous	none	none	Septa	common	cystidia C (Agerer)	1.76
	12	brownish-grey, slight sheen, spiny-short	C	straight	mono-podial pinnate	none	none	common	short hyphae	0.07
	13	beige white, slight sheen, cottony	C	straight	none	none	none	common		4.71
	14	brown, thin, slight sheen, smooth	H	straight	none	none	none	infrequent		0.07
	15	dark brown, white stringy hyphae	H	straight	none	few	none	common		0.01

16	brown, reflective, shiny-long	C	straight to bent	none	none	Septa/CC& H-bridges	common		0.01
17	yellow, shiny, lightly-cottony	A	straight	none	none	Septa/CC& H-bridges	common		4.56
18	white, reflective, lightly-cottony	C	straight	irregular	few	Septa/CC& H-bridges	common		5.14
19	white, shiny, grainy	C	straight	none	none	Septa/CC	few	short hyphae	1.47
20-22	immature mycorrhizal formation								2.65
23	white to teal blue, slight sheen, grainy	E	straight	none	none	Septa/CC	few		3.18
24	beige white, shiny, lightly-woolly	A	straight	none	none	Septa/CC& contact bridges	common		0.69

	25	beige white, slight sheen, cottony	H	straight to slightly bulbous	none	none	Septa/CC	common	rust color hyphae	5.61
	26	orange, mat, grainy	A	straight	none	none	Septa/CC& H-bridges	few		8.4
	27	black, smooth	N	straight	mono-podial pinnate	few	none	few		0.18
	28	orange, mat, grainy	A	straight	none	none	Septa	few		0.09
	29	light beige orange, spiny-short	C	straight	mono-podial pinnate	none	Septa	common	short hyphae	0.69
	30	blue teal, shiny, cottony	E	straight	none	none	Septa/CC	common		0.3
	31	beige grey, thin, smooth to slightly cottony	A	straight	none	none	Septa	common	bent hyphae	2.8
	32	white, felty	H	straight	none	none	Septa/CC	few	short hyphae	1.54

Note: descriptive terminology from Goodman et al. 1996 and Agerer 1991, mantle types from Agerer 1991
R*= rhizomorphs
CC†= clamp connections

Table 2. Identified Ectomycorrhizal Taxa

Taxonomic delineation	Method of Determination	Morphotype	RFLP type	Mushroom match	Ectomycorrhizal RFLP type relative abundance (%)
Russulaceae sp.	Seq. analysis	7, 26	JCt22	none	16.18
Pezizales sp.	Seq. analysis	M*1	JCt33	none	
Hebeloma sp	RFLP match & Seq. analysis	M1; 13	JCt7	S231	1.18
Tomentella sp.	Seq. analysis	M1	JCt44	none	
Pisolithus sp.	Seq. analysis	M2	JCt38	none	
Paxillus involutus	Seq. analysis	M2; 19	JCt9	none	1.89
Laccaria sp.	RFLP match & Seq. analysis	M1	JCt32	S169	
Cortinariaceae sp.	RFLP match & Seq. analysis	18	JCt30	S528	2.05
Helotiales sp.	Seq. analysis	9	JCt6	none	3.41
Cortinarius sp.	RFLP match & Seq. analysis	23, 30	JCt31	S42, S120, S471	3.48
Tuber sp.	Seq. analysis	26	JCt5	none	2.8
Russula sp.	RFLP match & Seq. analysis	7, 32	JCt28	S47, S449	3.45

Lactarius sp.	RFLP match & Seq. analysis	10, 7	JCt19	S400, S450, S200, S352	6.6
Pezizales sp.	Seq. analysis	17	JCt2	S162	4.56
Tuberaceae sp.	Seq. analysis	29	JCt16	none	1.17
Cortinarius sp.	RFLP match & Seq. analysis	M2; 18	JCt8	S42, S120, S471	3.08
Cenococcum sp.	Seq. analysis	9	JCt3	none	20.48
Cortinariaceae sp.	RFLP match	1	JCt43	S42, S120, S471	1.53
Russula mariae Pk.	RFLP match	25	JCt15	S249	1.4
Lactarius sp.	RFLP match	26	JCt20	S149, S195, S391	2.8
Bisporella citrina (Batsch ex Fr.) Korf & Carpenter syn.	morphological identification		JCt6		

* indicates mophotyped on seedlings

more abundant than the rest, together colonizing over 46% of the root tips, and occurring in more than 75% of the cores. (Table 1). Due to the variability in colonization rates by individual morphotypes, there were no significant differences detected between their levels of abundance.

Molecular Classification

DNA was successfully amplified from 17 of the 29 morphotypes and these 17 types encompassed 90% of the ectomycorrhizal tips quantified morphologically. The efficiency of amplification was about 50% for the 159 ectomycorrhizal genomic DNA samples, including at least 1 sample from each of the 17 morphotypes resulting in 28 RFLP types. (Table 2).

The RFLPs of 41% of the described morphotypes matched the RFLPs of mushrooms collected above ground. Several of the morphotypes separated into more than one RFLP type (Table 2) with the result that only 34% of the ectomycorrhizal tips identified by RFLP matched a mushroom RFLP type. An equal percentage of tips were identified by sequencing the two most abundant, unmatched, RFLP types (RFLP types #7 and #9) resulting in the identification, at least to genus, of the fungi on 70% of the root tips.

The most abundant ITS RFLP types from mature yellow birch and seedlings (Data from DeBellis, 2000) were sequenced, resulting in the ectomycorrhizal families grouping together upon construction of a phylogenetic cladogram (Fig. 7). Indicated on the cladogram are the abundant species on mature yellow birch roots versus seedlings. Seedlings showed higher colonization rates by *Hebeloma* and *Laccaria* species, while

Figure 7. Cladogram of sequenced ectomycorrhizal taxa.


Hebeloma sp.
Laccaria sp.
Cortinarius sp.
Cortinariaceae sp.
Cortinarius sp.
Boletales sp.
Tomentella sp.
Paxillus involutus
Pisolithus sp.
Pezizales sp.
Cenococcum sp.
Pezizales sp.
Helotiales sp
Bisporella citrina ITS AF335454
Bisporella citrina
Tuber sp.
Tuberaceae sp.
Lactarius sp.
Russulaceae sp.
Lactarius sp.
99
100
99
99
64
89
98
95
100
98
82
same Morphotype Seedlings
same Morphotype Trees
same RFLPtype
most abundant on seedlings
most abundant on trees

Figure 8. Diversity of ectomycorrhizae on mature trees and seedlings either using abundance or presence/absence data

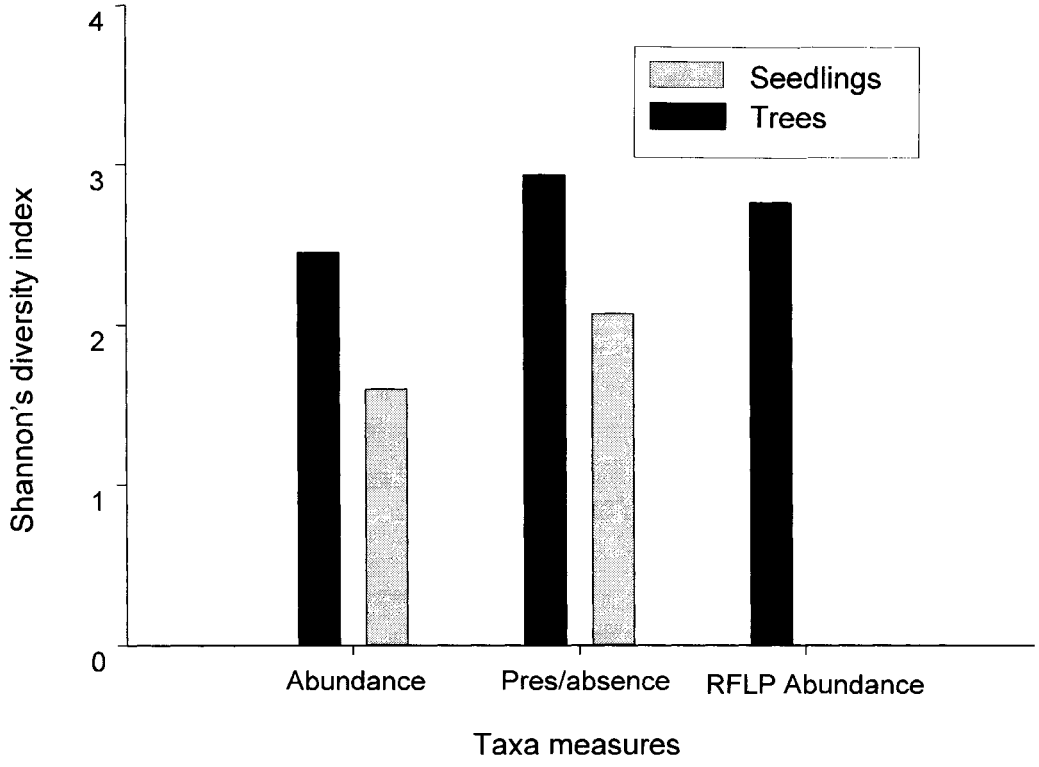
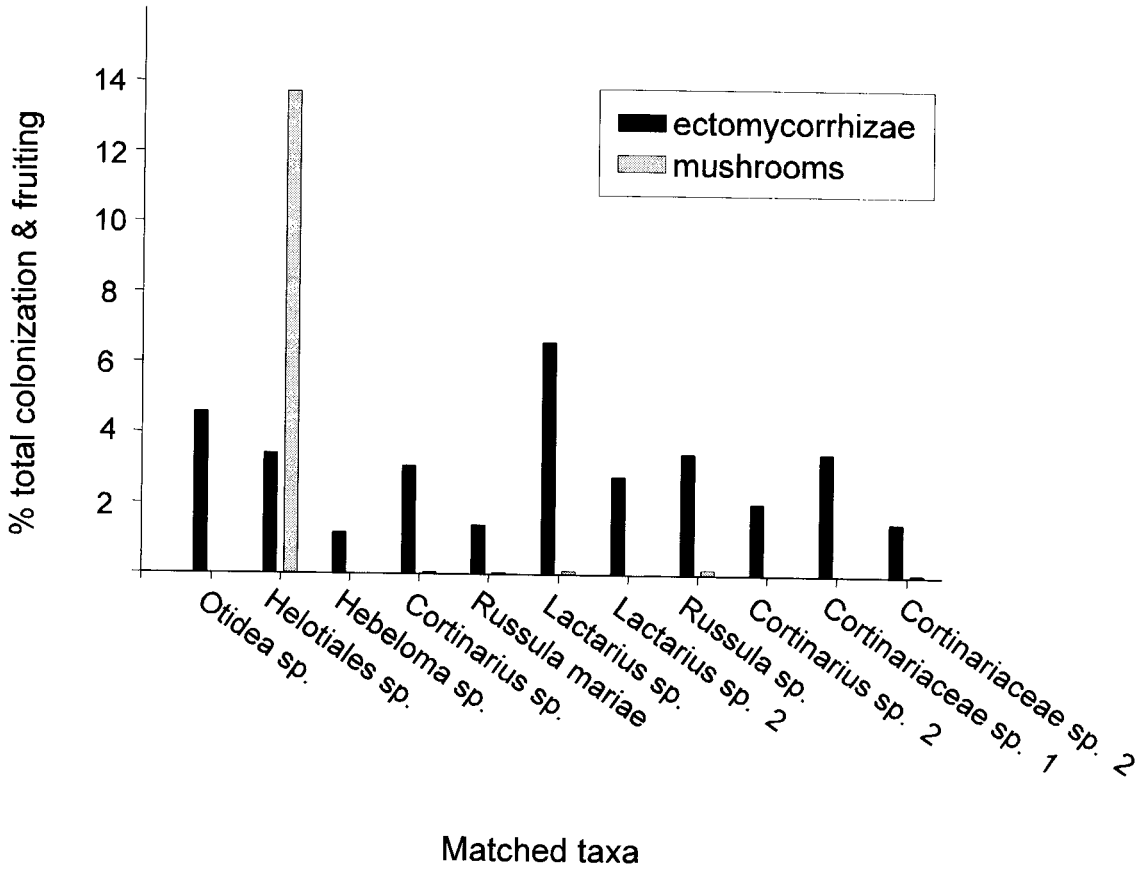

4
3
2
1
0
Shannon's diversity index
Seedlings
Trees
Abundance
Pres/absence
RFLP Abundance
Taxa measures

Figure 9. Abundance of fruiting and ectomycorrhizal colonization for mushrooms for which an RFLP match was obtained


% total colonization & fruiting
14
12
10
8
6
4
2
ectomycorrhizae
mushrooms
Otidea sp.
Helotiales sp.
Hebeloma sp.
Cortinarius sp.
Russula mariae
Lactarius sp.
Lactarius sp. 2
Russula sp.
Cortinarius sp. 2
Cortinariaceae sp. 1
Cortinariaceae sp. 2
Matched taxa

mature yellow birch trees were more highly colonized by species from the *Russulaceae*. The *Hebeloma* and *Laccaria* species colonized mature trees and seedlings but with differing rates, and both trees and seedlings have an abundant species from the Pezizales, though not the same species. Some infrequently colonizing species matched RFLP types between the seedlings and mature trees. The matches are generally within a family but not necessarily closer. The one weak branch on the cladogram separates two subphyla, the basidiomycotina from the ascomycotina.

Diversity of Ectomycorrhizal Fungi Colonizing Roots

The ectomycorrhizal diversity was higher on trees than it was on seedlings (Fig. 8). The diversity calculated using abundance of morphotypes was lower than the diversity calculated based on the abundance of RFLP types and lower still than the diversity calculated using presence/absence of a morphotype within a core.

Relative Abundance

For the fungi which matched between mycorrhizae and fruit bodies using RFLPs, it was possible to compare the relative abundances of the ectomycorrhizal root tip colonization with relative fruiting abundance. Thus, *B. citrina* had a much higher relative abundance based on fruiting compared with its abundance as a mycorrhiza (Fig. 9). All other fungi for which matches were obtained showed a higher relative abundance as mycorrhizae over fruit bodies. The evenness for the fungal community measured by mycorrhizal colonization was much higher than that based on the fruiting abundance of the different species, as observed using the relative abundance.

Discussion

Our data suggest that the ectomycorrhizal species richness in this region is quite high compared with other regions studied (Visser 1995, Gardes & Bruns 1996, Gehring et al. 1998, Horton & Bruns 1998, Taylor & Bruns 1999). Mushrooms were collected from at least 77 probable ectomycorrhizal taxa and 11 matched ectomycorrhizal RFLPs.

The ectomycorrhizal fungi on the root tips of mature yellow birch consisted of either 28 or 39 fungal taxa as determined by morphological characters and morphological and molecular data, respectively. The 11 RFLP types from mushrooms that could be matched with those of mycorrhizal tips identified 41% of the ectomycorrhizal RFLP types, a proportion similar to that found in other studies where only epigeous mushrooms have been collected (Stocchi et al. 1994, Gardes & Bruns 1996, Peter et al. 2001). The missing matches are probably due to the numerous ectomycorrhizal fungi which fruit below ground (Claridge et al. 2000a, Claridge et al. 2000b), which did not fruit within the study plots or during the study period. While the ectomycorrhizal species richness is high in this forest, it is consistent with data of Goodman & Trofymow (1998) who studied old-growth and mature douglas fir stands in BC and Byrd (2000) who studied cores near seedlings from clearcut and mature stands of lodgepole pine in Wyoming and Montana. They found over a hundred morphotypes or RFLP types by directly examining root tips. Morphotype richness has been shown to be an underestimate of the richness of ectomycorrhizal fungi as compared with mushroom taxa richness in several forest types (Visser 1995, Durall et al. 1999, Jansen & De Nie 1988). Mushrooms may represent epigeous ectomycorrhizal fungal species richness when sampling is intensively managed over several years (Bills et al. 1986, Villeneuve et al. 1989, Nantel & Neumann 1992) but

rarely does it represent the below ground ectomycorrhizal community distribution (Jansen & De Nie 1988, Gardes & Bruns 1996, Durall et al. 1999). In this study the relative abundances of mushroom fruiting and ectomycorrhizal colonization are not correlated. Gardes & Bruns (1996), who found a similar lack of correlation in a Californian bishop pine forest, suggested that the lack of correlation may be due to species differences in terms of resource allocation, varying environmental or ecological cues for fruiting, differences in the ability of species to take up carbon from the plant and varying levels of species specific saprophytic abilities.

The distribution of ectomycorrhizae on yellow birch seedlings has implications for their establishment. Yellow birch has a small seed with scant carbohydrate and protein reserves for the initial growth of germlings. The main competitors to yellow birch in this geographical region, maple and beech, have seeds with large carbon and nutrient reserves. This allows them to grow independent of the surrounding environment for the first few critical months at least. In this region, yellow birch makes up about 25% of the forest canopy cover (Ricard 1999). In natural conditions at least 50% of the germinating seeds of yellow birch in an undisturbed forest are found on rotting wood (White et al. 1985) with the rest germinating on perturbed soil (Erdmann 1990, Houle and Payette 1990). Yellow birch cannot penetrate the litter layer with such a small carbon reserve (Messier et al. 2002) and germination on disturbed soil presumably speeds the seedling's integration into the ectomycorrhizal network, which in turn would allow for a faster and more energy efficient access to increased levels of nutrients as well as increased water uptake and pathogen protection. Germination on rotting wood suggests another source of carbon to help in the establishment of seedlings (Colpaert & Van Laere

1996, Simard et al. 1997, Lindahl et al. 1999, Perez-Moreno & Read 2000). It has been proposed that yellow birch have a competitive advantage when the soil has been disturbed because this happens in congruence with a disturbance of possible competitors and an increase in light levels, such as with tree fall (Godman & Krefting 1960, Ricard et al. 2002). The occurrence of yellow birch seedlings on dead wood, however, suggests an alternative strategy to deal with a lack of light.

The evidence implying that yellow birch seedlings depend on ectomycorrhizal support lies in the distribution of *Hebeloma* sp., *Laccaria* sp. 2 and/or *Laccaria* sp. 1 which were the most abundant RFLP types within morphotype M1, the most abundant morphotype on seedlings. These mushrooms were found fruiting throughout most of the forest and in some of the gaps (personal observation). The CCAs (Fig. 4 & 5) show *Laccaria* sp. 1 located near the centre of the forest/gap separation and more correlated with the abundance of raspberry than any other factor while *Hebeloma* sp. was more affected by the gradient of soil water content than the ectomycorrhizal plant species cover. Last et al. (1984) found similar results when they collected fruit bodies around planted *Betula pendula* saplings and found that the early stage fungal species consisted of *Laccaria*, *Hebeloma* and *Inocybe* species. In contrast to the results and/or the interpretation of Jonsson et al. (1999) who found a similar distribution of fungal species colonizing both trees and seedling of *Pinus sylvestris* in Sweden, we found that while trees and seedlings did not differ in species richness or composition, there was a higher colonization by *Hebeloma* and *Laccaria* species on the seedlings while the mature yellow birch trees had a more even distribution of types with more domination by members of the *Russulaceae* (See fig. 7 and table 1). Yellow birch requires disturbance for

germination and regeneration (White et al. 1985, Erdmann 1990, Houle and Payette 1990). This, combined with the lack of fruiting of the main colonizers of mature yellow birch in gaps and the dominance of the abundant fruiting species on the seedlings certainly suggests that the fungal partners are important for the establishment of yellow birch seedlings and that the active fungi, in turn, must have robust growth parameters and thus probably tend to be more ruderal taxa.

The diversity of ectomycorrhizal taxa, as determined from root tips, was higher on mature yellow birch roots than on the seedlings studied from the same area by DeBellis et al. (2002). This seems to be due to the increase in the evenness of species distribution on mature yellow birch roots. Jonsson et al. (1999) did not find differences in the evenness of distribution or species richness of ectomycorrhizal taxa between mature trees and seedlings. They state that the distribution of ectomycorrhizal fungi on *Pinus sylvestris* seedlings and trees is probably due to spatial variation of the inoculum potential in the soil but they sampled less than a quarter the number of root tips analysed in this study. In this study, based on morphotypes, the levels of colonization by the major taxa on yellow birch showed distinct differences between mature trees and seedlings. Gibson & Deacon (1988), using an experimental growth trough with *Betula pendula*, have shown that different species of ectomycorrhizal fungi have "preferences" for different parts of the root system depending on distance from the trunk. They suggest that this could be due to the amount of carbon different fungal species need, those closer to the trunk getting more than those at a distance. If this is true then seedlings, not producing very much photosynthate, would best be able to support only certain species with lower carbon demands. In this study, ectomycorrhizae on mature yellow birch roots

were more evenly distributed than those found on seedlings (DeBellis et al. 2002), resulting in a higher ectomycorrhizal diversity on mature yellow birch.

Conclusions:

This study confirmed the general trends seen in ectomycorrhizal community studies, 1) that there is a high abundance of a few ectomycorrhizal taxa colonising the roots of one tree species, 2) the lack of correlation between the abundance of mushrooms and the fungi occurring as mycorrhizae and 3) that the proportion of matches between the ectomycorrhizae and mushrooms fruiting, based on ITS RFLP matches, is low. The ectomycorrhizae fungal community colonising mature yellow birch are more evenly distributed than that on seedlings, resulting in a higher calculated diversity of fungi colonising the roots of mature yellow birch. These differences between trees and seedlings may reflect the importance of the fungal partners for the establishment and survival of yellow birch.

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