The mycorrhizal fungi involved in the tree invasion of lowland heathlands

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The work presented in this thesis is entirely my own, collaborative work has been specifically acknowledged (section 4.2.1) and those who offered less formal advice and assistance have been listed in the acknowledgements.

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Abstract

In England, the loss of lowland heathland, a habitat of high conservation importance, is primarily due to the invasion of birch and pine. This secondary succession has been researched in depth from a plant perspective but little is known about the role of mycorrhizal fungi, even though both trees and heather are mycorrhizal. In fact, tree encroachment onto lowland heathland can be regarded as the replacement of a resident ericoid mycorrhizal community by an invading ectomycorrhizal community.

I determined the identity and distribution of the ectomycorrhizal fungi associated with birch and pine encroachment onto lowland heathlands. I established whether there are mycorrhizal fungi that mediate the invasion by a) comparing the mycorrhizal inoculum potential of soil and ectomycorrhizal fungal diversity at three levels of invasion (uninvaded heathland, invaded heathland and woodland), b) comparing the fungi forming mycorrhizas on tree seedlings and trees across diverse sites, c) determining the effect of proximity to trees on mycorrhization and seedling biomass, and d) identifying fungal dispersal methods.

I established that in lowland heathlands i) seedlings have limited access to ectomycorrhizal fungi even within sapling rooting zones, ii) ectomycorrhizal inoculum potential increases as the level of tree invasion increases, iii) mycorrhizal seedlings accumulate more biomass than non-mycorrhizal seedlings, iv) there are five keystone ectomycorrhizal fungi that participate in tree invasion - *Rhizopogon luteolus*, *Suillus bovinus*, *S. variegatus* (pine symbionts), *Laccaria proxima* and *Thelephora terrestris* (primarily birch symbionts), v) some ectomycorrhizal fungi cannot colonise seedlings via spores, and vi) ectomycorrhizal communities differ between lowland heathland sites.

This study is the first to identify the mycorrhizal fungi that associate with tree seedlings on lowland heathlands and it is one of the first biome-level mycorrhizal studies of secondary plant succession. The data presented provide the stepping-stones required for future ecologically-relevant modelling and experimentation aimed at understanding mycorrhizal invasions.
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Chapter One - Introduction

1.1 Introduction to this project

This thesis investigates the role of mycorrhizal fungi in the invasion of trees onto lowland heathlands. It is the first study to establish the presence, diversity and distribution of ectomycorrhizal fungi on lowland heathlands and in neighbouring woodlands. This study is based on surveys of diverse English lowland heathlands, using molecular methods to identify the fungi that form mycorrhizas on birch and pine encroaching onto lowland heathlands.

1.1.1 Lowland heathlands

Lowland heathland is a habitat of global importance and high conservation priority in the UK, characterised by dwarf ericaeous shrubs occurring below 300m altitude. Heathlands only occur in Northwest Europe in areas with mild winters on oligotrophic, acid soils, deficient in nitrogen and phosphorus (Gimingham 1972). Heathlands develop naturally where trees cannot establish, primarily where a history of grazing has prevented tree encroachment following the removal of trees by humans during the Neolithic and Bronze Age periods (circa 3000 B.C. – 500 B.C.). Heathlands can also occur in coastal areas where climatic conditions prevent tree establishment. This study will focus on the lowland heathlands of England where the primary ericaeous components are heather or ling (Calluna vulgaris L.), crossed-leaf heath (Erica tetralix L.) and bell heather (Erica cinerea L.). Lowland heathlands in England are also the primary habitat of many of Britain’s rarest plants and animals such as the marsh gentian (Gentiana pneumonanthe L.), natterjack toad (Bufo calamita Laurenti), sand lizard (Lacerta agilis L.), nightjar (Caprimulgus europaeus L.) and silver studded blue butterfly (Plebius argus L.) (Anon 2002).

Of the lowland heathland present in 1800 only 16% remains today and the UK holds 20% (approximately 60,000 hectares) of the world’s remaining lowland heathland (Anon 2002). During the 19th and 20th centuries large areas of lowland heathland were lost due to land use changes. For example, of the 30,000 hectares of lowland heathland in East Dorset and Hampshire in 1811, 28% had been converted to agricultural land, 23% lost to urban development and 20% converted...
to forestry by 1960 (Moore 1962). The same heathland area was broken up into 100 fragments leading to the local extinction of the black grouse (*Lyrurus tetrix* Latham) and natterjack toad (Moore 1962). Lowland heathlands are now protected from further loss through land use changes by conservation site designations: SACs (Special Areas of Conservation), NNRs (National Nature Reserves) and SSSIs (Sites of Special Scientific Interest) legislated through the EU Habitats Directive (1986), Wildlife and Countryside Act (1981), and Countryside and Rights of Way Act (2000). The habitat as a whole is covered by a UK Habitat Action Plan. Today, therefore, it is not the conversion to other land uses that threatens lowland heathlands but the loss of heathlands due to the unmanaged encroachment of native trees that has been noted since the first half of the 20th century (Summerhayes *et al.* 1924, Summerhayes *et al.* 1926, Pickworth-Farrow 1941).

Historically, heathlands were used by local communities for livestock grazing which prevented scrub establishment. Currently, in the absence of grazing, scrub invasion is the principal cause of the loss of lowland heathlands (Marrs *et al.* 1986, Rose *et al.* 2000). The main species encroaching into heathlands are native birch (*Betula pendula* Roth. and *B. pubescens* Ehrh.) and Scots pine (*Pinus sylvestris* L.); other native species invading include gorse (*Ulex europaeus* L.) and bracken (*Pteridium aquilinum* L.) (Gimingham, 1972). The non-native *Rhododendron ponticum* (L.) also invades lowland heathlands. Invasions of both native and non-native species can have severe ecological and economic impacts (Collier & Wentworth 2008). Preventing heath to woodland succession and encouraging heather regeneration have emerged as primary management goals (Marrs 1987). Controlled prescribed burning combined with livestock grazing may achieve these goals effectively in larger northern upland heathlands (Khoon & Gimingham 1984), but this form of management is often impractical in the fragmented lowland heathlands of the densely populated Southern England.

Due to the high conservation status of lowland heathlands, research has focused on managing and restoring heathlands: nutrient removal (Mitchell *et al.* 2000), mowing or burning (Barker *et al.* 2004) and cutting of invading trees, followed by herbicide treatment (Marrs 1987) studies have all been undertaken. Currently scrub removal is the most common form of management yet it is costly; the cost of recreating heathland from woodland is ten times more than managing an
existing heathland (£250 ha\(^{-1}\) yr\(^{-1}\) and £20 ha\(^{-1}\) yr\(^{-1}\), respectively, Michael 1994) and in the case of birch (which re-sprouts from stumps) management is rarely cost-effective (Mitchell et al. 2000). Even after removal the effect of birch on soil chemical properties, microarthropod communities and decomposition rates can last for 20 years (Mitchell et al. 2007). Nitrogen deposition is proving an additional threat to restoration and management projects (Mitchell et al. 2000), however, research on the effect of nitrogen deposition has focused upon grass rather than tree invasion onto heathlands (for example Barker et al. 2004, Terry et al. 2004).

Birch and pine encroachment in British heathlands has not escaped the attention of ecologists and consequently it is relatively well characterised from a plant perspective (Gimingham 1978, Miles & Kinnaird 1979) although soil ecology has remained a “black-box”. The factors known to determine susceptibility to tree invasion within heathlands include: seed availability (Hester et al. 1991a, Mitchell et al. 1997, Manning et al. 2004, 2005), soil nutrients (Hester et al. 1991b, Mitchell et al. 2000, Manning et al. 2004, 2005), disturbance and gap formation (Marrs 1987, Manning et al. 2004, 2005), burning and heath successional stage (Khoon & Gimingham 1984). Tree invasion affects the ericoid plant community through increased shading (Hester et al. 1991a), increased soil fertility (Hester et al. 1991a, Mitchell et al. 2007), by altering microarthropod community and increasing decomposition rates (Mitchell et al. 2007).

Despite the broad array of studies on the tree invasion of heathlands virtually nothing is known about the role of mycorrhizal fungi in the invasion, even though both trees and heather are obligately mycorrhizal. Understanding the basic mycorrhizal processes involved may aid efforts put into heathland conservation and improve modelling of the invasion process. This study is the first to focus on the role of mycorrhizas in the invasion of birch and pine onto lowland heathlands. The following chapters are based on the analysis of birch and pine roots from 1,145 naturally-occurring field seedlings, 1,440 seedling bioassays, 588 outplanted seedlings and 60 soil cores from eight lowland heathlands in England.
1.1.2 Mycorrhizal fungi

1.1.2.1 Description and current knowledge

Approximately 80% of terrestrial plants and several members of three fungal phyla (Ascomycota, Basidiomycota and Glomeromycota) form mycorrhizas (Wang & Qiu 2006, Smith & Read 2008). In 2004, Brundrett defined mycorrhizas as “a symbiotic association essential for one or both partners, between a fungus (specialised for life in soil and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer. Mycorrhizas are a specialised plant organ where intimate contact results from synchronised plant–fungus development” in the formation of diagnostic symbiotic structures (mantle, Hartig net, arbuscule and/or hyphal coil). This broad definition allows the grouping of multiple independently evolved but generally functionally convergent “mycorrhizal” symbioses. The mycorrhizal symbiosis is typically mutualistic; the plant partner exchanges photosynthesised carbon for fungal-acquired soil minerals such as nitrogen and phosphorus. Mycorrhizal fungi increase the extent and efficiency of soil exploitation with their long thin tubular cells that produce exoenzymes (e.g., proteases and/or phosphatases). Mycorrhizal fungi have also been reported to improve water uptake for the plant (Marjanovic et al. 2005), protect against soil pathogens (Marx 1973, Newsham et al. 1995) and increase tolerance towards heavy metals (Blau dez et al. 2000, Colpaert et al. 2000). A review of laboratory-culture studies and field studies revealed that ectomycorrhizal fungi receive up to 22% of the total net primary production of the plant (Hobbie 2006). Further field research found that ectomycorrhizal fungi provide 61% to 68% of the nitrogen in plants and fungi in return receive 8% to 17% of the plant’s photosynthetic carbon (Hobbie & Hobbie 2006).

There are different types of mycorrhizas; ecto- (ECM), ericoid, arbuscular, ectendo-, arbutoid, monotropoid, and orchid mycorrhizas have been described (Smith & Read 2008). Heathlands are dominated by ericoid mycorrhizal fungi, predominately Helotiales (Ascomycota). These fungi penetrate the epidermal root cells of ericaeous plants forming dense hyphal complexes (coils) and do not form a sheath around the root (typical of other forms of mycorrhizas). Ericoid mycorrhizal fungi can assimilate complex organic forms of nitrogen and phosphorus, benefiting the ericaeous plants of nutrient poor heathlands. The trees
that invade heathlands form ectomycorrhizas (predominately with Basidiomycete fungi). Ectomycorrhizas are characterised by three structural components: a sheath of fungal tissue around the plant root (mantle), the growth of hyphae between the root’s cortical cells (Hartig net), and the outward growth of hyphae forming connections with soil, other plants’ roots and fungal fruit bodies (extraradical mycelium) (Smith & Read 2008). Unlike in all other mycorrhizas, there is no intracellular invasion by ectomycorrhizal fungi.

Over recent years the importance of mycorrhizal symbioses in developing and maintaining terrestrial ecosystems has been identified (van der Heijden et al. 2008), including their role in CO$_2$ release from soil (Högberg & Read 2006), plant invasions (Fitter 2005), phosphorous and nitrogen acquisition by plants, soil aggregation (van der Heijden et al. 2006), plant community structure (Stampe & Daehler 2003) and the maintenance of plant diversity and ecosystem functioning (van der Heijden et al. 1998). Mycorrhizal fungi may also aid the restoration of priority conservation habitats such as lowland heathlands (Diaz et al. 2006).

Despite the potentially vast significance of these organisms there is still a general lack of knowledge of mycorrhizal fungal ecology. Significant advances in the study of mycorrhizas have however, occurred since the advent of molecular techniques (Horton & Burns 2001); in particular Gardes & Bruns (1993) paper describing the fungal-specific nuclear ribosomal internal transcribed spacer primer ITS1F. The primer ITS1F allows the direct amplification and sequencing of fungal DNA from a mycorrhiza whereas previously mycorrhizal fungi had to be identified using mycorrhizal morphology. The primer pair ITS1F/ITS4 has been used in many ecological studies because it provides species level recognition that can agree well with fungal phylogenetic species concepts (Taylor et al. 2000) based on concordance of protein-encoding genes (Hedh et al. 2008). This ITS region is now becoming universally recognised as a “barcode” for identifying fungi (Seifert & Crous 2008) and using this region provides the best opportunity to identify fungi through comparisons to the fungi currently represented in GenBank (http://www.ncbi.nlm.nih.gov/Genbank). Unfortunately, most fungi are still poorly represented or not represented at all in GenBank thus restricting its full potential for fungal identification (Brock et al. 2008). Our knowledge in several aspects of ectomycorrhizal ecology has developed considerably over recent years but improvements are still needed in many areas including: biodiversity and
distribution (Fitter 2005), the effect of the abiotic environment (Erland & Taylor 2002), autecology of dominant taxa (Horton & Bruns 2001), impact upon plant invasions (Desprez-Loustau et al. 2007), interactions between fungal species (Kennedy & Bruns 2005) and sampling methods (Horton & Bruns 2001).

1.1.2.2 Mycorrhizal fungi on lowland heathlands

The primary invaders of lowland heathland, birch and pine trees, are obligately ectomycorrhizal (commonly >90% of fine root tips are colonised) whereas the native ericaceous shrubs are obligately ericoid mycorrhizal (over 90% of epidermal root cells are typically colonised). Therefore, the invasion of birch and pine into lowland heathland may be viewed as an invasion of ectomycorrhizal fungi into an ericoid mycorrhizal community. This form of mycorrhizal invasion may be less widespread than plant invasions involving plants that form the relatively non-specific arbuscular mycorrhizas (AM; Richardson et al. 2000). Only 7% of the plants on a representative list of alien invasive species form ectomycorrhizas (Cronk & Fuller 1995). Ectomycorrhizal fungal invasions, both native and alien, do however, occur globally in other conservation priority, often AM dominated, habitats (Thiet & Boerner 2007) resulting in disproportionate ecosystem effects because they only involve trees (e.g., pines and eucalypts, Richardson et al. 1994).

Unfortunately, even simple criteria, such as the rate and thresholds required, for fungal invasions of natural environments are largely unknown (Otten et al. 2004). Consequently, the potentially key role of ectomycorrhizal fungi in native or alien plant invasions remains essentially a matter of speculation (Richardson et al. 2000, Simberloff et al. 2002, Vellinga et al. 2009), even though both the study of positive biotic interactions such as facilitation and mutualism (Bruno et al. 2003) and ectomycorrhizal ecology (Peay et al. 2008) are burgeoning fields of research. To date, virtually nothing is known about the involvement of mycorrhizal fungi in the invasion of birch and pine onto lowland heathland. For example, for the fungi neither presence, diversity, distribution, dispersal, size, successional dynamics, population structure, demographics, nor competitive interactions are known.

A potentially key mycorrhizal player in heathland to woodland transition has been identified by studies suggesting that there are some shared ectomycorrhizal and ericoid mycorrhizal fungi. Vrålstad et al. (2000) found that
samples of the ectomycorrhizal morphotype “Piceirhiza bicolorata” shared 95% ITS1 sequence identity with the ericoid mycorrhizal fungus Rhizoscyphus ericae (= Hymenoscyphus ericae). Phylogenetic analysis placed P. bicolorata within the R. ericae aggregate clade. This result led to Vrålstad et al. (2000) proposing that some Ascomycete fungi (Rhizoscyphus ericae agg.) can form both ecto- and ericoid mycorrhizas. Read (2000) however, warns that simply because these fungi are genetically related they may not be functionally related. Since 2000, support for Vrålstad et al.’s (2000) hypothesis has been given by laboratory studies. Vrålstad et al. (2002a) isolated two identical genotypes within the R. ericae clade from co-occurring ericoid and ectomycorrhizal roots. Nonetheless, Koch’s postulates remained unsatisfied; Vrålstad et al. (2002b) failed to find a strain of the “Rhizoscyphus ericae aggregate” that could form both ectomycorrhizas and ericoid mycorrhizas. When they tested 12 strains of the R. ericae aggregate, five of the nine strains of ectomycorrhizal origin formed ectomycorrhizas and all three ericoid mycorrhizal strains formed ericoid mycorrhizas but no one strain could form both ecto- and ericoid mycorrhizas. Villarreal-Ruiz et al. (2004) were the first to demonstrate that a single fungal mycelium of R. ericae can simultaneously form both ecto- and ericoid mycorrhizas in vitro. The ecological relevance of this finding remains unknown. In 2000, Bergero et al. reported that fungi found on Quercus ilex roots could form typical ericoid mycorrhizas on Erica arborea. Although these fungi are unrelated to the R. ericae aggregate, they indicate the potential for further links between ectomycorrhizas and ericoid mycorrhizas. The hypothesis proposed by Vrålstad et al. (2000) may explain tree encroachment onto heathlands without the need for invasion by solely ectomycorrhizal fungi but is yet to be tested under field conditions (Dahlberg 2001).

1.2 Aims and outline of this thesis

The main objective for this thesis is to discover how obligately ectomycorrhizal plants can invade a habitat where the mycorrhizal fungi they depend on may not be present and a different mycorrhizal community is already established. My work will be based on the invasion of birch and pine onto lowland heathland. In 1979, Miles & Kinnaird hypothesised that 1) a lack of ectomycorrhizal inoculum in heathlands slows invasion by birch and pine and 2)
there are ectomycorrhizal fungal species-specific effects on the establishment of
tree seedlings that invade heathlands.

I will test three related, but more specific, hypotheses:

1. Ectomycorrhizal inoculum in uninvaded heathland is less abundant and less
diverse than in woodlands or invaded heathland.

2. Pioneer ectomycorrhizal fungi will be the predominant formers of
ectomycorrhizas on seedlings.

3. Where established saplings or trees are present, seedlings are colonised via
vegetative fungal growth rather than spore colonisation.

I will also be simultaneously testing the hypothesis proposed by Vrålstad et al.
(2000) that some fungi within the R. ericae agg. can form ectomycorrhizas. In
general, very few studies have focused on mycorrhizas in habitats where trees and
ericaceous plants co-occur, studies on ectomycorrhizas have been conducted in
woodlands and ericoid mycorrhizal studies in heathlands without trees (Vrålstad
2004). Since 2004 a notable exception has been Bougoure et al. (2007) on the
diversity of fungi associated, but not necessarily forming mycorrhizas, with ericoid
roots along a vegetation gradient from a pine woodland to a heathland. There have
been reports of typical-ectomycorrhizal seedlings forming arbuscular mycorrhizas -
in birch by Haigh (2001) and in pines (P. muricata) by Horton et al. (1998) - in
areas dominated by typical arbuscular mycorrhizal plants. Heathlands can contain
plants that form arbuscular mycorrhiza and therefore I will test for the presence of
arbuscular mycorrhizal fungi during this study.

This study further evaluates the biodiversity and early mycorrhizal
succession of ectomycorrhizal fungi in a natural environment. The most detailed
studies of ectomycorrhizas on birch were carried out during the 1980s in Scotland
Last et al. 1983, Mason et al. 1983, Dighton & Mason 1984, Fleming et al. 1984,
Fleming 1984, Last et al. 1984a, b, Mason et al. 1984, Fleming 1985, Fleming et
1988, Deacon & Fleming 1992). These studies established the successional
dynamics of “early” through to “late” stage fungi forming ectomycorrhizas with
birch. No other set of studies has focused on analysing an ectomycorrhizal
community to the same extent as these 1980s studies. The Bush Estate studies are
however, still a controversial mycorrhizal succession paradigm (Jumpponen &
Egerton-Warburton 2005). They took place in a relatively unnatural environment (an ex-agricultural site) and they relied mainly on fungal fruitbody surveys which can poorly represent a site’s ectomycorrhizal diversity (Taylor & Alexander 1990, Gardes & Bruns 1996, Jonsson et al. 1999, Horton & Bruns 2001, Taylor 2002, Nara et al. 2003b). The basis for this inconsistency between frequency of sporocarps and frequency of mycorrhizas of the same fungal taxa at the same site is unknown (Smith & Read 2008) but may reflect trade-offs between allocation to vegetative versus reproductive growth in fungi. Morphological identification of fungi from ectomycorrhizal roots was used to a lesser extent in the Bush Estate studies but it is a low-resolution and low-throughput approach compared to today's DNA-based identification (Peay et al. 2008). Molecular techniques have also significantly improved the list of fungi known to form ectomycorrhizas, particularly for those with cryptic sporocarps (e.g. Tomentella). The use of DNA sequencing in this study provides the opportunity to evaluate the Bush Estate studies and the list of fungi that form ectomycorrhizas with birch in Britain produced by Atkinson (1992, and references therein) for the description of *B. pendula* and *B. pubescens* in the Biological Flora of the British Isles, yet this list is also based on non-molecular studies. There are no equivalent studies to the Bush Estate studies for pine and the Biological Flora of the British Isles description for *P. sylvestris* does not even mention mycorrhizas (Carlisle & Brown 1968).

The main aims for this thesis are to:

i) Assess the diversity of fungi forming mycorrhizas on birch and pine on lowland heathlands and in neighbouring woodlands using molecular identification techniques.

ii) To evaluate where and when tree seedlings become mycorrhizal within heathlands (in relation to presence of established trees) during their first year.

iii) To evaluate whether tree seedlings become colonised with ectomycorrhizal fungi via spores or via hyphae from the mycelium of established trees.

**Additional aims include:** 1) to establish whether members of the *R. ericae* aggregate form ectomycorrhizas on birch and pine in lowland heathlands, 2) to establish whether arbuscular mycorrhizas form with tree seedlings in lowland heathlands, and 3) to gain knowledge on the geographic distribution of
ectomycorrhizal fungi. Recently, focus has been drawn to the lack of data on the “forgotten kingdom” in particular the geographic distribution and conservation status of fungi (Anon 2009).

This thesis consists of five chapters:

• Chapter Two describes a bioassay-based survey of mycorrhizal inoculum and ectomycorrhizal fungal diversity in three levels of tree invasion at eight lowland heathlands.

• Chapter Three reports the ectomycorrhizal fungi detected on naturally occurring seedlings, the effects of mycorrhization on naturally-occurring seedling mass and survival, and the ectomycorrhizal community on mature tree roots at six lowland heathland sites.

• Chapter Four describes the influence of established saplings on ectomycorrhizal colonisation of nearby outplanted seedlings and the ability of common ectomycorrhizal fungi to form ectomycorrhizas via spore inoculum.

• Chapter Five describes the major findings of these studies and implications for future work on ectomycorrhizal ecology.

1.3 Introduction to study sites and terms used in this study

Eight lowland heathlands in Surrey, Suffolk, Dorset, Hampshire and Merseyside were surveyed in this study (Figure 1.1). These sites encompass a wide variety of lowland heathland types (Table 1.1). All of these sites are, or are part of, National Nature Reserves (NNR). Freshfield Heath is a SSSI and part of Ainsdale Sand Dunes NNR, for all other sites the name of the NNR is used as the site name (Table 1.1). Six of the sites (Fd, Hw, Kw, Sw, Td and Tw) are being invaded by birch and pine, one (Cd) only by birch and one (Gd) only by pine. The sites closest to each other are Tw and Td at approximately 1.2 km apart and the greatest distance between sites is 380 km between Sw and Fd. I have designated heathlands as wet or dry depending upon the plants dominant near my study plots, although many sites contain both wet and dry heath areas. Wet heaths contain mire associated plants such as Drosera sp. and Molinia caerulea, and cross-leaved heath. Dry heaths typically contain a higher proportion of Calluna cover and bell heather. Cavenham heath is a dry Breckland heath, Breck heath is unique to East Anglia and consists of a mosaic of heather, grasses and sedges. In addition, two
dry dune heaths were sampled, these heaths are coastal and form part of the dune succession from uninhabited sand dunes to woodland.

*Pinus sylvestris* and *Betula* spp. will be referred by their common names, pine and birch. In field site studies I will not differentiate between *B. pendula* and *B. pubescens* due to their ecological and physiological similarities, however, generally drier areas are invaded by *B. pendula* and wetter areas by *B. pubescens* (Atkinson 1992). For studies in which I grew birch seedlings, *B. pendula* was used. Authorities for Latin names for all fungal species are as quoted by Legon & Henrici (2005).

Table 1.1. Description of the eight lowland heathland sites sampled. Abb. = Abbreviated name. Type refers to classifications described above.

<table>
<thead>
<tr>
<th>Abb.</th>
<th>Site</th>
<th>OS Grid reference</th>
<th>County</th>
<th>Type</th>
<th>Trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>Cavenham Heath</td>
<td>TL 753 724</td>
<td>Suffolk</td>
<td>Breckland, dry</td>
<td>Birch</td>
</tr>
<tr>
<td>Fd</td>
<td>Freshfield Heath</td>
<td>SD 295 090</td>
<td>Merseyside</td>
<td>Dune, dry</td>
<td>Birch, Pine</td>
</tr>
<tr>
<td>Gd</td>
<td>Studland and Godlingston Heath</td>
<td>SZ 014 826</td>
<td>Dorset</td>
<td>Dune, dry</td>
<td>Pine</td>
</tr>
<tr>
<td>Hw</td>
<td>Holt Heath</td>
<td>SU 065 045</td>
<td>Dorset</td>
<td>Wet</td>
<td>Birch, Pine</td>
</tr>
<tr>
<td>Kw</td>
<td>Kingston Great Common</td>
<td>SU 186 035</td>
<td>Hampshire</td>
<td>Wet</td>
<td>Birch, Pine</td>
</tr>
<tr>
<td>Sw</td>
<td>Stoborough Heath</td>
<td>SY 936 849</td>
<td>Dorset</td>
<td>Wet</td>
<td>Birch, Pine</td>
</tr>
<tr>
<td>Td</td>
<td>Thursley</td>
<td>SU 909 406</td>
<td>Surrey</td>
<td>Dry</td>
<td>Birch, Pine</td>
</tr>
<tr>
<td>Tw</td>
<td>Thursley</td>
<td>SU 907 418</td>
<td>Surrey</td>
<td>Wet</td>
<td>Birch, Pine</td>
</tr>
</tbody>
</table>

I sampled three areas within Thursley; two of dry heath (Td1 and Td2) approximately 300 m apart and one area of wet heath (Tw). On 14th July 2006 a wildfire burnt a large tract (approximately 2 km²) of Thursley. Samples of burnt heathland and unburnt heathland were obtained after this date and will be referred to in this thesis. The tree encroachment for one study site (Cd) has been reported; of the open heath present at Cd in 1946 only 71% (28.9 ha) was present in 1981.
whereas the amount of birch woodland had nearly quadrupled (from 3.5 ha to 13.0 ha) over the same time period (Marrs et al. 1986) indicating the vast loss of this ecologically important habitat.

I will use the terms uninvaded heathland, invaded heathland and woodland as defined in Table 1.2.

**Table 1.2. Definitions of invasion terms used throughout this study.**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninvaded heathland</td>
<td>An area that does not contain any birch or pine trees or saplings and is not within the rooting zone of any birch or pine tree or sapling.</td>
</tr>
<tr>
<td>Invaded heathland</td>
<td>An area of primary invasion* containing young isolated birch and/or pine saplings (normally between 0.5 and 1m tall).</td>
</tr>
<tr>
<td>Woodland</td>
<td>An area of secondary invasion* (saplings surrounding established trees) or within the rooting zone of established trees (over 1.5m tall) with a heath understory.</td>
</tr>
</tbody>
</table>

* Overall, heathland to woodland succession is a secondary invasion, but I find this terminology useful to distinguish between areas where saplings are present from areas where trees and saplings are present.

**Figure 1.1.** Location of field sites.
Chapter Two - Spatial distribution of fungi forming mycorrhizas on birch and pine on lowland heathlands assessed via inoculum potential bioassays

2.1 Introduction

Lowland heathlands are a threatened habitat of high conservation importance; their current loss is primarily due to invasion by scrub vegetation in particular birch and pine trees. Understanding the role of ectomycorrhizal fungi in the invasion of trees onto lowland heathland is critical as birch and pine are dependent upon their fungal symbionts for growth. Yet, as for many other habitats, we lack the most basic information on the role of ectomycorrhizal fungi in the invasion process. In this chapter I present the first study of ectomycorrhizal fungi on lowland heathlands determining their presence, diversity, spatial distribution and temporal variation using a seedling bioassay study.

Information on the role of mycorrhizal fungi in seedling establishment is rare (Horton & van der Heijden 2008). The ectomycorrhizal fungal community in the secondary invasion habitat of lowland heathlands may display similar patterns to the ectomycorrhizal fungal community in areas of primary invasion for trees such as dune systems and on volcanic scoria. There have been hallmark studies on the role of mycorrhizal fungi in primary invasions including work by Nara and colleagues post-eruption at Mount Fuji in Japan (Nara et al. 2003a,b, Nara & Hogetsu 2004, Nara 2006a,b, Nara 2008), post-fire by Bruns and colleagues at Point Reyes in California (Gardes & Bruns 1996, Baar et al. 1999, Grogan et al. 2000, Bruns et al. 2002, Kennedy et al. 2007) and also following glacial retreat by Jumpponen (2003) and on a primary successional dune system by Ashkannejhad & Horton (2006). There may also be similarities to the tree invasion into AM habitats (e.g. Dickie & Reich 2005, Thiet & Boerner 2007). However, these primary successional habitats bear little similarity to lowland heathlands both in terms of vegetation and geographic proximity (the closest site to my study is over 3,500 miles away). This raises the possibility that the mycorrhizal fungi involved and the dependence of seedlings upon mycorrhizal fungi may differ in the lowland...
heathlands of England compared to the habitats analysed so far. As far as I am aware this is the first study on the role of ectomycorrhizas in any tree invasion habitat in Britain.

The Bush Estate studies (Ford et al. 1980, Mason et al. 1982, Deacon et al. 1983, Fleming 1983, Fox 1983, Last et al. 1983, Mason et al. 1983, Dighton & Mason 1984, Fleming et al. 1984, Fleming 1984, Last et al. 1984a,b, Mason et al. 1984, Fleming 1985, Fleming et al. 1986, Last et al. 1987, Gibson & Deacon 1988, Gibson et al. 1988, Mason et al. 1988, Deacon & Fleming 1992) provide the best set of data on ectomycorrhizal fungi in the UK (as described in section 1.2). However, due to the caveats of these studies - 1) reliance on sporocarp data and 2) location in a relatively unnatural setting - our knowledge on the underground, ecologically-relevant ectomycorrhizal communities on birch in natural environments in the UK is still limited. The lack of an equivalent analysis to the Bush Estate studies for pine means an even greater knowledge gap about ectomycorrhizas in the UK. My study provides an opportunity to: i) evaluate the ectomycorrhizal fungi of birch (Atkinson 1992) and pine in the UK, ii) detect fungi with cryptic sporocarps that may have been missed in sporocarp surveys, and iii) identify the ectomycorrhizal community that exists belowground, by using high-resolution DNA-based identification (Peay et al. 2008). This study will also provide one of the first surveys of ectomycorrhizal diversity at a regional scale.

Based on the cumulative results of the Bush Estate studies, Newton (1992) and Bowen (1994, and references therein), I predict that relatively fast-growing and often spore-dispersed ectomycorrhizal fungi (pioneer or “r-selected”) such as Hebeloma, Laccaria, Paxillus and Thelephora should dominate birch seedlings. The relatively slow-growing ectomycorrhizal fungi that are normally reliant on vegetative dispersal ("K-selected") and are present on established trees, such as Lactarius, Leccinum, and Russula (Taylor & Bruns 1999, Nara et al. 2003a, Twieg et al. 2007) may not develop in the bioassays due to the severing of mycelia during the sampling procedure and their preference for mature hosts. Bioassays are useful in two respects; they allow all seedlings to be grown under the same light, temperature and humidity conditions, and they can be used in mycorrhizal studies to study sites where naturally-occurring seedlings are absent or uncommon. Bioassays and microcosm experiments are a frequently used tool in studying pine ectomycorrhizal fungal ecology (Baar et al. 1999, Taylor & Bruns 1999,
Bidartondo et al. 2001a, Ashkannejhad & Horton 2006). Naturally-occurring seedlings were sampled from study sites when available (see Chapter Three) yet they were absent or infrequent at most sites, particularly within uninvaded and invaded heathland.

Establishing the presence or absence of mycorrhizal fungi will test my hypothesis that inoculum is rare in uninvaded heathlands and that the ectomycorrhizal community (if present) in heathland is less diverse than that in woodlands. Mycorrhizal seedlings may be at a competitive advantage over non-mycorrhizal seedlings, as suggested by their consistently higher biomass than non-mycorrhizal seedlings (Karst et al. 2008); this is probably due to the benefits that mycorrhization brings to plants in the form of increased nutrient (Hobbie & Hobbie 2006) and water uptake (Marjanovic et al. 2005), and protection from soil pathogens (Marx 1973, Newsham et al. 1995). The presence of ectomycorrhizal fungal inoculum in areas of heathland that have few or no birch or pine trees may increase the rate of tree invasion. Testing this may provide support for a hypothesis originally proposed by Miles & Kinnaird (1979): lack of ectomycorrhizal fungi slows tree invasion of heathlands. I will also be able to test whether members of the ericoid mycorrhizal clade, *Rhizoscyphus ericae*, can form ectomycorrhizas with pine seedlings as proposed by Vrålstad et al. (2000).

This study will determine the inoculum potential, diversity, and spatial heterogeneity of ectomycorrhizal fungal inoculum across invasion levels (see Table 1.2), between study sites (see Table 1.1), and between birch and pine trees on lowland heathlands using assessments carried out in the years 2005, 2006 and 2007. I hypothesise that i) ectomycorrhizal inoculum and ectomycorrhizal fungal diversity will increase as the level of invasion increases from uninvaded heathland to woodland, ii) seedlings will be colonised by pioneer fungi, iii) the ectomycorrhizal fungal community will be different pre- to post-fire and iv) mycorrhizal seedlings will be heavier than non-mycorrhizal seedlings. I provide one of the first molecular-based surveys of the diversity of ectomycorrhizal fungi on lowland heathlands, a priority conservation habitat for the UK and globally.
Chapter Two

2.2 Methods

2.2.1 Study sites

Eight lowland heathlands in England (abbreviated Cd, Fd, Gd, Hw, Kw, Sw, Td and Tw see Table 1.1 for details) were surveyed for the presence of ectomycorrhizal fungi. The sites were surveyed and sampled once a year between 2005 and 2007 (Table 2.1) except for Fd which was only surveyed in 2005 and Gd which was surveyed in 2006 and 2007 only. Td1 and Td2 are areas approximately 300m apart within the dry part of Thursley Common NNR and for the purposes of this study they are referred to as different sites.

Table 2.1. Sampling dates of eight lowland heathlands.

<table>
<thead>
<tr>
<th>Site</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>28th June</td>
<td>15th June</td>
<td>18th June</td>
</tr>
<tr>
<td>Fd</td>
<td>29th July</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Gd</td>
<td>N/A</td>
<td>26th July</td>
<td>29th June</td>
</tr>
<tr>
<td>Hw</td>
<td>13th June</td>
<td>22nd June</td>
<td>14th June</td>
</tr>
<tr>
<td>Kw</td>
<td>24th May</td>
<td>22nd June</td>
<td>29th May</td>
</tr>
<tr>
<td>Sw</td>
<td>14th July</td>
<td>13th June</td>
<td>26th June</td>
</tr>
<tr>
<td>Td1</td>
<td>6th May</td>
<td>25th September</td>
<td>16th July</td>
</tr>
<tr>
<td>Td2</td>
<td>18th May</td>
<td>18th September</td>
<td>19th July</td>
</tr>
<tr>
<td>Tw</td>
<td>7th July</td>
<td>2nd October</td>
<td>9th July</td>
</tr>
</tbody>
</table>

On 14th July 2006, a large wildfire burnt approximately 2km² of Td and some parts of Tw. I had intended to sample Td on this date but fortunately decided the weather was too hot for fieldwork! Due to this fire, results for Td1 and Td2 in 2006 and 2007 are presented separately to other sites for those years as the fire may have effects upon the ectomycorrhizal community. Plots sampled for bioassays at Tw were not affected by the fire.

2.2.2 Mycorrhizal inoculum potential bioassays

Six 1.5m² plots; two in each of uninvaded heathland, invaded heathland and woodland were selected at each site, except for Td2, where three 0.75 m² plots
were set up in each of invaded heathland and woodland, these plots were extended to 1.5 m$^2$ in 2006. Locations of the plots were determined by level of invasion (uninvaded heathland, invaded heathland and woodland; based upon the descriptions in Table 1.2) and were set up away from footpaths to avoid disturbance. At Td2 plots had previously been set up in areas of high birch seedling density in 2005 (see Chapter Three). Plots are between 5 and 45 m apart (average = 20 m) in uninvaded heathland, 4.5 and 92 m apart (average = 35 m) in invaded heathland and 7.5 and 25m apart (average 14 m) in woodland, the variation is due to difficulty in finding areas with appropriate levels of invasion and similar vegetation. From within each plot, five soil cores, approximately 2.5 cm in diameter and 20 cm in depth were removed at arbitrary positions. The soil corer was cleaned with household bleach between sampling each plot to avoid cross contamination. At each site the uninvaded plots were sampled first to further minimise the risk of contamination from the woodland plots, which were presumed to contain a higher diversity and density of fungi. Soil samples were stored in sealed plastic bags at 4°C until use.

Bioassays were set up within nine days of soil collection (average of four, three and two days in 2005, 2006 and 2007, respectively). Each soil core was manually homogenised in a sterile plastic bag as there can be spatial heterogeneity in ectomycorrhizal root and hyphal distribution between soil layers (Dickie et al. 2002b, Genney et al. 2006). The soil from each core was divided between two bioassay tubes (RLC-3UV Ray Leach Container; Stuewe & Sons Inc., Carvallis, OR, USA); each containing approximately 45 cm$^3$ of soil. Control bioassays were set up using autoclave-sterilised soil originating from a mix of soil cores including woodland cores. The soil was sterilised twice, 48 hours apart, to eliminate spores that may germinate in response to heat exposure. A small amount of porous, non-organic fabric (non-woven polypropylene sold as a protective plant fleece) was placed in the bottom of each bioassay tube to prevent the soil from pouring out and to allow excess water to drain. For each soil core, one bioassay tube was planted with birch (*Betula pendula*) seeds and one with two pine (*Pinus sylvestris*) seeds (Forestart, Shrewsbury, UK). Pine and birch seeds were surface-sterilised in dilute bleach (0.25%) and then rinsed before use. If seeds did not germinate, additional seeds were added on subsequent days with the aim of having two pine seedlings and two or three birch seedlings present in bioassays at harvest. Excessive
numbers of birch seedlings were trimmed to prevent competition but often new seeds germinated when space was created. In 2006 and 2007 a cutting of *Calluna vulgaris* was added to each bioassay tube to more closely reproduce the heathland environment. *Calluna vulgaris* was obtained from uninvaded heathland when soil samples were removed (before sporocarp production) to reduce the possibility of ectomycorrhizal spore contamination. The *C. vulgaris* cutting used was from the same site the soil was taken from to retain any local effects. On occasions when the cutting did not take it was replaced with a cutting from the same heathland where possible, otherwise it was replaced with a cutting from the nearest available heathland. Seedlings were grown in a controlled environment (20°C for 16 hours light and 16°C for 8 hours dark at 60% humidity). Bioassays were watered as necessary to retain soil moisture. Birch bioassays in 2006 and both birch and pine bioassays in 2007 were grown in small polyethylene bags to prevent desiccation.

### 2.2.3 Sampling of mycorrhizas

Seedlings were grown for approximately four months after which time they were removed from the bioassay tubes, loose soil was rinsed away and the remaining soil and *C. vulgaris* root systems were removed with tweezers under a dissecting microscope. Roots were viewed using a dissecting microscope. Bioassays were recorded as mycorrhizal if an ectomycorrhiza was present on at least one seedling within the bioassay or non-mycorrhizal if no ectomycorrhizas were detected on any seedlings in the bioassay. Some seedlings had roots that were not ectomycorrhizal but had morphological feature that indicated the root may be at an early stage of mycorrhization such as a lack of root hairs, bioassays that contained seedlings within this category and contained no typical ectomycorrhizas were initially recorded as potentially-mycorrhizal and following DNA sequence analysis the bioassay was placed within the mycorrhizal or non-mycorrhizal category. A sample of each mycorrhizal or potentially-mycorrhizal morphotype present in an individual bioassay tube was taken. Roots were sampled based on several morphological features including a lack of root hairs, presence of a mantle with or without rhizomorphs, dichotomous branching, and/or iridescent smooth surface which may be indicative of early stages of mycorrhizal colonisation. In 2005 and 2006 root samples were stored in CTAB at -20°C or -80°C until use. The DNA from sampled roots was extracted immediately in 2007.
In 2007, if other roots of the same morphotype were present within the same bioassay tube, representatives were collected and stored in water at -80°C. On some occasions additional samples were stored in 95% ethanol.

In 2005 and 2006 DNA was extracted from sampled roots using the CTAB protocol described in Gardes & Bruns (1993) with modifications. Briefly, samples were thawed, re-frozen and thawed again, ground with a micropstle using an electric drill, then warmed to 65°C for ten minutes to disrupt cell walls and membranes. Chloroform was then added and samples mixed to denature proteins. Samples were spun for 15 minutes (13.2 rpm) and the supernatant removed into a new 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany). After removal from chloroform, 600µl of 6M NaI and 12µl of glassmilk (Qbiogene, Irvine, CA, USA) were added to the supernatant. Samples were mixed at room temperature for 5 minutes and centrifuged for 2 minutes to form a pellet. The supernatant was removed and the pellet washed twice in NEWWash (Qbiogene). The pellet was then dried, resuspended in 45µl of TE buffer and centrifuged for 1 minute. The supernatant was removed from the silica pellet and stored at -20°C until use.

In 2007, the DNA was extracted using Extract-N-Amp (Sigma-Aldrich, St Louis, MO, USA); sampled roots were placed in 10µl of extraction solution in a 96-well plate, heated to 95 °C for 10 minutes and then allowed to cool to room temperature. Finally, 10µl of dilution solution was added to each well and mixed. If a DNA sequence could not be obtained from DNA extracted using Extract-N-Amp, root tips stored in water at -80 °C were freeze-dried and extracted using the CTAB protocol stated above. In 2007, seedlings were freeze-dried and weighed after harvest.

2.2.4 Molecular analysis

Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region using the fungal-specific primer ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) was attempted for all samples. An aliquot of 2µl of extracted DNA was combined with 8µl of PCR mix. For samples extracted using the CTAB protocol the PCR was performed using either Amplitaq Gold (Applied Biosystems, Foster City, CA, USA) or PicoMaxx reaction mix (Stratagene, Cedar Creek, TX, USA). For samples extracted using the Extract-N-Amp protocol, 8µl
of 2x Extract-N-Amp amplification mix was used. Amplifications were performed with an initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 53°C for 55 seconds then 72°C for 50 seconds and a final extension of 72°C for 7 minutes. PCR products were visualised on a 2% agarose gel with ethidium bromide. If PCR products had low yield, a nested PCR of the sample was performed using primers ITS1 and ITS4 from a 1:100 H2O dilution, including the original negative control and the number of cycles was reduced to 28. If multiple bands were visualised on the agarose gel (i.e., a strong band and a weaker band) the PCR was repeated with the hot-start enzyme JumpStart (Sigma-Aldrich, St Louis, MO, USA). After successful amplification, the PCR products were purified using the QIAquick Multiwell PCR purification protocol (Qiagen, Crawley, UK) or ExoSAP-IT (USB, Cleveland, OH, USA) and cycle sequenced using BigDye v3.1 (Applied Biosystems). The cycle sequenced products were electrophoresed using an ABI3730 Genetic Analyzer (Applied Biosystems).

The DNA sequences were analysed in Sequence Navigator (Applied Biosystems) or Sequencher (GeneCodes, Ann Arbor, MI, USA). Preliminary identification was achieved by conducting a BLASTn search on GenBank (http://blast.ncbi.nlm.nih.gov). Identifications were confirmed by aligning sequences within the same genera with named examples from GenBank using CLUSTALX (version 1.83, Jeanmougin et al. 1998) or MUSCLE (Edgar 2004) and the alignment was checked visually using MacClade (version 4.08, Maddison & Maddison 2003). Checked alignments were used in a neighbour-joining analysis in PAUP version 4.0b10 (Swofford 2002) and/or an analysis with DOTUR (Schloss & Handelsman 2005) to infer within genus groups. To increase the reliability of identification the name obtained by genetic analysis was compared with the description I made of the ectomycorrhizal morphotype for each sample and published morphotype descriptions (Ingleby et al. 1990, Agerer 1987-2002, Agerer & Rambold 2004–2009). Representative DNA sequences have been submitted to GenBank.

2.2.5 Intracellular colonisation

In 2006 root samples of birch and pine seedlings and C. vulgaris cuttings were removed from bioassays representing all sites (except for Tw in case of pine and C. vulgaris) and all invasion levels to test for the presence of arbuscular or
ericoid mycorrhizas. Roots were stained with acid fuchsin following the method in Appendix A of Peterson & Massicotte (2004). Briefly, roots were fixed in 50% ethanol for at least 24 hours, rinsed in deionised water then cleared in KOH (5% KOH at room temperature overnight for *C. vulgaris* roots, 10% KOH for 2 hours in a 90°C water bath for pine roots, and 10% KOH at room temperature overnight for birch). Roots were then rinsed in deionised water, acidified with 2% HCl for 1-2 minutes and stained with 0.1% acid fuchsin for 2-3 hours at 90°C. Excess stain was removed by placing in 50% glycerin for 24 hours. Stained roots were mounted on 50% glycerin and viewed under a light microscope to detect endomycorrhizal structures (i.e., arbuscules, coils).

### 2.2.6 Statistical analysis

Differences between sites and invasion level in the proportion of bioassays within a plot that were mycorrhizal versus non-mycorrhizal were tested in R version 2.7.2 (R Development Core Team 2008) by applying a generalised linear model (GLM) with binomial errors or quasibinomial errors if needed, to account for over-dispersion. I also tested for an interaction between site and invasion level with respect to the proportion of bioassays which were mycorrhizal within each plot. This analysis was conducted separately for birch bioassays and pine bioassays. All models reported are the minimum adequate models; models were tested for potential simplification by testing for significant differences between models with ANOVA.

All richness and diversity indice calculations omitted the few samples of fungi that could not be unambiguously assigned to a taxon, e.g., *Suillus* spp. were excluded when samples could not be assigned to *S. bovinus* or *S. variegatus*. When no other fungi were present within the same higher level taxon, such as Atheliaceae sp., the samples were included. Fungal richness estimators and diversity indices were calculated using fungi found at all levels of invasion (uninvaded heathland, invaded heathland and woodland) within a site to compare sites or within the same level of invasion at all sites to compare levels of invasion within the same year. Shannon index (H’) takes into account the number of species and the evenness of the species and was calculated using the equation:

\[
H' = -\sum (p_i \log_{10} p_i)
\]

\(p_i\) = relative abundance of each fungus, calculated as the proportion of samples of a
given fungus to the total number of samples in the community (either level of invasion or site).

Simpson's Index (D) measures the probability that two fungi randomly selected from a sample will be identical, taking into account the diversity and the relative abundance of each fungus. I report Simpson’s reciprocal index (1/D); the higher the value, the greater the diversity.

Simpson’s Index (D) is calculated as

\[ D = \sum \left( \frac{(n(n-1))}{(N(N-1))} \right) \]

n = the total number of bioassays containing a particular fungus within the same invasion level or site.
N = the total number of bioassays containing all fungi within the same invasion level or site.

Fungal richness estimates - first-order Chao (Chao1; Chao 1984), second-order Chao (Chao2; Chao 1987), first order Jackknife (Jack1; Burnham & Overton, 1978, 1979) and second-order Jackknife (Jack2; Burnham & Overton, 1978, 1979) - were calculated using EstimateS (Colwell 2005) using a soil core as sample unit; i.e. if the same fungus occurred within both the birch and pine bioassay the occurrence within that core would be two. Each estimate was based on 50 randomisations of sample order without replacement (unless it is stated the classic method is used). Ectomycorrhizal fungal accumulation curves were calculated in EstimateS using the same data set as that used to calculate estimated richness.

Richness estimators were calculated again using ten soil cores from within the same level of invasion at the same site in the same year to test for an effect of invasion level, site and any interaction between invasion level and site on each of the four richness estimators using a two-way ANOVA in R version 2.7.2 (R Development Core Team 2008) for 2006 and 2007 data. I did not conduct this analysis using 2005 results due to missing data. All models reported are the minimum adequate models; models were tested for potential simplification by testing for significant differences between models with ANOVA. The effect of the wildfire on ectomycorrhizal richness was tested for with a paired t-test by testing for a difference in estimated richness values from pre-fire to immediately post-fire and between immediately post-fire to one year post-fire.

The effect of site, level of invasion, mycorrhizal status and any interactions on average seedling mass in 2007 bioassays was tested for with a three-way
ANOVA. This analysis was conducted separately for pine bioassays containing two seedlings, birch bioassays containing two seedlings and birch bioassays containing three seedlings. It was not conducted for birch or pine bioassays containing any other number of seedlings. Nearly all pine bioassays contained two seedlings (n = 163), and the majority of birch bioassays contained two (n = 41) or three seedlings (n = 62). The difference in biomass between birch and pine seedlings was large hence requiring separate analyses. I transformed birch mass data using the natural log to normalise the residuals. All models reported are the minimum adequate models; models were tested for potential simplification by testing for significant differences between models with ANOVA. Fungal-specific effects on seedling mass were tested using Kruskal-Wallis non-parametric tests or t-tests depending upon the distribution of the data by comparing the mass of seedlings with a specific fungus to all other mycorrhizal seedlings, when the bioassay contained the same number of mycorrhizal seedlings at harvest. I conducted this analysis when the number of bioassays containing an individual-fungus was greater than five.

A relationship between area sampled and number of fungi detected within each specified area was tested for using a linear model in R version 2.7.2 (R Development Core Team 2008) using combined data from woodland plots in 2006 and 2007. Levels of area sampled were in hierarchical order: soil core within a woodland plot, plot within a woodland, woodland (approximate woodland area sampled was calculated by the difference in easting and northing OS grid references and multiplying these values together), geographic area (Hw and Kw were within and typical of the New Forest, Gd and Sw were within and typical of the Isle of Purbeck, the areas of the New Forest and the Isle of Purbeck were used) and England. Mantel tests were used to test for spatial and environmental (tree invading, whether the heathland was wet or dry and the year of sampling) effects on ectomycorrhizal community composition with data from all three levels of invasion (uninvaded heathland, invaded heathland and woodland) pooled within each site and within each year (Mantel 1967). Mantel tests were conducted using The R package version 4 (Casgrain & Legendre 2001). Non-metric multidimensional scaling (a non-parametric method) was used to test for grouping of sites on the basis of the presence and absence of ectomycorrhizal fungi (binary data) and abundance of ectomycorrhizal fungi (number of bioassays containing
each fungus) both between sites within years (2006 and 2007) and at the same sites between years (2006 and 2007) pooling data from three levels of invasion (uninvaded heathland, invaded heathland and woodland). Using the same dataset I also tested for the grouping of uninvaded heathland, invaded heathland and woodland ectomycorrhizal communities both within and between years by pooling the number of bioassays of each ectomycorrhizal fungus in each invasion level each year from all sites.

The similarity in ectomycorrhizal community composition between sites and at the same site between years was calculated using 2006 and 2007 data from all three invasion levels (uninvaded heathland, invaded heathland and woodland). Three similarity indices (Sørensen index, Bray-Curtis index and Morisita-Horn index) discussed in Magurran (2004) were calculated in EstimateS (Colwell 2005) using the number of bioassays containing each fungus at each site. A linear model of change in similarity index against distance between sites was calculated and tested in R version 2.7.2 (R Development Core Team 2008).

2.3 Results

2.3.1 Bioassays in 2005

In total 480 experimental and 14 control bioassays were set up. Seventy-eight out of 240 birch bioassays, from three sites (Kw, Td1 and Td2) had a live seedling at harvest (Figure 2.1a). None of the uninvaded heathland birch seedlings were mycorrhizal, 6% of the invaded heathland and 41% of the woodland birch seedling bioassays were mycorrhizal (n = 2, n = 12, respectively). Over twice as many pine bioassays than birch bioassays had surviving seedlings at harvest (n = 183) and bioassays from all eight sites produced pine seedlings (Figure 2.1b). For pine, 7%, 14% and 26% of bioassays from uninvaded, invaded and woodland bioassays, respectively, were mycorrhizal (n = 4, 10, 15, respectively). Site had no significant effect on the proportion of birch or pine bioassays that were mycorrhizal. For both birch and pine bioassays the level of invasion had a significant effect on the proportion of bioassays that were mycorrhizal (GLM with binomial errors; birch $\chi^2 = 19.2_{2.15}$, P < 0.001; pine, $\chi^2 = 7.8_{2.42}$, P < 0.05). All control birch bioassays died, and the three control pine bioassays with live seedlings at harvest were non-mycorrhizal.
In total 176 root samples were used for DNA analysis. Overall, thirteen ectomycorrhizal fungi were identified (Figure 2.2) from birch and pine bioassays. *Cenococcum geophilum* and *Lactarius rufus* were the most prevalent fungi identified and were detected in both birch and pine bioassays (Figure 2.2). *Lactarius rufus* was only detected at Td1, whereas *C. geophilum* was detected at two sites (Kw and Td2, Figure 2.3). Most fungi (n = 10) were detected in woodland bioassays, four in invaded heathland and two in uninvaded heathland. Only suilloid fungi (*Suillus* and *Rhizopogon*) were detected in uninvaded heathland. The suilloid fungi were also present in invaded heathland along with *Leccinum holopus* and *Thelephora terrestris*. Despite the proximity between Td1 and Td2 only *T. terrestris* and *Leccinum holopus* were detected at both sites; *Lactarius rufus* and *Scleroderma citrinum* were detected at Td1 only and *Cenococcum geophilum*, *Tomentella subilacina*, *Paxillus involutus* and *Russula* sp. were detected at Td2. *Thelephora terrestris* was detected at more sites than any other fungus (Cd, Td1 and Td2) and half of the fungi detected were present at only one site. No ectomycorrhizal fungi were detected at Fd; although this may be due to the high mortality rate of seedlings from this site, in particular no seedlings grown in soil from the woodland plots survived (in total only eight pine seedlings survived; five from uninvaded heathland and three from invaded heathland).

The identity of ectomycorrhizal fungi from 11 bioassays was not obtained because of poor DNA sequence results; the CTAB I used was not buffered thereby damaging the DNA and preventing further attempts to sequence the DNA. Rough morphotype descriptions were not detailed enough to identify these mycorrhizal fungi unambiguously. The 11 unidentified samples were from uninvaded heathland (n = 1), invaded heathland (n = 5) and woodland (n = 5) pine bioassays from Cd, Kw, Td2 and Tw. None of the mycorrhizal fungi at Tw were identified. The identity of two fungi from two bioassays was ascribed using the morphotype description (*Rhizopogon* sp.) and in another bioassay a partial DNA sequence (*Laccaria* sp.). Two fungi were identified to genus level; the only *Suillus* sp. sample was detected at Kw where *S. variegatus* was detected in bioassays but *S. bovinus* was also present at this site (see 2006 and 2007 data). The *Russula* sample occurred at Td2 where *Russula ochroleuca* was detected on naturally occurring seedlings (see Chapter Three) but the DNA sequence was too poor to assign this sample to a species-level group.
Based on birch and pine bioassays of all sites, fungal diversity and estimated richness increased as the level of tree encroachment increased (Table 2.2). The richness estimates for all habitats indicate that not all fungi present were detected; twelve fungi were detected but the estimated richness is between 17 and 22.
Figure 2.1. Percentage of a) birch and b) pine bioassays recorded as non-mycorrhizal (open bars), mycorrhizal (closed bars) and possibly mycorrhizal (grey bar) in 2005. Numbers on bars are actual numbers for each type of bioassay.
Figure 2.2. Number of a) birch and b) pine bioassays from each level of invasion containing each ectomycorrhizal fungus identified in 2005 at three sites for birch bioassays and six sites for pine bioassays. Black bars indicate woodland, grey is invaded heath and open is uninvaded heath.
Table 2.2. Ectomycorrhizal fungal richness and diversity at each level of invasion in bioassays in 2005 at six sites.

<table>
<thead>
<tr>
<th>Level of invasion</th>
<th>Number of fungi detected</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shannon ($H'$)</td>
<td>Simpson’s reciprocal $1/D$</td>
</tr>
<tr>
<td>Uninvaded</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Invaded</td>
<td>4</td>
<td>0.555</td>
<td>5.25</td>
</tr>
<tr>
<td>Woodland</td>
<td>10</td>
<td>0.892</td>
<td>8.21</td>
</tr>
<tr>
<td>All</td>
<td>12</td>
<td>0.980</td>
<td>10.44</td>
</tr>
</tbody>
</table>

Figure 2.3. Number of soil bioassays at each site for each ectomycorrhizal fungus identified in 2005 based on all levels of invasion (uninvaded heathland, invaded heathland and woodland).

Fungal diversity and estimated richness was highest at Kw despite not all fungi being identified (Table 2.3) and lowest at Hw and Sw despite all ectomycorrhizal fungi present in bioassays being identified.
Table 2.3. Ectomycorrhizal fungal richness and diversity at each site where ectomycorrhizal fungi were detected in bioassays in 2005 in all levels of invasion (uninvaded heathland, invaded heathland and woodland).

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of fungi detected</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Simpson's reciprocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shannon ($H'$)</td>
<td>1/D</td>
</tr>
<tr>
<td>Cd</td>
<td>2</td>
<td>0.276</td>
<td>3.0</td>
</tr>
<tr>
<td>Hw</td>
<td>1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Kw</td>
<td>5</td>
<td>0.616</td>
<td>5.0</td>
</tr>
<tr>
<td>Sw</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Td1</td>
<td>4</td>
<td>0.487</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* Value reported is Abundance-based Coverage Estimator (ACE); Td1 species richness was estimated using the classic method rather than the bias-corrected method because CV for abundance distribution was > 0.5 thus the highest value out of Chao1 and ACE was reported, as recommended by A. Chao in EstimateS (Colwell 2005).

The sampling design at Td2 was different to that of the other sites with no uninvaded heathland plots and an additional plot in each of invaded heathland and woodland; therefore, I am not able to directly compare the fungal diversity and richness of this site with the other sites. This additional sampling in areas of higher fungal diversity may be the reason for the higher fungal diversity and richness at Td2 than the other sites (Tables 2.3 and 2.4).
Table 2.4. Ectomycorrhizal fungal richness and diversity in Thursley (Td2) bioassays in 2005.

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of fungi detected</th>
<th>Shannon $(H')$</th>
<th>Simpson’s reciprocal $1/D$</th>
<th>ACE*</th>
<th>ICE*</th>
<th>Jack1</th>
<th>Jack2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Td2</td>
<td>6</td>
<td>0.640</td>
<td>4.13</td>
<td>16.09</td>
<td>16.71</td>
<td>9.87</td>
<td>12.7</td>
</tr>
</tbody>
</table>

* ACE and Incidence-based Coverage Estimator (ICE) are reported instead of Chao1 and Chao2, respectively; estimated species richness values were calculated using the classic method rather than bias-corrected method because CV for abundance and incidence distribution was > 0.5 then the highest value out of Chao1 and ACE, and Chao2 and ICE was reported, as recommended by A. Chao in EstimateS (Colwell 2005).

2.3.2 Bioassays in 2006

Here, I report results from bioassays carried out in 2006 of sites which had not been burnt; I report the results of the post-fire Td bioassays later in this chapter. In 2006, 180 experimental birch, 180 experimental pine and 10 control birch bioassays and 10 control pine bioassays were set up.

For birch bioassays the level of mycorrhizal inoculum increased as the level of tree encroachment increased; none of the birch bioassays from uninvaded heathland were mycorrhizal, 14 % (n = 8) of invaded heathland birch bioassays and 53% (n = 30) of woodland birch bioassays were mycorrhizal (Figure 2.4a). Site, as well as invasion level, had a significant effect on the proportion of birch bioassays that were mycorrhizal but there was no interaction between site and invasion level (GLM with binomial errors; site, $\chi^2 = 17.2 \, 5,30, \, P < 0.01$; invasion level, $\chi^2 = 61.2 \, 2,28, \, P < 0.001$). The level of inoculum for pine seedlings was higher than birch in all invasion levels; a third (n = 19) of uninvaded heathland bioassays were mycorrhizal and 82 % and 84 % of bioassays in invaded heathland and woodland, respectively, were mycorrhizal (Figure 2.4b). Site had no effect on the proportion of pine bioassays that were mycorrhizal but invasion level had a highly significant effect (GLM with quasibinomial errors; $F = 43.4 \, 2,33, \, P < 0.001$). Five control pine bioassays, ten experimental pine bioassays from Tw and nine birch from five different sites (Gd, Hw, Kw, Sw and Tw) had no surviving seedlings at harvest. None of the surviving control bioassay seedlings were mycorrhizal. The C.
vulgaris cuttings died in all pine bioassays, five of the control birch bioassays and seven of the experimental birch bioassays.

Figure 2.4. Number of a) birch and b) pine bioassays recorded as non-mycorrhizal (open bars) seedlings and mycorrhizal (closed bars) from six sites and three levels of invasion in 2006.
Overall, 561 individual root samples were obtained, of these 102 were replicates taken as back up in case the DNA sequence obtained from the first root was not usable. In total, 488 sequences were obtained, some sequences were not clean, hence reserve samples were used and I was able to identify nearly all fungi based on the sequences obtained and morphological descriptions recorded when sampling. The three most prevalent fungi (*Rhizopogon luteolus*, *Suillus bovinus* and *S. variegatus*) were found at all three invasion levels (Figure 2.5). These fungi are Pinaceae-specific. The most prevalent fungi forming mycorrhizas with birch were *Laccaria proxima* and *Thelephora terrestris*, these fungi along with *Lactarius rufus* were detected in both invaded heathland and woodland. All other fungi were only in woodland plots. Level of invasion had a highly significant effect on all richness estimators (Chao1 $F = 4.22_{2,15}, P < 0.05$; Chao2 $F = 4.40_{2,15}, P < 0.05$; Jack1 $F = 8.32_{2,15}, P < 0.01$; Jack2 $F = 7.47_{2,15}, P < 0.01$).

One pine seedling from invaded heathland from Cd was mycorrhizal with *Rhizopogon luteolus*, this is probably contamination within the bioassay as *R. luteolus* is Pinaceae-specific and there are not any pine trees within the vicinity of Cd, this sample has been removed from further analyses. There was no other contamination; none of the five surviving pine control bioassays became mycorrhizal and only one of the 30 pine bioassays from Cd became mycorrhizal with Pinaceae-specific fungi despite these fungi occurring in 86 pine bioassays from other sites. The identity of mycorrhizal fungi in twelve bioassays has not been resolved (one from Cd, three from Hw, three from Kw, one from Sw and four from Tw). No DNA sequence was obtained for the Sw sample but morphotype descriptions match a suilloid fungus, DNA sequences were obtained for one Hw sample and one Kw sample but there were no significant matches on GenBank. The other nine samples yielded poor DNA sequences and consequently no close matches were found on GenBank. The following analyses exclude these twelve bioassays.
Figure 2.5. Number of a) birch and b) pine bioassays from each level of invasion for each ectomycorrhizal fungus identified in 2006 at six sites. Black bars indicate woodland, grey is invaded heathland and open is uninvaded heathland. Note difference in scale on y-axes.
Ectomycorrhizal fungal accumulation curves for invasion level indicate that all mycorrhizal fungi in uninvaded heathland and nearly all mycorrhizal fungi in invaded heathland were detected (Figure 2.6); however not all woodland fungi may have been detected.

Estimated fungal richness indices also indicate that not all woodland mycorrhizal fungi were detected; 14 were detected but richness estimators predict the presence of between 17 and 22 different fungi (Table 2.5).

Figure 2.6. Ectomycorrhizal fungal accumulation curves for each level of invasion in bioassays in 2006 based on six sites (Cd, Gd, Hw, Kw, Sw and Tw).
Table 2.5. Ectomycorrhizal fungal richness and diversity at each level of invasion in bioassays in 2006 at six sites.

<table>
<thead>
<tr>
<th>Level of invasion</th>
<th>Number of fungi detected</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shannon ($H'$)</td>
<td>Simpson's reciprocal</td>
</tr>
<tr>
<td>Uninvaded</td>
<td>3</td>
<td>0.466</td>
<td>3.19</td>
</tr>
<tr>
<td>Invaded</td>
<td>6</td>
<td>0.672</td>
<td>4.52</td>
</tr>
<tr>
<td>Woodland</td>
<td>14</td>
<td>0.990</td>
<td>8.90</td>
</tr>
<tr>
<td>All</td>
<td>14</td>
<td>0.916</td>
<td>6.66</td>
</tr>
</tbody>
</table>

* Woodland species richness was estimated using the classic method rather than bias-corrected method because CV for abundance distribution was > 0.5, thus the highest value out of Chao1 and ACE was reported as recommended by A. Chao in EstimateS, Chao1 is reported (Colwell 2005).

The two most common fungi, *R. luteolus* and *S. bovinus* were both detected at Gd, Hw, Kw and Sw, *S. bovinus* was additionally detected at Tw (Figure 2.7). The site invaded by birch only (Cd) was dominated by *L. proxima* and *T. terrestris*, the prevalence of *L. proxima* at Cd means it is the fifth most common fungus despite only being detected at Cd. Gd, which is only invaded by pine, only had two Pinaceae-specific fungi (*R. luteolus* and *S. bovinus*) and all birch bioassays at Gd were non-mycorrhizal. Perhaps surprisingly, fungal diversity was higher on pine bioassays at Cd than on birch despite Cd being invaded only by birch trees; two fungi (*L. proxima* and *T. terrestris*) were detected on birch, whereas four were detected on pine bioassays (*Lactarius tabidus, Laccaria proxima T. terrestris* and *Tomentella subilicacia*). There was no significant difference in fungal richness estimators between sites.
Ectomycorrhizal fungal accumulation curves indicate that all fungi present at Gd and Sw were detected (Figure 2.8) along with most from Cd and Hw as these curves are close to saturation. Fungal accumulation curves for Kw and Tw are far from saturation indicating not all fungi present were detected.

The higher estimated richness than actual number of fungi detected also indicates that not all fungi present at Kw and Tw were detected (Table 2.6). Sw had the highest and Gd the lowest Shannon’s and Simpson’s reciprocal indices.
Figure 2.8. Ectomycorrhizal fungal accumulation curves for bioassays from six sites in 2006 based on all three levels of invasion (uninvaded heathland, invaded heathland and woodland).

Table 2.6. Ectomycorrhizal fungal richness and diversity at six sites where ectomycorrhizal fungi were detected in bioassays from three levels of invasion in 2006.

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of fungi detected</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shannon ($H'$)</td>
<td>Simpson’s reciprocal</td>
</tr>
<tr>
<td>Cd</td>
<td>4</td>
<td>0.462</td>
<td>2.61</td>
</tr>
<tr>
<td>Gd</td>
<td>2</td>
<td>0.300</td>
<td>2.08</td>
</tr>
<tr>
<td>Hw</td>
<td>6</td>
<td>0.682</td>
<td>4.56</td>
</tr>
<tr>
<td>Kw</td>
<td>7</td>
<td>0.699</td>
<td>4.42</td>
</tr>
<tr>
<td>Sw</td>
<td>6</td>
<td>0.763</td>
<td>6.75</td>
</tr>
<tr>
<td>Tw</td>
<td>6</td>
<td>0.628</td>
<td>3.89</td>
</tr>
</tbody>
</table>

* Value reported is ICE; Tw species richness was estimated using the classic method rather than bias-corrected method because CV for incidence distribution was > 0.5 thus the highest value out of Chao2 and ICE was reported, as recommended by A. Chao in EstimateS (Colwell 2005).
2.3.3. Bioassays in 2007

In 2007, 360 experimental and 10 control bioassays were set up using soil from unburnt sites, of which 359 experimental bioassays and all control bioassays yielded live seedlings at harvest. The level of invasion and site had a highly significant effect on the proportion of birch bioassays containing mycorrhizal seedlings (GLM with binomial errors; Invasion level, $\chi^2 = 65.3_{2,28}, P<0.001$; Site, $\chi^2 = 27.9_{5,30}, P < 0.001$, Figure 2.9a) but there was no interaction between site and level of invasion in the proportion of mycorrhizal birch bioassays. The proportion of mycorrhizal birch bioassays increased as the level of invasion increased; 2%, 24%, 57% in uninvaded heathland, invaded heathland and woodland, respectively (n = 1, 14, 34, respectively). There was no significant effect of site on the overall proportion of non-mycorrhizal pine bioassays but level of invasion had an effect on the overall proportion of non-mycorrhizal bioassays with 13%, 25% and 42% of bioassays containing mycorrhizal pine seedlings in uninvaded heathland, invaded heathland and woodland, respectively (n = 8, 15, 25, respectively, GLM with quasibinomial errors, $F = 3.11_{2,33}, P = 0.058$, Figure 2.9b). All ten control bioassays produced at least one live seedling at harvest and all were non-mycorrhizal. The *C. vulgaris* cuttings survived in 83% of the experimental bioassays, but only 20% of the control bioassays. The site and level of invasion had no effect on *Calluna* survival. Whether the bioassay contained birch or pine affected the survival of *Calluna* (GLM with quasibinomial errors, $F=8.67_{1,34}$, P<0.01) with *Calluna* surviving in 75% of pine bioassays and 90% of birch bioassays.

There were site-dependent differences in the proportion of birch and pine seedlings becoming mycorrhizal. Cavenham heath (Cd) is invaded solely by birch trees resulting in nine of the ten birch bioassays and one of the ten pine bioassays grown in woodland soil being mycorrhizal at harvest. The reverse effect occurred at Gd, which is invaded by pine trees only (Figure 2.9).

A total of 404 root samples were taken for DNA sequencing and 394 sequences obtained. Thirteen ectomycorrhizal fungi were identified belonging to eleven genera. The most prevalent fungi forming mycorrhizas in the bioassays overall were *Rhizopogon luteolus*, *Lactarius hepaticus*, *Laccaria proxima*, *Thelephora terrestris* and *Suillus variegatus* (Figure 2.10). *Laccaria proxima*,
Rhizopogon luteolus, Suillus bovinus and S. variegatus formed mycorrhizas on seedlings grown on soil from uninvaded heathland (Figure 2.10). The only mycorrhizal birch bioassay from uninvaded heath soil was colonised by Laccaria proxima. The identity of fungi forming mycorrhizas in two bioassays was not established, one birch from invaded heathland at Sw and one birch from woodland at Gd because there were no close matches on GenBank, these fungi have been removed from further analysis.
Figure 2.9. Number of a) birch and b) pine bioassays recorded as non-mycorrhizal (open bars) seedlings and mycorrhizal (black bars) at three levels of invasion at six sites in 2007.
For all levels of invasion the ectomycorrhizal fungal accumulation curves do not reach saturation although the woodland curve is steeper than the uninvaded and invaded heathland projections (Figure 2.11).
Figure 2.11. Ectomycorrhizal fungal accumulation curves for each level of invasion in bioassays in 2007 based on six sites (Cd, Gd, Hw, Kw, Sw and Tw).

Estimated richness indices indicate that between one and four fungi from each of uninvaded and invaded heathland were not detected, along with between four and eight fungi from within the woodland areas (Table 2.7). The level of invasion had a significant effect on each richness estimator calculated (Chao1 $F = 7.28_{2,15}$, $P < 0.01$; Chao2 $F = 7.32_{2,15}$, $P < 0.01$; Jack1 $F = 7.42_{2,10}$, $P < 0.05$; Jack2 $F = 4.50_{2,10}$, $P < 0.05$). Overall, mycorrhizal fungal diversity and estimated richness increases as the level of invasion increases from uninvaded heathland to woodland (Table 2.7). Uninvaded and invaded heathland had similar Simpson’s reciprocal indices but diversity was higher in the woodland.
Table 2.7. Ectomycorrhizal fungal richness and diversity in uninvaded and invaded heathland and neighbouring woodland detected on bioassay seedlings from six sites in 2007.

<table>
<thead>
<tr>
<th>Level of invasion</th>
<th>Number of fungi detected</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shannon ($H'$)</td>
<td>Simpson's reciprocal 1/$D$</td>
</tr>
<tr>
<td>Uninvaded</td>
<td>4</td>
<td>0.528</td>
<td>4.00</td>
</tr>
<tr>
<td>Invaded</td>
<td>6</td>
<td>0.660</td>
<td>4.40</td>
</tr>
<tr>
<td>Woodland</td>
<td>13</td>
<td>0.917</td>
<td>7.03</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>0.930</td>
<td>7.58</td>
</tr>
</tbody>
</table>

* Value reported is ACE; total richness was estimated using the classic method rather than bias-corrected method because CV for abundance distribution was > 0.5 thus the highest value out of Chao1 and ACE was reported, as recommended by A. Chao in EstimateS (Colwell 2005).

Half of the fungi identified in the bioassays were only detected at one study site (Figure 2.12), and yet, three of the most prevalent fungi were detected at three sites (Rhizopogon luteolus, Suillus variegatus) or four sites (Thelephora terrestris). Laccaria proxima was prevalent in Cd bioassays forming mycorrhizas on thirteen of the seventeen mycorrhizal birch bioassays but only colonised one of the pine bioassays at Cd. The proportion of mycorrhizal pine bioassays grown on uninvaded heathland soil from Kw was high compared to the other uninvaded heathland soil bioassays. The diversity of fungi in uninvaded heath at Kw was also relatively high, comprising three fungi (Rhizopogon luteolus, Suillus bovinus and S. variegatus). These fungi are all specific to Pinaceae and accordingly, all birch bioassays from the same soil cores were non-mycorrhizal. Site significantly affected two out of four species richness estimators calculated (Chao1 – no significant difference; Chao2 – no significant difference; Jack1 F = 3.64_{5,10}, P<0.05; Jack2 F = 3.44_{5,10}, P<0.05).
Ectomycorrhizal fungal accumulation curves are saturated for Cd, Gd and Tw but not for Hw, Kw and Sw (Figure 2.13). Cd and Gd are invaded by only birch (Cd) or pine (Gd) and they have the lowest diversity of ectomycorrhizal fungi.

The estimated fungal richness estimates agree with the ectomycorrhizal fungal accumulation analysis for Cd, Gd and Hw the number of fungi detected equals the estimated fungal richness (Table 2.8).
Figure 2.13. Ectomycorrhizal fungal accumulation curves for bioassays from six sites in 2007 based on three levels of invasion.

Table 2.8. Ectomycorrhizal fungal richness and diversity at each site where ectomycorrhizal fungi were detected in bioassays in 2007 based on three levels of invasion.

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of fungi detected</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shannon $(H')$</td>
<td>Simpson’s reciprocal $1/D$</td>
</tr>
<tr>
<td>Cd</td>
<td>2</td>
<td>0.281</td>
<td>1.90</td>
</tr>
<tr>
<td>Gd</td>
<td>2</td>
<td>0.254</td>
<td>1.77</td>
</tr>
<tr>
<td>Hw</td>
<td>3</td>
<td>0.261</td>
<td>1.53</td>
</tr>
<tr>
<td>Kw</td>
<td>5</td>
<td>0.586</td>
<td>3.88</td>
</tr>
<tr>
<td>Sw</td>
<td>6</td>
<td>0.689</td>
<td>5.25</td>
</tr>
<tr>
<td>Tw</td>
<td>6</td>
<td>0.740</td>
<td>6.58</td>
</tr>
</tbody>
</table>

* ACE and ICE are reported instead of Chao1 and Chao2, respectively; Hw estimated richness values were calculated using the classic method rather than bias-corrected method because CV for abundance and incidence distribution was > 0.5 then the highest value out of Chao1 and ACE, and Chao2 and ICE was reported, as recommended by A. Chao in EstimateS (Colwell 2005).
2.3.3.1 Mass of seedlings

For birch bioassays containing two seedlings site and mycorrhizal status had significant effects on seedling biomass (site F = 8.35,34, P < 0.001; mycorrhizal status F = 13.21,34, P < 0.001; Figure 2.14). In the bioassays from the four sites where mycorrhizal seedlings were present at harvest the biomass of mycorrhizal seedlings was always greater than that of non-mycorrhizal seedlings.

![Figure 2.14](image.png)

**Figure 2.14.** Average mass (g) per mycorrhizal seedling (closed bars) and non-mycorrhizal seedling (open bars) in 2007 for bioassays containing two birch seedlings. Number on bars indicates number of seedlings within each category.

For birch bioassays containing three seedlings, the site, level of invasion and mycorrhizal status had significant effects on seedling biomass (site F = 13.55,53, P < 0.001; level of invasion F = 9.52,53, P < 0.001; mycorrhizal status F = 5.51,53, P < 0.05; Figure 2.15). On four out of five occasions when mycorrhizal and non-mycorrhizal seedlings were present at the same level of invasion at the same site, the mycorrhizal seedlings were heavier than non-mycorrhizal seedlings.
Figure 2.15. Average mass (g) per mycorrhizal seedling (closed bars) and non-mycorrhizal seedling (open bars) in 2007 for bioassays containing three birch seedlings. Number on bars indicates number of seedlings within each category.

For pine bioassays, site and mycorrhizal status independently influenced biomass and there were multiple significant interactions (Table 2.9, Figure 2.16). Mycorrhizal seedlings in uninvaded heathland were heavier than non-mycorrhizal seedlings. In invaded heathland at three sites mycorrhizal seedlings were heavier than non-mycorrhizal seedlings and at three sites non-mycorrhizal seedlings were heavier. At five sites mycorrhizal pine seedlings in woodland bioassays were heavier than non-mycorrhizal pine seedlings. No fungal-specific effects on seedling biomass were detected for *Rhizopogon luteolus*, *S. variegatus*, *S. bovinus* or *L. hepaticus* on pine seedling biomass or for *Laccaria proxima* on birch seedling biomass.
Table 2.9. Significant results (F value, DF and P value) of a three-way ANOVA for effects of site, level of invasion and mycorrhizal status and their interactions on average pine seedling biomass in 2007 bioassays.

<table>
<thead>
<tr>
<th>Factor</th>
<th>F</th>
<th>DF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>2.6</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Mycorrhizal status</td>
<td>8.9</td>
<td>2</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Site : Level of invasion</td>
<td>2.4</td>
<td>10</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Site : Mycorrhizal status</td>
<td>4.3</td>
<td>5</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Site : Level of invasion : Mycorrhizal status</td>
<td>2.3</td>
<td>6</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Figure 2.16. Average mass (g) per mycorrhizal seedling (closed bars) and non-mycorrhizal seedling (open bars) in 2007 for bioassays containing two pine seedlings. Number on bars indicates number of seedlings within each category.

2.3.4 Temporal variation in ectomycorrhizal fungi

Direct comparisons between all three years are difficult as methodologies varied slightly with use of polyethene bags to decrease evaporation and cross-contamination in 2006 for birch and 2007 for birch and pine. This was the result of a process of optimising bioassay growth conditions which led to nearly 100% seedling survival and greatly increased Calluna survival in 2007. The use of bags however, appears to have had no effect on the fungi detected.
2.3.4.1 Birch bioassays

For birch bioassays, *Laccaria proxima* and *Thelephora terrestris* were the first and second most prevalent fungi in both 2006 and 2007. *Laccaria proxima* was detected in all invaded heathland and woodland plots at Cd in 2006 and 2007 and additionally in one uninvaded heathland plot at Cd and one woodland plot at Tw in 2007. *Thelephora terrestris* was in the same five plots at Cd, Hw, Sw and Tw in 2006 and 2007 with an additional plot in 2007 at Tw. The prevalence of some fungi changed slightly between years as would be expected from the lack of saturation in several accumulation curves; for instance, *Lactarius hepaticus* was the third most prevalent on birch bioassays in 2006 and 2007 being detected in 11 bioassays from the woodlands of Hw and Kw, but it was only detected three times in the same woodlands in 2006. *Tomentella sublilacina* was detected five times from two sites (Kw and Sw) in 2006 but not in 2007. Seven fungi were detected in one year but not in both.

2.3.4.2 Pine bioassays

Direct comparison between years for pine bioassays is more difficult due to the high levels of mortality and a relatively high number of samples with poor DNA sequences in 2005 and the use of bags in 2007 but not in 2005 or 2006. However, *Rhizopogon luteolus* was the most prevalent Pinaceae-specific fungus in all years, the most prevalent overall in 2006 and 2007, and the third most prevalent in 2005. The different methodologies appear to have little or no effect on the fungi detected; at Gd, *S. bovinus* and *R. luteolus* were the only fungi detected on pine in 2006 and 2007; but, the number of occurrences varied between years with 11 mycorrhizal bioassays in 2007 and 21 in 2006. No Pinaceae-specific fungi were detected at Hw, where pines are present near both woodland and invaded heathland plots in 2007 but they were prevalent in 2006 at this site. *Lactarius hepaticus*, *S. bovinus*, *S. variegatus* and *R. luteolus* were detected in pine bioassays at Kw in 2006 and 2007. In 2005, three of these four re-occurring fungi (*Lactarius hepaticus*, *S. variegatus* and *R. luteolus*) were detected along with a *Suillus* sample whose DNA sequence was too poor to attribute to a to either *S. bovinus*, *S. variegatus*. The same three fungi were detected on pine bioassays at Tw in 2006 and 2007 (*C. geophilum*, *S. bovinus* and *S. variegatus*) and in the case of *C. geophilum* in the same plot in both years. *S. variegatus* was far more widespread
in 2006 occurring in five out of six plots compared to just two in 2007. *Rhizopogon luteolus* was present at Sw in 2005, 2006 and 2007. *S. variegatus* and an unknown Atheliaceae sp. were present in both 2006 and 2007.

### 2.3.5 Spatial and temporal effects on ectomycorrhizal fungal communities

#### 2.3.5.1 Species Area Relationship (SAR)

The number of fungi detected in woodlands was positively associated with the area sampled (Figure 2.17) with the following relationship:

\[
\ln(\text{number of fungal taxa} + 1) = 1.13 + 0.055 \times \ln(\text{area}) \quad F = 192.1,160, \, P<0.01.
\]

![Figure 2.17](image.png)

*Figure 2.17. Relationship between number fungi detected in woodland bioassays in 2006 and 2007 at six sites (Cd, Gd, Hw, Kw, Sw and Tw) and area sampled (m²).*

#### 2.3.5.2 Similarity in ectomycorrhizal communities between sites

The similarity of ectomycorrhizal communities decreased as distance between sites increased for three similarity indices (Sørensen index, Morisita-Horn
index and Bray-Curtis index). Ectomycorrhizal communities are more similar at the same site between years than between different sites in the same year (Figure 2.18). Linear models predicted for each similarity index are as follows:

\[
\ln(\text{Sørensen index + 1}) = 0.409 - (0.0010 \times \text{distance})
\]
\[
F = 38.83_{1,64}, P<0.0001;
\]

\[
\ln (\text{Morisita-Horn index +1}) = 0.435 - (0.0015 \times \text{distance})
\]
\[
F = 51.32_{1,64}, P<0.0001;
\]

\[
\ln (\text{Bray-Curtis index +1}) = 0.362 - (0.0012 \times \text{distance})
\]
\[
F = 53.99_{1,64}, P<0.0001.
\]

**Figure 2.18.** Change in similarity indices (Sørensen, Morisita-Horn and Bray-Curtis) over distance for 2006 and 2007 bioassays across uninvaded heathland, invaded heathland and woodland at six sites.

Mantel tests confirmed a spatial relationship in ectomycorrhizal community composition \((r = 4.798, P < 0.0001)\) using data from 2006 and 2007. There was no effect of year on the spatial relationship. When a partial Mantel test was conducted to control for the effect of year, the spatial relationship was still significant \((r = 0.756, P < 0.001)\). The environmental conditions at sites (tree species invading and
whether the site was wet or dry) were also correlated with ectomycorrhizal community composition ($r = 3.157$, $P < 0.001$). A partial Mantel test on the relationship between distance and ectomycorrhizal community indicated the relationship between distance and community composition was still present when environmental conditions were controlled for ($r = 0.667$, $P < 0.001$).

Non-metric multidimensional scaling using presence and absence of ectomycorrhizal fungi data generally grouped together the same site between years (Cd, Gd, Kw and Tw; Figure 2.19) but sites did not group based on proximity, e.g. Gd and Sw did not cluster together. Hw and Sw grouped closer within 2006 than at the same site between years.

![Figure 2.19](image)

**Figure 2.19.** Non-metric multi-dimensional scaling of presence and absence of ectomycorrhizal fungi in bioassays at six sites (Cd, Gd, Hw, Kw, Sw and Tw) in 2006 (06) and 2007 (07) across uninvaded heathland, invaded heathland and woodland.
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When a NMDS analysis using abundance of ectomycorrhizal fungi is conducted (Figure 2.20) generally sites that clustered together with presence/absence data (Figure 2.19) still do so, Cd06 and Cd07 move closer and Gd06 and Gd07 move further apart. Hw06 and Sw06, which clustered together in presence/absence data analysis (Figure 2.19) move further apart in the abundance dataset analysis (Figure 2.20) and Sw06 moves slightly closer to Sw07. Hw06 and Hw07 are not close in either presence/absence data or abundance data analysis. The similarity in ectomycorrhizal community composition between sites on the basis of proximity is less evident in the NMDS (Figures 2.19 and 2.20) than in the linear models in Figure 2.18. Cd does however, tend to be isolated from other sites in the NMDS analysis as it is geographically, and the closest site to Cd both in the NMDS and geographically is Tw.
Figure 2.20. Non-metric multi-dimensional scaling of abundance of ectomycorrhizal fungi in bioassays at six sites (Cd, Gd, Hw, Kw, Sw and Tw) in 2006 (06) and 2007 (07) across uninvaded heathland, invaded heathland and woodland.

2.3.5.3 Effect of level of invasion on ectomycorrhizal community composition

In non-metric multidimensional scaling using presence and absence of ectomycorrhizal fungi data, the distance between 2006 and 2007 data is the same for each level of invasion (Figure 2.21). Invaded heathland community is nearly equidistant between uninvaded and woodland communities.
Figure 2.21. Non-metric multi-dimensional scaling of presence and absence of ectomycorrhizal fungi detected in bioassays at three levels of invasion across six sites (Cd, Gd, Hw, Kw, Sw and Tw); uninvaded heathland (U), invaded heathland (I) and woodland (W), in 2006 (06) and 2007 (07).

Taking into account abundance of each ectomycorrhizal fungus, the woodland habitats in 2006 and 2007 cluster very close together as does uninvaded heathland in 2006 and 2007 (Figure 2.22). Invaded heathland in 2006 clusters with uninvaded heathland however, rather than with invaded heathland in 2007; this pattern may be explained by specific fungi such as *Suillus bovinus*: in 2006 *Suillus bovinus* was present in high numbers, similar to that of uninvaded heathland in 2006 but was absent from invaded heathland in 2007.
Figure 2.22. Non-metric multi-dimensional scaling of abundance of ectomycorrhizal fungi detected in bioassays at three levels of invasion; unininvaded heathland (U), invaded heathland (I) and woodland (W), in 2006 (06) and 2007 (07) at six sites (Cd, Gd, Hw, Kw, Sw and Tw). In the lower right-hand corner W06 and W07 overlap and in the lower left-hand corner U06, U07 and I06 overlap.

2.3.6 Post-fire results

2.3.6.1 Effects of wildfire on site

In 2006, soil was collected for bioassays two months after a wildfire at Thursley Common. Most plots were relocated through the presence of burnt remains of flags, metal tags or powdered metal. Invaded heathland plots at Td2 were easiest to relocate as the metal pegs used to wind string round to create a grid in 2005 (see Chapter Three) had formed a white powder which was noticeable against the burnt black ground (Figure 2.23). The most difficult plot to relocate
was a woodland plot at Td1 where only the remains of one corner marker could be found, this plot was set up again from memory of its location (Figure 2.24).

**Figure 2.23.** Invaded heathland plot at Thursley (Td2) after fire. Yellow flags mark corners of the plot.

**Figure 2.24.** Woodland plot at Thurlsey (Td1) after fire. Yellow flag marks where the remains of an original corner flag were found.
2.3.6.2 Effect of fire on seedling survival

Bioassays conducted two months after the fire (2006) yielded slightly fewer live seedlings at harvest (98 bioassays) compared to before the fire (2005; 110 bioassays) and a year after the fire (2007; 118 bioassays); however, this difference was not significant and site, level of invasion and whether it was a birch or pine bioassay also had no effect on seedling survival (tested for with a quasibinomial-distribution GLM).

2.3.6.3 Post-fire bioassays in 2006

There was no detectable ectomycorrhizal inoculum post-fire in uninvaded or invaded heathland at Td1 but 44% (n = 7) of woodland soil bioassays yielded mycorrhizal seedlings (Figure 2.25). At Td2 post-fire ectomycorrhizal inoculum was present in invaded heathland and in the woodland (17% and 50% of bioassays yielded mycorrhizal seedlings, n = 4, 14, respectively, Figure 2.25). Level of invasion had a significant effect on inoculum for birch bioassays ($\chi^2 = 22.8_{2,9}$, $P < 0.01$) with more inoculum as the level of invasion increases (Figure 2.25). For pine, the level of invasion had no effect on inoculum but site had an effect with significantly more inoculum at Td2 than Td1 ($\chi^2 = 6.7_{1,10}$, $P < 0.01$, Figure 2.25). There was no significant difference in the amount of inoculum for birch or pine at Td1 or Td2 pre-fire (2005) versus post-fire (2006; tested for with GLM with binomial error distribution).
Fungal diversity was far higher at Td2 than Td1 with only two fungi (*Lactarius rufus* and *Thelephora terrestris*) detected at Td1 compared with eight at Td2 (Figure 2.26). *Lactarius rufus* was also the only fungus detected with both birch and pine bioassays; it was present in one pine bioassay and three birch bioassays.
2.3.6.4 Post-fire bioassays in 2007

In 2007, one year after the wildfire, ectomycorrhizal inoculum potential for both birch and pine bioassays was highest in the woodland and lowest in the uninvaded heathland (Figure 2.27). In 2007, at Td1 and Td2 combined, 33%, 20% and 78% of birch bioassays from uninvaded heathland, invaded heathland and woodland, respectively (n = 3, 5, 17, respectively), were mycorrhizal compared to 0%, 9% and 59% in 2006 (n = 0, 1, 13, respectively). Level of invasion had a significant effect on inoculum for birch bioassays ($\chi^2 = 12.6_{2,9}, P < 0.01$) with more inoculum as the level of invasion increases (Figure 2.27). There was a significant interaction between level of invasion and site on the level of inoculum for pine bioassays ($\chi^2 = 5.1_{3.7}, P < 0.05$) the amount of inoculum increased consistently as tree encroachment increased at Td1 whereas at Td2 there was more inoculum in...
invaded heathland than in the woodland (Figure 2.27). There was significantly more inoculum for birch at Td1 in 2007 than 2006 ($\chi^2 = 22.0_{2,10}, P < 0.05$). There was a significant interaction between year and invasion level for pine at Td2 with more inoculum in invaded heathland than woodland in 2007, but more inoculum in the woodland than in invaded heathland in 2006 ($\chi^2 = 5.4_{1,9}, P < 0.05$), there was no significant effect of year or site independently.

![Figure 2.27. Percentage of non-mycorrhizal (open bars) and mycorrhizal (closed bars) birch and pine bioassays from Thursley (Td1 and Td2) one year post-fire in 2007, number on bars indicate actual numbers.](image)

Two fungi were detected in uninvaded heathland at Td one year after the fire (*Laccaria proxima* and *Thelephora terrestris*, Figure 2.28). Over half of the fungi detected in 2007 at Td were present in invaded heathland and all were present in at least one woodland plot (Figure 2.28). Two fungi were detected with both birch and pine bioassays; *Laccaria proxima* was detected in two pine and eight birch bioassays, *Lactarius rufus* was detected in one pine and three birch bioassays.
Figure 2.28. Number of soil bioassays from each level of invasion for each ectomycorrhizal fungus identified at Thursley (Td) in 2007, one year after the fire. Black bars indicate woodland, grey is invaded heath and open is uninvaded heathland. Above the bar it is indicated whether the fungus was detected at Td1, Td2 or both (*). Letters on the bars indicate whether fungi were detected in birch (B), pine (P) or both birch and pine bioassays (BP).

2.3.6.5 Effect of fire on the ectomycorrhizal community

One year after fire at Td, in 2007, a different mycorrhizal community was present compared with that detected in 2006, immediately post-fire or pre-fire in 2005. *Amanita muscaria* and *Elaphomyces muricatus*, both detected in 2006 were not detected in 2007 although they were rare in 2006 occurring only once each. In addition, *Tomentella subilacina*, *Rhizopogon luteolus* and *Suillus variegatus* were detected in 2007 but were not present in 2006. The fungal community at Td1 was more diverse in 2007 than 2006; only two fungi were detected in 2006 (*Thelephora terrestris* and *Lactarius rufus*) and six in 2007 (Figure 2.26 and 2.28).

Immediately post-fire (2006), at Td1, all fungal richness indicators were lower than pre-fire (2005, Figure 2.29) but not significantly so. Differences in Jack1 and Jack2 estimators between 2005 and 2006 data were close to significance (Jack1 \( t = 2.60, P = 0.081 \); Jack2 \( t = 2.59, P = 0.089 \)). A year after the fire (2007)
ectomycorrhizal richness increased to levels slightly higher than pre-fire and significantly higher than immediately following the fire (for Chao1, Chao2, Jack1 and Jack2 $t = 4.33, 4.5, 5.88, \text{ and } 3.81$, respectively, for each test $P < 0.05$). Due to poor DNA sequence results from Td2 in 2005, I am not able to directly compare pre- and post-fire ectomycorrhizal richness at this site.

![Figure 2.29. Estimated ectomycorrhizal inoculum fungal richness (Chao1 - circles, Chao2 - squares, Jack1 - diamonds, and Jack2 - triangles) for Td1 in 2005 (pre-fire), 2006 and 2007 (post-fire) for uninvaded heathland (Uninvaded, open symbols), invaded heathland (Invaded, grey symbols) and woodland (Woodland, solid symbols).](image)

### 2.3.7 Cumulative results

The cumulative results reported below include all fungi detected at all sites over all years. The post-fire data from Td have been included because fires, both accidental and managed, are common on heathlands (in addition to the fire at Td there was an accidental fire at Gd on 20th May 2008) and therefore the post-fire ectomycorrhizal community will often be a component of the overall community on heathlands.

#### 2.3.7.1 Birch bioassays 2005-2007

A total of fifteen fungi was detected with birch bioassays and all were detected in woodland plots within at least one site (Figure 2.30). Over all bioassay
studies *Laccaria proxima* and *Thelephora terrestris* were the most prevalent fungi in birch bioassays and the only to occur in all three invasion levels and, along with *Lactarius rufus*, *Leccinum holopus* and *Paxillus involutus*, they were the only fungi to occur in invaded heathland. Over half of the fungi detected on birch were detected in multiple years; five fungi were detected every year of the study (*T. terrestris*, *Lactarius hepaticus*, *L. rufus*, *Tomentella sublilacina*, *Cenococcum geophilum* and *L. rufus*, Figure 2.30).

![Figure 2.30](image)

**Figure 2.30.** Number of birch bioassays from each level of invasion for each identified ectomycorrhizal fungus detected 2005-2007 across seven sites (Cd, Gd, Hw, Kw, Sw, Td and Tw). Black bars indicate woodland, grey is invaded heath and open is uninvaded heath. Numbers above the bars indicate the number of years each fungus was detected, letters on or above bars indicate whether the fungus was detected in birch and pine bioassays (BP) or just birch bioassays (B).

### 2.3.7.2 Pine bioassays 2005-2007

Twelve fungi were detected with pine bioassays and all were detected in the woodland within at least one site (Figure 2.31). Three suilloid fungi (*Rhizopogon luteolus*, *Suillus bovinus* and *Suillus variegatus*) were the most prevalent fungi and
the only to occur at all three invasion levels. All fungi were detected in multiple years and half of the fungi in pine bioassays were detected every year.

Figure 2.31. Number of pine bioassays from each level of invasion for each identified ectomycorrhizal fungus detected 2005-2007 across seven sites (Cd, Gd, Hw, Kw, Sw, Td and Tw). Black bars indicate woodland, grey is invaded heath and open is uninvaded heath. Numbers above the bars indicate the number of years each fungus was detected, letters on or above bars indicate whether the fungus was detected in pine and birch bioassays (PB) or just pine bioassays (P).

2.3.8 Intracellular colonisation

Ninety pine root samples were obtained from Cd, Gd, Hw, Kw, Sw and control bioassays, 95 birch root samples from Cd, Gd, Hw, Kw, Sw, Td1, Td2 and control bioassays and 67 *Calluna* hair root samples from Cd, Gd, Hw, Kw, Sw and control bioassays. No intracellular mycorrhizal structures were detected in any birch or pine roots. Nine pine and eight birch roots had dark-septate fungal endophytes visible on the root surface, 43 birch and 19 pine root samples had hyphae on them but they did not form an ectomycorrhizal sheath, four pine and 13 birch samples were ectomycorrhizal, seven birch and 44 pine roots had no fungal hyphae on their surface. Records were not taken for three birch samples and 17 pine samples. Of the 67 *Calluna* hair root samples, nine samples did not contain
erico mycorrhizal structures, seven samples had erico mycorrhizal structures but the staining was too faint to approximate how many cells were colonised, 19 had $\geq 50\%$ of cells colonised and the remainder had $< 50\%$ of cells colonised. The staining of one sample did not work.

2.4 Discussion

Overall, I found that ectomycorrhizal inoculum in lowland heathlands is limited particularly for birch; nearly all seedlings grown in soil from heathlands were non-mycorrhizal. There is an increase in mycorrhization and fungal diversity as the level of tree invasion into heathlands increases agreeing with the first hypothesis of this chapter; ectomycorrhizal inoculum and ectomycorrhizal fungal diversity will increase as the level of invasion increases from uninvaded heathland to woodland. Overall three dominant fungi were detected using pine bioassays ($\textup{Rhizopogon luteolus, Suillus bovinus}$ and $\textup{S. variegatus}$), and two using birch bioassays ($\textup{Laccaria proxima}$ and $\textup{Thelephora terrestris}$), in abundance graphs these taxa are followed by a tail of less frequent taxa present at fewer sites, a common pattern in ectomycorrhizal studies (Peay et al. 2008). Different sites differ in their ectomycorrhizal community composition and diversity; most of the fungi detected were detected at only one or two sites. The same abundant fungal taxa were present each year concurring with previous studies that have shown ectomycorrhizal community structure is generally stable between years at larger spatial scales (Izzo et al. 2005, Smith et al. 2007). Species within the $\textup{R. ericae}$ aggregate do not form ectomycorrhizas on birch or pine seedlings invading the lowland heathlands sampled. The same applied to arbuscular mycorrhizal fungi even though formation of arbuscular mycorrhizal structures has been reported previously in birch by Haigh (2001) and in pines ($\textup{P. muricata}$) by Horton et al. (1998).

2.4.1 Woodlands have abundant and diverse ectomycorrhizal inoculum

The proportion of mycorrhizal to non-mycorrhizal bioassays increased as the level of tree invasion increased from uninvaded heathland to woodland. Mycorrhizal fungal inoculum levels are higher in areas where host plants dominate and decrease as distance from host plants increases or as their dominance decreases.
Over twice as many fungi were detected in woodlands than in heathlands and fungal accumulation curves for the woodland did not saturate. Ectomycorrhizal diversity is often higher with increased proximity to trees or in established woodland compared with scrub (Horton et al. 1998, Dickie et al. 2002a, Cline et al. 2005, Ashkannejad & Horton 2006). There are several potential reasons for this higher diversity. Woodland soils contain a higher diversity of inoculum types (spores and mycorrhizas) compared to uninvaded heathland that only contains spores. Fungi differ in their abilities to form ectomycorrhizas from spores (Deacon et al. 1983, Fox 1983, 1986, Ishida et al. 2008) and as woodland soils contain alternative sources of inoculum it allows fungi that cannot form mycorrhizas from spores to form mycorrhizas from vegetative mycelia. In particular, woodlands contain common mycelial networks (CMNs) in which radiating ectomycorrhizal mycelia connect the root systems of trees and seedlings (Brownlee et al. 1983, Cline et al. 2005, Dickie & Reich 2005, see reviews by Simard et al. 2002 and Selosse et al. 2006). CMNs are the determining factor controlling seedling survival and establishment in primary invasion areas (Nara & Hogetsu 2004, Nara 2006a,b) and they can increase the chances of ectomycorrhizal invasion from forest edges into old fields (Dickie & Reich 2005), praries and barrens (Thiet & Boerner 2007). Woodlands also contain more microhabitats, which mycorrhizal fungi are known to be sensitive to (Iwanski & Rudawska 2007, Tedersoo et al. 2008), due to a more diverse plant community - rather than the virtual Calluna monoculture of uninvaded heathlands - increasing the number of potential niches through changes in soil composition. Woodlands can contain more mycophagus animals such as invertebrates and their predators (Lilleskov & Bruns 2005), small mammals (Johnson 1996) and deer (Ashkannejhad & Horton 2006). Spores eaten and transported by these animals are likely to remain in the woodland where there is more cover and provisions for animals. It may also be that as a stand ages fungal diversity increases because woodlands have contained trees for a longer period of time than invaded heathland allowing species to accumulate. Jumpponen et al. (2002) detected a higher fungal diversity on land which had been deglaciated for the longest period. Trees in woodlands tend to be older and larger than those in invaded heathland and an increase in tree age size can also increase ectomycorrhizal fungal diversity (Visser 1995, Nara et al. 2003b).
2.4.2 Ectomycorrhizal inoculum is rare and not diverse in lowland heathland

Mycorrhizal inoculum was often absent from lowland heathland and when present its abundance and diversity was low. In uninvaded heathland, the only sources of ectomycorrhizal inoculum are wind- or animal-dispersed spores from fruiting bodies in nearby wooded areas whereas in the woodland itself sources of vegetative inoculum are also present. Five fungi were detected in uninvaded heathland; three Pinaceae-specific, Rhizopogon luteolus, Suillus bovinus and S. variegatus, and two that are able to form mycorrhizas with both birch and pine, Laccaria proxima and Thelephora terrestris. Suillus and Rhizopogon are frequently reported as the main genera detected forming ectomycorrhizas on pine seedlings growing in uninvaded areas, scrub habitat, and areas with few ectomycorrhizal competitors (Borchers & Perry 1989, Horton et al. 1998, Read 1998, Ashkannejad & Horton 2006). They can be prevalent in deer faeces and survive desiccation, thus suggesting a method of dispersal and potential for formation of a spore bank. Consequently, these two closely related fungi are uniquely adapted for dispersal and survival in isolated areas (Ashkannejad & Horton 2006). Their spore survival and ability to form a spore bank unlike most ectomycorrhizal fungi (Ishida et al. 2008) may be key to their prevalence in uninvaded heathlands. Spores of Rhizopogon can remain viable for at least four years in the field, with progressively decreasing dormancy (Bruns et al. 2009). Suillus bovinus may be particularly important to invading plants as it has been reported to have more proteolytic activity than other mycorrhizal species (Bending & Read 1995, Read 1989). This could provide seedlings with a competitive advantage, particularly in characteristically nutrient-poor heathlands. As well as the potentially deer-dispersed suillloid fungi, ectomycorrhizal inoculum in uninvaded areas can be wind-dispersed (Allen 1987). Ectomycorrhizal fungal spores are relatively small and well-adapted to dispersal by wind compared to arbuscular mycorrhizal spores. Laccaria proxima is wind-dispersed (Horton & van der Heijden 2008) and Laccaria has been reported as one of the first mycorrhizal fungi in primary invasion habitats (Nara et al. 2003a). Thelephora terrestris, a wind-dispersed fungus, is known to frequently form mycorrhizas from spores and it is commonly found in field saplings and as a contaminant in forestry nurseries,
glasshouses and growth chambers (Colpaert 1999, Menkis et al. 2005); however, the absence of mycorrhizas in control bioassays and the lack of *Thelephora* mycorrhizas in most uninvaded heathland bioassays implies the presence of *T. terrestris* in my study is not due to contamination. A reason for the lack of ectomycorrhizal fungi in heathland may be due to a poorer competitive ability of ectomycorrhizal fungi in the nutrient poor heathland which the ericoid mycorrhizal fungi are adapted to (Smith & Read 2008). The lack of ectomycorrhizal inoculum may perhaps be exacerbated by the reported allelopathic effects of *C. vulgaris* root secretions that can inhibit ectomycorrhizas (Robinson 1972). Ericoid mycorrhizas are also known to suppress arbuscular mycorrhizas via unknown mechanisms (Genney et al. 2000) however allelopathy could be one cause.

### 2.4.3 Ectomycorrhizal inoculum differs across lowland heathlands

Similarity in ectomycorrhizal community composition increases as proximity between sites increases and sites have a more similar ectomycorrhizal community between years compared to sites nearby within the same year. This indicates a greater influence of space rather than time. Variation in the fungal taxa detected at different sites, may be partly due to different tree species at different sites; Gd contained only Pinaceae-specific fungi and is being invaded only by pine. Differences in ectomycorrhizal community composition between sites may be due to different microhabitats at different sites, such as a pine needle litter layer over the soil where *Calluna* is not present at Tw versus a birch leaf litter layer at Cd, or to different geographic ranges of ectomycorrhizal fungal species within England.

The geographic distributions of fungi forming ectomycorrhizas clearly merit investigation and mapping. *Hebeloma* and *Inocybe* are frequently reported taxa in primary invasion habitats (Jumpponen et al. 2002, Nara et al. 2003a,b, Ashkannejad & Horton 2006) and on birch (Fleming 1984, Watling 1984, Atkinson 1992). Neither *Hebeloma* nor *Inocybe* were however, detected in this study despite *Inocybe lacera*, a common fungus in the Bush Estate studies (Fleming 1984), being a common and widespread species fruiting in heathlands in the UK (Legon & Henrici 2005). The Bush Estate birch ectomycorrhizal succession took place in the ecologically unrealistic conditions of an agricultural soil (Molina et al. 1992) so that the rare studies that were carried out in native forest settings contradicted it.
(Fleming et al. 1986, unpublished data mentioned in Deacon & Fleming 1992 and Fleming 1983); therefore, the presence of different taxa is not entirely unexpected. The *Lactarius* and *Leccinum* fungi detected in this study are also different to those reported in some Bush Estate studies (Fleming 1984, 1985). In particular, *Lactarius pubescens* was common at the Bush Estate was not detected in my study; nonetheless, *L. pubescens* fruitbodies are known to occur on neutral or calcareous soil, which can explain its absence in my characteristically acidic heathland sites (Legon & Henrici 2005). Despite the lack of some expected fungi, I detected an additional seven fungi to add to the list of fungi that form mycorrhizas with birch in Britain complied by Atkinson (1992) including a new genus (*Elaphomyces*). A study on ectomycorrhizas of mature pine at a site in Scotland found only seven species (Genney et al. 2006), three of which were found in this study (*Suillus variegatus*, *Lactarius rufus* and *Cenococcum geophilum*). The other four were in different genera and families to the fungi in my study.

### 2.4.4 What controls a heathland's ectomycorrhizal richness?

Ectomycorrhizal richness can vary greatly even at local scales (Horton & Bruns 2001). In this study richness estimates of between 16 and 22 fungi were predicted in 2006 and 2007. The estimated mycorrhizal fungal richness for individual plant species in a primary habitat was found to be between 21 and 28 by Nara (2006b). Scots’ pine stands have been reported to contain a similar diversity to that detected in this study (Jonsson et al. 1999) and pine seedlings in woodlands have an estimated fungal richness of between 4 and 12 fungi depending upon microhabitat (Iwanski & Rudawska 2007). Relatively low fungal richness in this study may be due to the island effect, where fungal richness increases as the size of the “island” increases (Peay et al. 2007); many of the woodlands sampled in my study were isolated from large woodlands.

Fungal diversity was higher overall than at any individual site due to a positive species-area relationship (Newton & Haigh 1998). In species-area relationships the slope of the line is commonly referred to as the z-value. The z-value in my study (*z* = 0.05) is lower than that found by Peay *et al.* (2007, *z* = 0.2) for ectomycorrhizal fungi in tree islands of various sizes. Very few studies have reported z-values for microorganisms, but of those which have, values are generally less than 0.1 and more rarely similar to the higher values reported for
macroorganisms (0.1 < z < 0.3, Green & Bohannan 2006). Two possible factors causing the discrepancy in z-value between my study and Peay et al. (2007) are:

1) My study used bioassays which can reduce fungal richness because a) seedlings are used and b) there is the potential for selectivity. Peay et al. (2007) used mature tree roots which have a higher fungal richness as mycorrhizal fungal richness increases as the size of the host increases (Nara et al. 2003b) and Peay et al. (2007) combined data from a root tip survey and a sporocarp survey; due to a lack of correspondence between fungi present on root tips and those present as sporocarps using both survey methods may result in a greater number of fungi being detected (Taylor & Alexander 1990, Gardes & Bruns 1996, Jonsson et al. 1999, Horton & Bruns 2001, Taylor 2002, Nara et al. 2003b).

2) My z-value is based on number of fungi detected per soil sample, whereas Peay et al. (2007) used estimated species richness per plot. I was unable to estimate fungal richness as individual soil cores were used as the smallest area unit. If fungal richness estimates had been used I would expect my z-value to be slightly higher but not as high as in Peay et al. (2007) as fungal accumulation curves saturated for several sites in my study.

It appears however, that tree species diversity was more important than the size of woodland surveyed; Cd and Gd (each invaded by just one tree type) had lower fungal richness than other sites in which woodlands formed islands such as Sw and Hw. It is difficult to compare Simpson and Shannon indexes to other studies due to differences in sampling; I have recorded the presence of a single fungus in a single bioassay as an individual whereas some studies record each mycorrhiza as an individual despite the fact that several roots can be colonised by the same fungal genet (Taylor 2002, Lian et al. 2006). The eveness (a component of species diversity which takes into account the abundance of individuals) of ectomycorrhizal fungi is rarely known because individuals (fungal genets) are not typically identified in mycorrhizal community studies (Bruns 1995). The diversity indices however, provide useful comparisons between the level of invasion and fungal richness estimates can be tentatively compared with other studies.

Soil samples were only taken between May and October leading to the possibility that intra-annual variation will be missed and some fungi may not be detected. Temporal partitioning between seasons has however, been reported for a minority of fungi in one study (Koide et al. 2007) and did not occur in another
(Smith et al. 2007). In addition, there was little inter-annual variation; generally individual sites and levels of invasion clustered between years indicating that ectomycorrhizal community composition is stable temporally (Izzo et al. 2005, Smith et al. 2007).

### 2.4.5 Fire affects ectomycorrhizas

Uncontrolled fires on heathlands such as the one at Thursley in 2006 reach higher temperatures than controlled fires used as a management tool and they have been demonstrated to increase the number of pine seedlings present by six to twelve times above that present after managed burns or no burning (Barker et al. 2004). This increased number of seedlings in addition to the continued presence of mycorrhizal fungi at similar levels of inoculum potential, may allow the increased invasion of pine trees following wildfire causing concern for heathland management.

Ectomycorrhizal community composition changes (Stendell et al. 1999) and stability (Horton et al. 1998) post-fire have been reported previously. I detected *Tomentella* and suillloid fungi one year post-fire but not pre-fire or immediately post-fire at Td1; these are amongst the most prevalent fungi detected post-fire in some coastal pine forest areas (Horton et al. 1998, Baar et al. 1999). Fungi that colonise seedlings via mycelia were not detected post-fire at Thursley despite their presence pre-fire (e.g. *Leccinum holopus* and *Paxillus involutus*). Wind-dispersed fungi such as *T. terrestris* and *Laccaria proxima* were however, detected post-fire. Despite the above mentioned changes in the ectomycorrhizal community I detected stability in part of the ectomycorrhizal community pre- and post-fire with ectomycorrhizal richness returning to pre-fire levels one year after the fire. *Lactarius rufus* was detected in four bioassays in the same woodland plot at Td1 in 2005, 2006 and 2007, it was also the only ectomycorrhizal fungus I detected one year post-fire on apparently dead mature tree roots in the same plot (full study not reported); however, the viability of this fungus on mature tree roots was not established. *Cenococcum geophilum* was detected both pre and post-fire. *Cenococcum geophilum* sclerotia have been reported to be more prevalent in woodlands that were burnt than in non-burnt woodlands (Miller et al. 1994) despite *C. geophilum* colonising fewer seedlings with heat treatment than without (Izzo et al. 2006). Changes to the ectomycorrhizal community post-fire gives some support
to the third hypothesis in this chapter; the ectomycorrhizal fungal community will be different pre- to post-fire.

2.4.6 Mycorrhization affects tree seedling biomass

Generally mycorrhizal seedlings are significantly heavier than non-mycorrhizal seedlings; this finding concurs with results presented in Chapter Three and with the meta-analysis of 36 publications by Karst et al. (2008) which found that total biomass is greater when seedlings are mycorrhizal and the fourth hypothesis of this chapter. The biomass of seedlings varied between sites and sometimes between levels of invasion, probably due to variation in nutrient levels. Fungal species-specific effects on mass have been reported in other studies (Baxter & Dighton 2001, Jonsson et al. 2001, Nara 2006a) yet I detected none in this bioassay study with birch or pine among five fungi.

2.4.7 Are bioassays selective?

One caveat of using bioassays is that taxa which specialise in forming mycorrhizas on established trees such as Russulaceae (Lactarius and Russula; Termorshuizen 1991, Ryan & Alexander 1992, Nara et al. 2003a, Twieg et al. 2007) and Amanita (Fox 1986) may not form mycorrhizas on the seedlings used in bioassays due to either host age preference or their inability to form mycorrhizas from spores which are presumed to form the majority of the inoculum in a bioassay. The presence of certain fungal taxa in birch seedling bioassays indicates this study was not completely selective; Amanita sp. have been reported as not developing mycorrhizas on seedlings in bioassay conditions (Deacon et al. 1983, Taylor & Bruns 1999) but formed mycorrhizas, although rarely, in my study. Russulaceae were infrequent in my study but not excluded from bioassays; particular exceptions are Lactarius hepaticus and L. rufus which were the third and fourth most common fungi detected in birch bioassays. Some Russulaceae can maintain mycorrhizas on seedlings in primary successional habitats when artificially inoculated (Nara 2006a) but do not occur naturally until vegetation patches containing plant hosts are approximately 5m² (Nara 2003a). Leccinum is not a common primary colonising fungus (Nara et al. 2003a) and it has been reported not to form mycorrhizas on birch under glasshouse conditions (Deacon et al. 1983), yet it was detected in six bioassays in this study despite its dependence
upon connections to an established plant (Fleming 1984). Newton & Piggott (1991) and Newton (1991) identified *Paxillus involutus* as the most common species on birch seedlings in woodland soil of low nutrient availability, however in this study it was infrequent. The rarity of *Leccinum* and *Paxillus* in bioassays may be due to their reliance on rhizomorphs (root-like aggregations of hyphae), rather than spores, to colonise new roots (Newton 1992). The presence of these fungi, presumed to colonise vegetatively, indicates the presence of at least some viable ectomycorrhizas and/or mycelia, as well as spores, in the bioassays. The presence of these fungi also goes against the second hypothesis within this chapter; seedlings will be colonised by pioneer fungi. Although pioneer fungi such as Thelephora and Suillus were prevalent.

This study indicates that ectomycorrhizal inoculum is limited in lowland heathlands and only a few fungi form mycorrhizas on seedlings in uninvaded heathland. It begins to chart the geographic distribution of ectomycorrhizal fungi and provides further evidence that mycorrhizal fungi do not concur with the Baas-Becking (1934) view of microbial ecology that “everything is everywhere but the environment selects” (Hawksworth & Mueller 2005, Peay *et al.* 2007).
Chapter Three - *In situ* mycorrhizas on naturally-occurring seedlings in heathlands and woodlands and on mature tree roots in woodlands

3.1 *Introduction*

So far within this thesis I have reported on the *ex situ* studies I have conducted to determine the distribution and diversity of ectomycorrhizal fungi on lowland heathlands; in this chapter I describe the distribution and diversity of ectomycorrhizal fungi *in situ* on the roots of naturally established seedlings and mature trees.

To date, there is limited information on the potentially crucial role of mycorrhizal fungi in the establishment of seedlings (Horton & van der Heijden 2008). For many habitats, including heathlands, we lack the most basic information concerning early symbiotic events such as the presence and diversity of ectomycorrhizal fungi. There are notable exceptions in the case of primary invasions (Gardes & Bruns 1996, Baar *et al*. 1999, Grogan *et al*. 2000, Bruns *et al*. 2002, Jumpponen 2003, Nara *et al*. 2003a,b, Nara & Hogetsu 2004, Ashkannejhad & Horton 2006, Nara 2006a,b, Kennedy *et al*. 2007, Nara 2008). The primary successional habitats that have been the focus of the above mentioned studies (see section 2.1 for further details) however, bear little similarity to lowland heathlands both in terms of vegetation and geographic proximity. This raises the possibility that the mycorrhizal fungi involved and the dependence of seedlings upon mycorrhizal fungi may differ in the lowland heathlands of England compared to the habitats considered so far.

The analysis of naturally occurring seedlings will determine whether seedlings are dominated by pioneer fungi, whether there is inoculum in heathland and may contribute to testing a hypothesis originally proposed by Miles & Kinnaird (1979): lack of ectomycorrhizal fungi slows tree invasion of heathlands. Mycorrhizal seedlings may be at a competitive advantage due to the benefits that mycorrhization brings to plants such as increased nutrients (Hobbie & Hobbie 2006) and water (Marjanovic *et al*. 2005), and protection from soil pathogens (Marx 1973, Newsham *et al*. 1995) potentially resulting in the higher biomass of
mycorrhizal compared to non-mycorrhizal seedlings (mainly in glasshouse settings: Karst et al. 2008 and see Chapter Two). On the other hand, it has also been reported that early mycorrhization can place a cost on seedlings (Jones & Smith 2004). So far, studies on the cost or benefit of the mycorrhizal symbiosis to plants have involved limited plant-fungal species combinations and more work is needed to evaluate the energy demand or benefits of mycorrhizal fungi towards seedlings under field conditions (Horton & van der Heijden 2008). To address this lack of knowledge, I report in this chapter, the survival and biomass of mycorrhizal and non-mycorrhizal naturally-occurring seedlings and identify the mycorrhizal fungi present on naturally-occurring seedlings.

There is limited information on the diversity and distribution of mycorrhizal fungi in natural environments in the UK (Anon 2009). For lowland heathlands it is not known which ectomycorrhizal fungi are present in neighbouring woodlands to form a potential source of inoculum for invading seedlings. The Bush Estate studies in Scotland, the greatest array of studies of any mycorrhizal fungal community used a young Betula pendula plantation as a model system (Ford et al. 1980, Mason et al. 1982, Deacon et al. 1983, Fleming 1983, Fox 1983, Last et al. 1983, Mason et al. 1983, Dighton & Mason 1984, Fleming et al. 1984, Fleming 1984, Last et al. 1984a,b, Mason et al. 1984, Fleming 1985, Fleming et al. 1986, Last et al. 1987, Gibson & Deacon 1988, Gibson et al. 1988, Mason et al. 1988, Deacon & Fleming 1992) but as described previously (see sections 1.2 and 2.1) has major caveats. My study also provides an opportunity to re-evaluate ectomycorrhizal species lists for the UK. Atkinson (1992) listed the mycorrhizal species associated with birch in Britain, drawing on data based on sporocarp surveys. For pine, no comprehensive survey has been completed within the UK equivalent to the Bush Estate studies causing a gap in the knowledge of the fungi which associate with pine in the UK. Genney et al. (2006) conducted a molecular based study in one pine forest in Scotland yet this study focused on microsite, local distribution rather than biome diversity. In this study, the fungal DNA of mycorrhizas was sequenced to identify fungi, to provide the first survey of ectomycorrhizal diversity on birch and pine trees on lowland heathlands at a regional scale.

In addition to advancing knowledge on the role of mycorrhizas in seedling establishment and on ectomycorrhizal fungal diversity, a primary reason for
conducting in situ studies is that previous studies have indicated a potential for mycorrhizal communities to differ between glasshouse-grown and field-grown settings. Newton (1991) found that pot-grown and field-grown birch seedlings in soil from the same site differed in the presence and abundance of many ectomycorrhizal morphotypes. Similarly, Taylor & Bruns (1999) reported a different ectomycorrhizal community on laboratory-grown seedlings than on mature tree roots. Naturally-occurring, “primary” invading seedlings are however, generally not available for sampling in uninvaded heathland and they are sparse in invaded heathland and woodland; therefore, bioassays were necessary to establish the diversity and spatial heterogeneity of inoculum across all invasion levels and sites (see Chapter Two).

Based on the cumulative results of the Bush Estate studies, Newton (1992) and Bowen (1994, and references therein), I would predict that relatively fast-growing and putatively spore-dispersed ectomycorrhizal fungi (pioneer or “r-selected”) such as Hebeloma, Laccaria, Paxillus and Thelephora should dominate birch seedlings. Results from Chapter Two however, suggest that Hebeloma may be lacking on lowland heathlands, Paxillus is infrequent, and the dominant genera on pine seedlings are Suillus and Rhizopogon. These early fungal colonists should be subsequently replaced and therefore I expect the relatively slow-growing ectomycorrhizal fungi that are reliant on vegetative dispersal (“K-selected”), such as Lactarius, Leccinum, and Russula to dominate mature tree roots (Taylor & Bruns 1999, Nara et al. 2003a, Twieg et al. 2007). My survey also provides the opportunity to determine whether Rhizoscyphus ericae forms ectomycorrhizas with pine or birch trees in nature as proposed by Vrålstad et al. (2000) based on results from studies in vitro.

The study reported in this chapter describes the occurrence and diversity of ectomycorrhizal fungi on naturally-occurring seedlings and on mature trees in situ on lowland heathlands. Furthermore, I examine the effect of mycorrhization on seedling survival and mass both spatially and temporally and I provide one of the first molecular-based surveys of the diversity of ectomycorrhizal fungi associated with trees in the UK. I hypothesise that i) a smaller proportion of seedlings in heathland and away from trees will be mycorrhizal compared to those in woodland or near trees, ii) mycorrhizal seedlings will be heavier than non-mycorrhizal seedlings, iii) pioneer fungi will be the dominant fungi on ectomycorrhizal
seedlings and “K-selected” fungi will be the dominant fungi on ectomycorrhizal mature tree roots.

3.2 Methods

3.2.1 Sites

Five lowland heathlands in England (Hw, Kw, Sw, Td, Tw, see Table 1.1 for details) were surveyed for naturally-occurring seedlings and six lowland heathlands (Cd, Gd, Hw, Kw, Sw and Tw) for the presence of mycorrhizal fungi on mature tree roots (Table 3.1).

Table 3.1. Dates of sampling for seven lowland heathland sites. Abbr. = Abbreviated name. N/A indicates where sampling did not take place.

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Date of naturally-occurring seedling sampling</th>
<th>Date of mature tree root sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>N/A.</td>
<td>18/6/07</td>
</tr>
<tr>
<td>Gd</td>
<td>N/A.</td>
<td>29/6/07</td>
</tr>
<tr>
<td>Hw</td>
<td>13/6/05</td>
<td>14/6/07</td>
</tr>
<tr>
<td>Kw</td>
<td>24/5/05</td>
<td>29/5/07</td>
</tr>
<tr>
<td>Sw</td>
<td>14/7/05</td>
<td>26/6/07</td>
</tr>
<tr>
<td>Td</td>
<td>various *</td>
<td>N/A.</td>
</tr>
<tr>
<td>Tw</td>
<td>various *</td>
<td>9/7/07</td>
</tr>
</tbody>
</table>

* Naturally-occurring seedlings were collected from Td and Tw at various dates from May 2005 to October 2006, and July 2005 to April 2007, respectively.

3.2.2 Naturally-occurring seedling sampling

3.2.2.1 Sampling within grids at Thursley

Six grids (0.75m$^2$ divided into twenty-five 0.15m$^2$ squares) were set up, three in invaded heathland and three in neighbouring woodland (Figure 3.1) at Td2 (see Chapter Two). Grids were set up in areas containing high densities of naturally-occurring birch seedlings at the two-cotyledon stage on the date of my first site visit (18$^{th}$ May 2005).
Figure 3.1. Examples of grids in a) woodland and b) invaded heathland. Flags indicate squares containing birch seedlings.

Every ten days, the number of seedlings within the same two squares within each grid was recorded. An additional square within each grid was removed and taken to the laboratory. The seedlings from this soil block were manually extracted from the soil and the dense ericoid root mass using tweezers under a dissecting microscope and classified as 1) mycorrhizal or non-mycorrhizal, and 2) dead or alive. Grids were sampled five times between 18\textsuperscript{th} May and 27\textsuperscript{th} June 2005 by which date all seedlings had been removed from the grids or had died.

On several dates, representative samples of ectomycorrhizas were removed, stored in CTAB buffer and frozen at -20\textdegree C or -80\textdegree C until extraction. DNA was extracted using the method stated in Gardes & Bruns (1993) with modifications (see Chapter Two for full method details). Briefly, after extraction with chloroform, 600\mu l of 6M NaI and 10\mu l of glassmilk (Qbiogene, Cambridge, UK) were added. This was mixed at room temperature for 5 minutes and centrifuged for 2 minutes. The supernatant was removed and the pellet washed three times in NEWWash (Qbiogene). The pellet was then dried, re-suspended in 45\mu l of TE buffer and stored at -20\textdegree C until use. All seedlings were freeze-dried and weighed.

3.2.2.2 Sampling outside of grids at Thursley

From the third grid sampling visit (6\textsuperscript{th} June 2005) onwards, birch and occasionally pine seedlings outside of the sampling grids, but within the Td2 area, in invaded and unininvaded heathland (areas containing only small seedlings, less than two years old, with no saplings or large seedlings) were sampled.
opportunistically 11 times (last sampling date 17th October 2005). An average of 30 seedlings were sampled in each visit with a minimum of ten on the last visit because no other seedlings could be found. I recorded the distance from the seedling to the nearest tree/sapling. Seedlings were classified according to their distance from the nearest tree/sapling. Seedlings closer to a tree than the height of that tree were classified as being “near to trees”. Seedlings further away from a tree than the height of that tree were classified as being “away from trees”. Note that seedlings classified as “away from trees” were often two or three times further away from a sapling or tree than the sapling or tree’s height.

For sampling, seedlings and surrounding soil were cut out with a soil knife. The seedlings and entire root systems were then carefully and thoroughly extricated from the dense mass of ericoid hair roots under a dissecting microscope using tweezers. All seedlings were recorded as mycorrhizal or non-mycorrhizal. On several dates, potentially mycorrhizal roots were sampled and stored in CTAB until extraction. DNA extractions followed the same method as stated in section 3.2.2.1. All seedlings were freeze-dried and weighed.

3.2.2.3 Additional tree seedling sampling

Birch and pine seedlings were sampled opportunistically from five lowland heathlands (Hw, Kw, Sw, Td and Tw) between 2005 and 2007. For each seedling the level of tree invasion the seedling was found in was recorded. Some seedlings are reported as being sampled from uninvaded heathland; this may appear illogical but means that other than the seedlings sampled being present there were no other seedlings or saplings in the area. Seedlings and potentially mycorrhizal roots were analysed as described in section 3.2.2.1. Seedlings were freeze-dried and weighed on some occasions.

3.2.3 Establishing date of germination

During the winter/spring of 2006, surface soil samples from Thursley (Td2) were obtained once a month (between January and May) to determine a date when birch seedlings first germinated. Blocks of soil approximately 15cm² containing high numbers of birch seeds were taken from the woodland grids used in the spring/summer 2005 survey or nearby areas. Soil blocks were examined within 24
hours of collection under a dissecting microscope for evidence of germinating seeds.

### 3.2.4 Mature tree root sampling

In summer 2007, soil cores were removed from the woodland plots established for the bioassay experiment (Chapter Two) to examine *in situ* ectomycorrhizas. The plots were sampled between 29th May and 9th July, 2007 (Table 3.1). From within each plot, five soil cores (approximately 2.5cm diameter x 20cm depth) were removed from arbitrary positions. The depth of the soil core allows for the maximum possible number of ectomycorrhizal roots to be obtained; typically 90% of mycorrhizas occur in the top 10cm of soil (Dahlberg 2001). The soil corer was cleaned with household bleach between sampling each plot. Soil samples were stored in sealed plastic bags at 4°C until use within one week. The contents of these soil samples were individually washed through a series of sieves of decreasing pore size and sieves of at least 500 µm mesh size were viewed under a dissecting microscope. The DNA of one root per morphotype per soil core was extracted immediately using Extract-N-Amp (Sigma-Aldrich, St Louis, MO, USA); each root was placed in 10µl of extraction solution in a 96-well plate, heated to 95°C for 10 minutes and then allowed to cool to room temperature. Finally, 10µl of dilution solution were added and mixed. Any remaining samples of each morphotype were stored in deionised water at -80°C and lyophilised if needed. The roots of birch and pine were not distinguished between when sampling from mixed woodlands.

### 3.2.5 Fungal identification

Polymerase chain reaction amplification of the internal transcribed spacer region was conducted using fungal-specific primer ITS1F (Gardes & Bruns 1993) and generic-primer ITS4 (White *et al.* 1990). An aliquot of 2µl of extracted DNA was combined with 8µl of AmpliTag Gold reaction mix (Applied Biosystems, Foster City, California) or Extract-N-Amp amplification solution depending upon DNA extraction method. Amplifications were performed with an initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 53°C for 55 seconds and 72°C for 50 seconds, with a final extension of 72°C for 7 minutes. PCR products were visualised on a 2% agarose gel with ethidium
bromide. For amplifications conducted using Amplitaq Gold reaction mix if the gel indicated PCR products had low yield or there was minimal contamination (i.e. a stronger band and weaker band) the extraction was repeated using 8µl of PicoMaxx reaction mix (Stratagene, Cedar Creek, TX, USA) or JumpStart (Sigma-Aldrich), respectively. If after amplification with PicoMaxx or Extract-N-Amp there was still a low yield, a nested PCR of the sample was performed using ITS1 and ITS4 from a 1:100 H2O dilution including the original negative control. If amplification of a sample using Extract-N-Amp was unsuccessful the DNA of a replicate freeze-dried root was extracted using Extract-N-Amp and/or CTAB and the PCR repeated with the new sample. After amplification, the PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA) or QIAquick Multiwell PCR purification protocol (Qiagen, Crawley, UK) and cycle sequenced using BigDye v3.1 (Applied Biosystems). The cycle sequencing products were electrophoresed using an ABI3730 Genetic Analyzer (Applied Biosystems). The DNA sequences were edited in Sequence Navigator (Applied Biosystems) or Sequencher (GeneCodes). Preliminary fungal identification was achieved by conducting a BLASTn search on GenBank (www.ncbi.nlm.nih.gov/blast/). Identifications were confirmed by aligning sequences within genera with named examples from GenBank using CLUSTALX (version 1.83, Jeanmougin et al. 1998) or MUSCLE (Edgar 2004) and the alignment was checked visually using MacClade (version 4.08, Maddison & Maddison 2003). Checked alignments were used in a neighbour-joining analysis in PAUP version 4.0b10 (Swofford 2002) and/or an analysis with DOTUR (Schloss & Handelsman 2005) to infer within genus groups. To ensure there was no DNA contamination at the PCR stage, names obtained from genetic analysis were checked against the rough morphotype description I recorded for each root when sampling. Potential names for each ectomycorrhizal morphotype were obtained from: Agerer (1987-2002), Ingleby et al. (1990) and Agerer & Rambold (2004-2009). Representative DNA sequences have been submitted to GenBank.

3.2.6 Statistical analysis

Unless otherwise stated statistical analyses were conducted in R version 2.7.2 (R Development Core Team 2008). The change in the number of seedlings over time within the grids and the difference between heathland and woodland
grids at Td2 was analysed with a linear model. The number of seedlings in the two squares counted per grid was averaged for each plot to remove pseudoreplication. To test for a change over time and potential difference between plots in the proportion of seedlings which were mycorrhizal in the woodland plots, I specified a generalised linear model with quasi-binomial errors to account for overdispersion.

A change in mass over time and between mycorrhizal and non-mycorrhizal seedlings in the woodland grids and the difference in mass between non-mycorrhizal seedlings in heathland and woodland grids over time were tested with linear models. The mass of seedlings of the same mycorrhizal status on the same date was averaged to remove pseudoreplication. On some dates, this was done at the weighing stage, occasionally several seedlings of the same mycorrhizal status on the same date were freeze-dried together and shattered during processing preventing me from weighing them individually.

I tested for a change over time and difference in the proportion of seedlings that were mycorrhizal between “near to trees” and “away from trees” categories at Td using a generalised linear model with a quasibinomial errors. The mass of seedlings on each sampling date was averaged within the categories i) “near to trees” and mycorrhizal, ii) “near to trees” and non-mycorrhizal, iii) “away from trees” and mycorrhizal, and iv) “away from trees” and non-mycorrhizal. To test for a change in mass over time in relation to distance from trees and mycorrhizal status, I specified a linear model in which the mass of the seedlings was transformed using a Box-Cox transformation (Box & Cox 1964) to normalise the data, where:

\[ f(x) = (x^\lambda - 1) / \lambda \]

A GLM with quasibinomial errors was used to test for the effect of the level of invasion on the proportion of seedlings which were mycorrhizal. I tested for potential model simplification using ANOVA and all models reported are the minimal adequate models.

Species richness estimates (Chao1, Chao2, Jack1, Jack2) were calculated with EstimateS (Colwell 2005) using a soil core as sample level, i.e., presence in a soil core counted as one individual. Shannon index (H’) takes into account the number of species and the evenness of species:

\[ H' = -\sum (p_i \log_{10} p_i) \]
where \( p_i \) = relative abundance of each fungus, calculated as the proportion of soil cores with a given fungus to the total number of soil cores containing fungi within the same site.

Simpson's Index (D) measures the probability that two fungi randomly selected from a soil core will be the same, it takes into account the diversity and the relative abundance of each fungus:

\[
D = \sum \left( \frac{(n(n-1))}{(N(N-1))} \right)
\]

where \( n \) = the total number of soil cores containing a particular fungus within the same site.

and \( N \) = the total number of soil cores containing all fungi within the same invasion level or site.

I report Simpson’s reciprocal index (1/D), where the higher the value, the greater the diversity.

Three similarity indices discussed in Magurran (2004) were calculated in EstimateS (Sørensen index, Bray-Curtis index and Morisita-Horn index) using number of cores containing each ectomycorrhizal fungus to identify differences in ectomycorrhizal community composition on mature tree roots between sites. A linear model of change in similarity index against distance between sites was tested in R version 2.7.2 (R Development Core Team 2008). To test for spatial and environmental (based on tree species invading and whether the heathland was wet or dry) effects on ectomycorrhizal community composition Mantel tests (Mantel 1967) were conducted using The R package version 4 (Casgrain & Legendre 2001). Non-metric multidimensional scaling (a non-parametric method) was used to test for clustering of sites on the basis of abundance (number of cores) of ectomycorrhizal fungi using R version 2.7.2 (R Development Core Team 2008).

3.3 Results

3.3.1 Naturally-occurring seedling studies

I examined 1,145 naturally-occurring seedlings: 984 birch seedlings in the 2005 studies at Td, 67 additional birch seedlings from Kw, Td and Tw and 94 pine seedlings from Hw, Kw, Sw, Td and Tw.
3.3.1.1 Sampling within grids at Thursley

The survival rate of naturally-occurring birch seedlings is extremely poor with all seedlings having died before day 55 (11/7/05) and only two remaining on day 40 (27/6/05, Figure 3.2). The loss of seedlings over time is highly significant ($F = 80.08_{1,26}, P < 0.001$). There is a small direct effect of habitat (heathland vs. woodland grids) on the loss of seedlings ($F = 3.05_{1,26}, P < 0.1$) and there is a significant effect of the interaction between habitat and date of sampling ($F = 5.16_{1,26}, P < 0.05$). Initially, there are significantly more seedlings in woodland grids (average number = 41) than in heathland grids (average number = 27; $P < 0.01$) and the loss of seedlings over time is higher in woodland grids than heathland grids ($P < 0.05$).

![Figure 3.2. Average number of live birch seedlings in two blocks of six grids at Thursley (Td) over a 40 day period. Day 1 is 18/5/05. Solid symbols indicate woodland grids and open symbols indicate invaded heathland grids. Differently shaped symbols indicate different grids.](image)

A total of 242 seedlings were sampled to assess mycorrhizal status from the heathland grids and 388 from the woodland grids. None of the seedlings collected from the heathland grids at Td between 18th May and 27th June 2005 were
mycorrhizal (Figure 3.3). In contrast, on the first day of collection (18th May 2005) 49% of seedlings (70 seedlings) in the woodland plots were mycorrhizal (Figure 3.3). The percentage of mycorrhizal seedlings in the woodland grids was high over the next two collection dates: 89% (164 seedlings, 27th May) and 78% (36 seedlings, 6th June). On 16th and 27th June 2005, there were only a few live seedlings remaining giving more skewed results (3/6 and 2/2 mycorrhizal, respectively). There was no significant effect of date or plot on the proportion of seedlings which were mycorrhizal.

**Figure 3.3.** Number of naturally occurring mycorrhizal and non-mycorrhizal birch seedlings sampled from woodland and invaded heathland grids at Thursley (Td) between 18th May 2005 (Day 1) and 27th June 2005 (Day 41). There were no mycorrhizal birch seedlings present in the heathland grids.

Within the woodland, mycorrhizal seedlings were significantly heavier than non-mycorrhizal seedlings (9.16 x 10^{-4} g and 4.93 x 10^{-4} g respectively; F = 9.53_{1, 21}, P < 0.01). Seedlings in the woodland grids did not become heavier over time; there is no significant interaction between mycorrhizal status and date of sampling. The change in mass of non-mycorrhizal seedlings over time is significantly different for seedlings in the heathland and woodland (t = 2.27, P < 0.05; Figure 3.4).
Five ectomycorrhizal fungal species were detected in the woodland plots. *Paxillus involutus* was present in all three woodland plots and *Tomentella sublilacina* was detected in two plots. *Cenococcum geophilum, Laccaria laccata* and *Russula ochroleuca* were also detected.

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**Figure 3.4.** The mass of naturally occurring seedlings over time for non-mycorrhizal birch in heathland (open markers, dashed line) and woodland (solid markers, solid line) grids at Thursley (Td) between 18th May 2005 (Day 1) and 27th June 2005 (Day 41).

3.3.1.2 Sampling outside of grids at Thursley

Outside of the grids, 354 seedlings were sampled, 205 “away from trees” and 149 “near to trees”, 83 were mycorrhizal and 271 non-mycorrhizal. There was no change over time in the proportion of seedlings that were mycorrhizal but significantly more seedlings “near to trees” were mycorrhizal than those “away from trees” (43% and 9% respectively, t = 4.1, P < 0.001, Figure 3.5).

---

Linear model for woodland grids;
mass of seedlings = 5.277x10^-4 - 2.84x10^-6 x day.

Linear model for heathland grids;
mass of seedlings = 3.403x10^-4 + 1.5x10^-5 x day.
Figure 3.5. Number of mycorrhizal and non-mycorrhizal naturally-occurring birch seedlings sampled between 6th June 2005 (Day 20) and 17th October 2005 (Day 153) both near to and away from trees at Thursley (Td).

Seedling mass data were not normally distributed; therefore, a Box-Cox transformation (Box & Cox 1964) was used where \( \lambda = -1.17 \). The average mass of the seedlings sampled was not significantly affected by the distance from the nearest sapling/tree. Mycorrhizal seedlings were significantly heavier than non-mycorrhizal seedlings (F = 15.651,32, P < 0.001, Figure 3.6). The increase in mass of mycorrhizal seedlings over time was significantly faster than for non-mycorrhizal seedlings (F = 4.061,32, P = 0.052).

Five ectomycorrhizal fungi were detected “near to trees”: *Laccaria laccata*, *Paxillus involutus*, *Scleroderma citrinum*, *Tomentella bryophila* and *Tomentella sublilacina*. One fungus was detected on two seedlings “away from trees”, *Leccinum holopus*. 
Figure 3.6. Increase in naturally-occurring seedling mass, a) with Box-Cox transformed data and b) untransformed data, (when available error bars are ± 1 S.E.) of mycorrhizal (closed symbols) and non-mycorrhizal (open symbols) birch seedlings “near to trees” (triangles) and “away from trees” (squares) between 6\textsuperscript{th} June 2005 (day 20) and 17\textsuperscript{th} October 2005 (day 153).

3.3.1.3 Additional tree seedling sampling

In addition to the seedlings sampled from Thursley (Td2) described above a further 67 birch seedlings were sampled from three other heathland sites (Kw, Td1, Tw), 14 of these were mycorrhizal and 53 non-mycorrhizal (Table 3.2). All
seedlings were in their first year of growth. Seedlings sampled from Td reported in this section were sampled either outside of the dates stated in the survey above or outside of the sampling area used.

Table 3.2. The number of naturally-occurring mycorrhizal (myc.) and non-mycorrhizal (non-myc.) birch seedlings sampled between May 2005 and October 2006 at three sites (Kw, Td and Tw) in invaded heathland (inv.) and woodland (wood.) and the ectomycorrhizal fungi detected.

<table>
<thead>
<tr>
<th>Date</th>
<th>Site</th>
<th>Level of invasion</th>
<th>Number myc.</th>
<th>Number non-myc.</th>
<th>Ectomycorrhizal fungi detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/5/05</td>
<td>Td</td>
<td>inv.</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>24/5/05</td>
<td>Kw</td>
<td>wood.</td>
<td>4</td>
<td>12</td>
<td><em>Paxillus involutus, Lactarius hepaticus, Leccinum holopus</em></td>
</tr>
<tr>
<td>7/7/05</td>
<td>Tw</td>
<td>wood.</td>
<td>4</td>
<td>0</td>
<td><em>Amanita fulva, Cenococcum geophilum</em></td>
</tr>
<tr>
<td>13/5/06</td>
<td>Td</td>
<td>wood.</td>
<td>5</td>
<td>0</td>
<td><em>Cenococcum geophilum, Paxillus involutus, Russula ochroleuca</em></td>
</tr>
<tr>
<td>18/9/06*</td>
<td>Td</td>
<td>wood.</td>
<td>1</td>
<td>5</td>
<td><em>Cenococcum geophilum</em></td>
</tr>
<tr>
<td>2/10/06*</td>
<td>Td</td>
<td>wood.</td>
<td>0</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

* samples obtained after a wildfire at Td.

At Kw mycorrhizal birch seedlings on average were heavier than non-mycorrhizal seedlings (0.83mg and 0.53mg, respectively).

Forty-eight pine seedlings in their first growing year (cotyledons present) were sampled from five sites (Table 3.3). All six seedlings sampled from uninvaded heathland were non-mycorrhizal. Of the 19 seedlings sampled from invaded heathland six were mycorrhizal and nine of the 22 seedlings sampled from woodland were mycorrhizal.
Table 3.3. The number of naturally-occurring mycorrhizal (myc.) and non-mycorrhizal (non-myc.) pine seedlings under one year old sampled between May 2005 and October 2006 at five sites (Hw, Kw, Sw, Td and Tw) in uninvaded heathland (uninv.), invaded heathland (inv.) and woodland (wood.) and the ectomycorrhizal fungi detected.

<table>
<thead>
<tr>
<th>Date</th>
<th>Site</th>
<th>Level of invasion</th>
<th>Number myc.</th>
<th>Number non-myc.</th>
<th>Ectomycorrhizal fungi detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/6/05</td>
<td>Hw</td>
<td>uninv.</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7/7/05</td>
<td>Tw</td>
<td>uninv.</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>27/5/05</td>
<td>Td</td>
<td>inv.</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6/6/05</td>
<td>Td</td>
<td>inv.</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>27/6/05</td>
<td>Td</td>
<td>inv.</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>14/7/05</td>
<td>Sw</td>
<td>inv.</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>24/5/05</td>
<td>Kw</td>
<td>wood.</td>
<td>4</td>
<td>11</td>
<td>\textit{Lactarius tabidus, Tomentella subilacina, unknown} \textit{Tomentella}</td>
</tr>
<tr>
<td>7/7/05</td>
<td>Tw</td>
<td>wood.</td>
<td>5</td>
<td>0</td>
<td>\textit{Cenococcum geophilum, Russula emetica, Xerocomus sp.}</td>
</tr>
<tr>
<td>2/10/06*</td>
<td>Td</td>
<td>Wood.</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* samples obtained after a wildfire at Td.

An additional 46 pine seedlings beyond their first growing season were also collected from four sites (Table 3.4). These seedlings were in their second or third growing season but no more than three years old. Fifteen such seedlings were sampled from uninvaded heathland of which four were mycorrhizal with \textit{Suillus bovinus}. Six out of nine seedlings from invaded heathland were mycorrhizal and the 22 seedlings sampled from woodland were mycorrhizal.
Table 3.4. The number of naturally-occurring mycorrhizal (myc.) and non-mycorrhizal (non-myc.) pine seedlings between one and three years old sampled between May 2005 and April 2007 at four sites (Hw, Kw, Td and Tw) from uninvaded heathland (uninv.), invaded heathland (inv.) and woodland (wood.) and the ectomycorrhizal fungi detected.

<table>
<thead>
<tr>
<th>Date</th>
<th>Site</th>
<th>Level of invasion</th>
<th>Number myc.</th>
<th>Number non-myc.</th>
<th>Ectomycorrhizal fungi detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/6/05</td>
<td>Hw</td>
<td>uninv.</td>
<td>4</td>
<td>5</td>
<td>Suillus bovinus</td>
</tr>
<tr>
<td>7/7/05</td>
<td>Tw</td>
<td>uninv.</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3/10/05</td>
<td>Td</td>
<td>uninv.</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>27/6/05</td>
<td>Td</td>
<td>inv.</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>11/7/05</td>
<td>Td</td>
<td>inv.</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17/10/05</td>
<td>Td</td>
<td>inv.</td>
<td>1</td>
<td>0</td>
<td>Scleroderma sp.</td>
</tr>
<tr>
<td>7/7/06</td>
<td>Tw</td>
<td>inv.</td>
<td>4</td>
<td>1</td>
<td>Suillus variegatus</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Tw</td>
<td>inv.</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>24/5/05</td>
<td>Kw</td>
<td>wood.</td>
<td>19</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7/7/05</td>
<td>Tw</td>
<td>wood.</td>
<td>1</td>
<td>0</td>
<td>Russula emetica</td>
</tr>
<tr>
<td>7/04/07</td>
<td>Tw</td>
<td>wood.</td>
<td>2</td>
<td>0</td>
<td>Suillus bovinus</td>
</tr>
</tbody>
</table>

As with birch seedlings, pine seedlings collected in their first year of growth, from several sites indicate that mycorrhizal seedlings are heavier than non-mycorrhizal seedlings (Table 3.5). These seedlings were weighed collectively so I was unable to conduct statistical analyses to test for significant differences.
Table 3.5. Average mass (milligrams) of naturally-occurring mycorrhizal and non-mycorrhizal pine seedlings at four lowland heathland sites (Sw, Tw, Kw, Td) collected between May and July 2005. All seedlings appeared to have germinated in 2005. Numbers in parentheses indicate the number of seedlings collected.

<table>
<thead>
<tr>
<th>Site</th>
<th>Average mass (mg) of mycorrhizal seedlings (n)</th>
<th>Average mass (mg) of non-mycorrhizal seedlings (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stoborough (Sw)</td>
<td>40 (5)</td>
<td>37.6 (5)</td>
</tr>
<tr>
<td>Thursley (Tw)</td>
<td>26.5 (5)</td>
<td>24.4 (6)</td>
</tr>
<tr>
<td>Kingston (Kw)</td>
<td>15.4 (4)</td>
<td>8.0 (11)</td>
</tr>
<tr>
<td>Thursley (Td)</td>
<td>85 (1)</td>
<td>15.3 (8)</td>
</tr>
</tbody>
</table>

For the purposes of comparison below, seedlings detected “away from trees” in the sampling at Td are recorded as being in uninvaded heathland, and seedlings “near to trees” and in the heathland plots are recorded as being in invaded heathland. Although these labels do not exactly conform with the distinctions used for these levels of invasion on all other occasions in this thesis, I feel that they are appropriate for this particular study.

Levels of mycorrhization increase as the level of tree invasion increases (Figure 3.7) and the effect of invasion on the proportion of seedlings which are mycorrhizal is highly significant ($F = 22.58_{2.5}, P < 0.01$).
Figure 3.7. Percentage of naturally-occurring birch and pine seedlings less than one year old that were mycorrhizal (black bars) vs. non-mycorrhizal (open bars) at five sites for three levels of tree invasion in 2005 and 2006. Seedlings were not detected at all levels of invasion at all sites. Numbers above bars indicate the total number of seedlings.

In total 22 fungi were detected on naturally occurring seedlings and the diversity of fungi increased as the level of invasion increased (Table 3.6).
Table 3.6. Fungi forming ectomycorrhizas with naturally occurring birch and pine seedlings under three years old in uninvaded heathland (Uninv.), invaded heathland (Inv.) and woodland (Wood.) at Hw, Kw, Td and Tw in 2005, 2006 and 2007. Letters in parentheses indicates whether a fungus was detected on birch (B), pine (P), or birch and pine (BP) seedlings.

<table>
<thead>
<tr>
<th>Level of invasion</th>
<th>Ectomycorrhizal fungi detected</th>
</tr>
</thead>
</table>

### 3.3.2 Establishment of germination date

Top soil horizon samples were examined with a dissecting microscope once a month from January 2006 until May 2006 (when the first seedlings had germinated). Between January and April 2006 none of the soil samples contained germinating seeds. On 13th May 2006, six birch seedlings were found and all were already mycorrhizal even though two of the seedlings had yet to produce a true leaf.

### 3.3.3 Mature tree root sampling

In total, 219 DNA sequences were obtained from tree root samples in 48 soil cores. A few of these samples yielded sequences of non-mycorrhizal fungi but the ectomycorrhizal status of those roots based on morphology was ambiguous. There were also some duplicates when the same fungi were sampled from a single soil core more than once. This is likely to have occurred when the morphology of
the ectomycorrhizas varied due to differences in age. Ninety-three DNA sequences (from 40 soil cores) were used for analysis. The data was analysed as the number of fungi within each soil core with duplicates removed. The soil cores from Gd and Sw yielded no ectomycorrhizas and the total number of roots at these two sites was exceptionally low (only three cores from Gd and five from Sw contained any roots). In contrast, ectomycorrhizas were found in every soil core (ten cores per site) from Cd, Hw, Kw and Tw.

Overall, 22 ectomycorrhizal fungi were detected including five *Russula*, four *Lactarius*, four Thelephoraceae and two *Amanita* species. *Lactarius hepaticus* was the most prevalent on mature tree roots and was the only fungus found at three sites (Figure 3.8). All other fungi were only found at one or two sites. In addition to well-known ectomycorrhizal fungi, a *Ceratobasidium* was detected forming ectomycorrhizas in two soil cores from Kw. I know of only one previous report of *Ceratobasidium* ectomycorrhizas (Yagame et al. 2008). The ericoid mycorrhizal fungus *R. ericae* was detected in one soil core and other members of the *R. ericae* clade (predominantly *Meliniomyces variabilis*, a non-mycorrhizal species) were detected in three soil cores; however, these roots were sampled only due to a lack of root hairs and they had not formed true ectomycorrhizas.
Figure 3.8. Number of soil cores containing each ectomycorrhizal fungus detected on mature tree roots in woodlands neighbouring lowland heathlands at four sites in 2007.

At Tw fungal evenness and diversity was higher than at all other sites and it was similar among Cd, Hw and Kw (Table 3.7). Simpson’s reciprocal index is higher at Tw than at all other sites, it has a similar value at Cd and Kw and at Hw it is the lowest. Richness was highest at Tw based on estimated richness indices and it appears that all or nearly all fungi forming ectomycorrhiza at Cd, Hw and Kw were detected (Table 3.7, Figure 3.9). The overall estimated ectomycorrhizal fungal diversity on mature tree roots at all sites is between 23 and 26.
Table 3.7. Ectomycorrhizal fungal richness and diversity from mature tree roots at Cd, Hw, Kw and Tw in 2007.

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of fungi</th>
<th>Diversity indices</th>
<th>Estimated richness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Simpson’s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shannon $H'$</td>
<td>$1/D$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chao1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chao2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jack1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jack2</td>
</tr>
<tr>
<td>Cd</td>
<td>6</td>
<td>0.761</td>
<td>7.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.00</td>
</tr>
<tr>
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<td>Hw</td>
<td>7</td>
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<td></td>
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<td>5.86</td>
</tr>
<tr>
<td>Kw</td>
<td>8</td>
<td>0.789</td>
<td>7.65</td>
</tr>
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<td></td>
<td>7.50</td>
</tr>
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<td></td>
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<td>7.45</td>
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<td></td>
<td></td>
<td>8.80</td>
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<td>9.69</td>
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<td>14.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15.67</td>
</tr>
<tr>
<td>All</td>
<td>22</td>
<td>1.23</td>
<td>15.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>23.18</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>25.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.00</td>
</tr>
</tbody>
</table>

* Value reported is ICE; Hw richness was estimated using the classic method rather than bias-corrected method because CV for incidence distribution was > 0.5 thus the highest value out of Chao2 and ICE was reported, as recommended by A. Chao in EstimateS (Colwell 2005).

The ectomycorrhizal fungal accumulation curves saturated or nearly saturated for Cd and curves for Hw and Kw were closer to saturation than Tw, meaning that most fungi present at these sites on tree roots were detected (Figure 3.9). The fungal accumulation curve for Tw was not near saturation; this agrees with the higher estimated fungal richness detected.
Figure 3.9. Ectomycorrhizal fungal accumulation curves for Cd, Hw, Kw and Tw mature tree roots sampled in 2007.

There were no fungi in common between Kw and Cd. Across indices, the wet heaths at Kw and Hw are the most similar sites and they are also the geographically closest sites used in this study (Table 3.8).

Table 3.8. Similarity indices for ectomycorrhizal fungi on mature tree roots between sites Cd, Kw, Hw and Tw sampled in 2007.

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Site 2</th>
<th>Classic Sørensen</th>
<th>Bray-Curtis index</th>
<th>Morisita-Horn index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kw</td>
<td>Hw</td>
<td>0.429</td>
<td>0.244</td>
<td>0.364</td>
</tr>
<tr>
<td>Kw</td>
<td>Cd</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kw</td>
<td>Tw</td>
<td>0.222</td>
<td>0.213</td>
<td>0.297</td>
</tr>
<tr>
<td>Hw</td>
<td>Cd</td>
<td>0.154</td>
<td>0.093</td>
<td>0.063</td>
</tr>
<tr>
<td>Hw</td>
<td>Tw</td>
<td>0.222</td>
<td>0.308</td>
<td>0.514</td>
</tr>
<tr>
<td>Cd</td>
<td>Tw</td>
<td>0.235</td>
<td>0.204</td>
<td>0.174</td>
</tr>
</tbody>
</table>
Similarity in ectomycorrhizal communities increases as proximity increases (Figure 3.10). Linear models predicted for each similarity index are as follows:

- **Sørensen index**
  \[ S = 0.390 \text{ – } (0.0013 \times \text{distance}) \]
  \[ F = 11.45_{1,4}, \ P<0.05; \]

- **Morisita-Horn index**
  \[ S = 0.480 \text{ – } (0.0018 \times \text{distance}) \]
  \[ F = 9.55_{1,4}, \ P<0.05; \]

- **Bray-Curtis index**
  \[ S = 0.315 \text{ – } (0.0010 \times \text{distance}) \]
  \[ F = 8.13_{1,4}, \ P<0.05. \]

**Figure 3.10.** Change in similarity indices (Sørensen, Morisita-Horn and Bray-Curtis) of mature tree roots over distance for four sites (Cd, Hw, Kw and Tw).

Non-metric multi-dimensional scaling also indicates that the closest sites (Hw and Kw) are more similar in their ectomycorrhizal community composition than either site is to either Cd or Tw (Figure 3.11).
Mantel tests however, indicate that the similarity in the community composition of Hw and Kw may partly be due to their similarity in environmental conditions (tree invading and whether the site is wet or dry). Mantel tests indicate a weak spatial relationship in ectomycorrhizal community composition ($r = 1.30, P = 0.09$) and a weak relationship between environmental conditions and ectomycorrhizal community ($r = 1.48, P = 0.07$) but the spatial relationship is not significant when environmental factors are taken into account ($r = 0.01, P = 0.5$).

The average number of fungi found in a plot varied within site at Cd and Kw but it was similar for Hw and Tw (Table 3.9). Each soil core removed from the four sites yielding ectomycorrhizas contained between approximately one and
three fungi. Approximately a third of the fungi detected at each site were present in both woodland plots. *Lactarius hepaticus* was detected in nine soil cores at Hw whereas all other fungi at all other sites were found in between one and five soil cores each (mean = 2.8).

**Table 3.9.** Within-site heterogeneity in distribution of ectomycorrhizal fungi on mature tree roots showing the mean number of fungi detected in each core and the distribution of fungi amongst cores and plots. Number in parentheses show the number of fungi found in two plots

<table>
<thead>
<tr>
<th>Site</th>
<th>Plot 1</th>
<th>Plot 2</th>
<th>Total fungal taxa detected</th>
<th>Number of ectomycorrhizal fungi found in x cores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Cd</td>
<td>2.4</td>
<td>1.6</td>
<td>6</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Hw</td>
<td>2.4</td>
<td>2.2</td>
<td>7</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Kw</td>
<td>3.2</td>
<td>0.8</td>
<td>8</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Tw</td>
<td>3</td>
<td>2.8</td>
<td>11</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

**3.4 Discussion**

Overall, I found that seedling mycorrhization in lowland heathlands is severely limited and ectomycorrhizal fungal diversity increases as level of tree invasion increases. Mycorrhizal seedlings are significantly heavier than non-mycorrhizal seedlings concurring with hypothesis two within this chapter yet seedling recruitment is low regardless of mycorrhizal status. Woodlands with an ericoid understorey neighbouring lowland heathlands, differ between sites in their ectomycorrhizal community. Most of the fungi detected on mature trees were detected at only one or two sites and fungi within the *R. ericae* aggregate do not form ectomycorrhizas on birch or pine trees within these woodlands.

**3.4.1 Effect of mycorrhization on biomass**

Naturally-occurring mycorrhizal seedlings are heavier than non-mycorrhizal seedlings regardless of habitat. Studies which reported neutral (Baxter & Dighton 2001) and negative (Fleming 1985) responses in biomass to
mycorrhization for birch seedlings have not been supported. In my study the sample number was larger and I found that mycorrhizal birch seedlings are heavier than non-mycorrhizal seedlings in the field.

3.4.2 Effect of proximity to established trees on survival and mycorrhization

For seedlings growing in woodlands there are a mixture of costs and benefits. I found that non-mycorrhizal seedlings in woodland were heavier than those in heathland, this may be due to higher nutrient levels in the woodland compared to nutrient-poor heathland (Gimingham 1972) yet in woodlands there is increased root competition (Dickie et al. 2005) for these nutrients and shading from established trees. Shading by trees has been reported to be both detrimental (Dickie et al. 2005) and beneficial (Kennedy & Sousa 2006) to mycorrhizal seedlings indicating that habitat and location can alter the costs and benefits associated with increased proximity to trees. Seedlings near trees and in woodlands are more likely to be mycorrhizal than those further away from trees. It remains unclear what are the benefits of mycorrhization per se in nature (Taylor 2006). Are ectomycorrhizal seedlings heavier than non-mycorrhizal seedlings because: 1) they have symbiotic fungi, or 2) they have a larger rooting zone and thus it is more likely that their roots have reached fungal inoculum? The increased growth rate of mycorrhizal seedlings compared with non-mycorrhizal seedlings over the growing season indicates that the seedlings’ mycorrhizal status is the basis for their greater biomass. This would be expected due to the reported benefits of mycorrhization (Marx 1973, Newsham et al. 1995, Hobbie & Hobbie 2006) and has significant ecological implications in ectomycorrhizal inoculum-limited heathlands. Mycorrhization has been reported to improve seedling survival during their first growing season (Miles & Kinnaird 1979, Miller et al. 1998, Onguene & Kuyper 2002) and low establishment rates have been linked to a lack of suitable mycorrhizal partners (Haskins & Gehring 2005, Weber et al. 2005). Seedlings in the woodland have the potential to become mycorrhizal very early (in some cases before a first leaf is produced). This near immediate colonisation of seedlings when inoculum is available has also been reported by Fleming (1985), Newton & Pigott (1991) and Dickie et al. (2002a). Despite some seedlings benefiting from being mycorrhizal for the entire growing season seedling establishment was very
low; potentially due to hot, dry summer weather (Miles 1967). I would expect annual recruitment to be very low; of the few seedlings to survive the summer many will die over winter, birch mortality was 81-94% in a variety of habitats in upland Scotland (Miles 1973) and in the study reported in Chapter Four only 22% of birch and 39% of pine seedlings survived the winter. In addition to the low survival rate, there is a low rate of establishment from seed, 0-8% of birch seedlings established in heathland from sown seeds (Miles 1972). I found very low establishment rates after sowing approximately 6,400 pine seeds and 22,800 birch seeds at five lowland heathlands, fewer than twenty seedlings germinated (full experiment not reported); demonstrating how harsh the heathland environment is for seedlings and how fortunate I was to find the Td study site. The high viability of the seeds I sowed was confirmed by their germination rate of at least 50% for birch and near 100% for pine in bioassays. In addition, the time of birch seed sowing and rate of birch seeds sown followed recommendations for birch woodland regeneration in Willoughby et al. (2007).

Seedlings within heathlands but “near to trees” were not heavier than those “away from trees,” a relationship which was also reported by Dickie et al. (2005). However, seedlings “near trees” were more likely to be mycorrhizal and these seedlings were heavier than non-mycorrhizal seedlings (regardless of distance to trees) indicating that increased proximity to trees in heathlands benefits seedlings as long as they become mycorrhizal. The increased levels of mycorrhization concurs with hypothesis one of this chapter; a smaller proportion of seedlings in heathland and away from trees will be mycorrhizal compared to those in woodland or near trees. The increased proportion of mycorrhizal seedlings (Onguene & Kuyper 2002, Nara & Hogetsu 2004) or increased proportion of mycorrhizal roots (Dickie et al. 2002a, Dickie & Reich 2005 Dickie et al. 2007) near trees has been reported in a variety of habitats and it is probably due to seedlings near trees becoming part of a “common mycelial network”. CMNs are the radiating ectomycorrhizal mycelia that connect the root systems of trees and seedlings (Brownlee et al. 1983, Cline et al. 2005, Dickie & Reich 2005, see reviews by Simard et al. 2002, Selosse et al. 2006). They are often a more “vigorous and effective” way of colonising seedlings than via spores (Read 1998) and have strong positive effects on seedlings (Nara 2008). CMNs can determine the ability of seedlings to further colonise invaded areas during primary and secondary
succession (Nara & Hogetsu 2004, Dickie & Reich 2005, Nara 2006a,b, Thiet & Boerner 2007). By allowing fungi that do not readily colonise seedlings via spores to form mycorrhizas with seedlings, CMNs increase the inoculum potential and ectomycorrhizal diversity in woodlands to a level above that of uninvaded heathland which contains only spore inoculum (Simard et al. 1997, Dickie et al. 2002a, Cline et al. 2005).

### 3.4.3 Ectomycorrhizal fungi detected on seedlings

Common colonisers of seedlings including *Paxillus involutus* (Newton 1991), *Lactarius* (Fleming 1984, 1985), *Leccinum* (Fleming 1984, 1985), *Laccaria* (Deacon et al. 1983, Fleming 1984, Mason et al. 1984, Fleming 1985, Jumpponen 2003, Nara et al. 2003a, Nara & Hogetsu 2004, Ashkannejhad & Horton 2006), *Scleroderma* sp. (Newton 1991, Nara & Hogetsu 2004), *Cenococcum geophilum* (Fleming 1984, Borchers & Perry 1989, Dickie & Reich 2005, Ashkannejhad & Horton 2006) and *Tomentella* sp. (Nara 2003b) were all detected on the naturally-occurring seedlings at my study sites. *Laccaria*, whose spores are wind-dispersed and may form spore banks away from trees (Horton & van der Heijden 2008) was detected in invaded heathland where it may colonise seedlings via CMNs and spores. *Suillus bovinus* was the only fungus I detected with pine in uninvaded heathlands. Ashkannejad & Horton (2006) reported *Suillus* to be one of two genera to predominate on pine seedlings grown in primary coastal sand dunes. *Suillus*, and the also Pinaceae-specific related genus *Rhizopogon*, were prevalent in deer faeces and survived desiccation suggesting a method of dispersal and potential for formation of a spore bank; thus, these key fungi are uniquely adapted for dispersal and survival in isolated areas. *Suillus bovinus* is an important fungus in uninvaded heathland and along with *Paxillus involutus*, it is often more efficient at obtaining nutrients than other mycorrhizal species (Bending & Read 1995, Read 1989). Access to nutrients could provide seedlings associated with these species a competitive advantage in characteristically nutrient-poor heathland soils. *Leccinum* has been reported as a poor coloniser of seedlings via spores (Deacon et al. 1983, Fleming 1984, Fleming 1985) yet it was detected “away from trees” on two seedlings. As I discovered later (see Chapter Four) however, a limited number of seedlings under the “away from trees” classification in this study may still be within the rooting zone of trees and therefore *Leccinum* could have colonised the
seedlings via CMNs. *Paxillus involutus*, a common species on birch seedlings in woodland soil of low nutrient availability (Newton 1991, Newton & Piggott 1991) was detected in both invaded heathland and woodland. The reliance of *P. involutus* on rhizomorphs (root-like aggregations of hyphae), rather than spores, to colonise new roots (Newton 1992) indicates that rhizomorphs are able to spread out from the roots of invading trees as well as within the woodland. Post-fire at Thursley the only fungus detected on naturally-occurring seedlings was *C. geophilum*, one of three fungi detected by Miller et al. (1998) post-fire. However, *C. geophilum* actually colonised fewer seedlings with heat treatment than without (Izzo et al. 2006). In my study, virtually all seedlings were non-mycorrhizal three months after fire unlike those of Miller et al. (1998) which were all mycorrhizal after the same time period post-fire.

**3.4.4 Ectomycorrhizal fungi detected on established trees**

Russulaceae (*Lactarius* and *Russula*) are often a prevalent fungal family on established trees (Ryan & Alexander 1992) and were more frequent in the study on mature tree roots than on naturally-occurring seedlings. They were also more abundant on older seedlings than ones in their first year of growth further supporting evidence that *Russula* species are late-successional fungi (Termorshuizen 1991, Visser 1995, Taylor & Bruns 1999, Nara et al. 2003a, Twieg et al. 2007). They do however, colonise naturally-occurring seedlings along with another typical late-stage fungus, *Amanita*. *Amanita* is often reported as a poor coloniser of seedlings, particularly by spore (Taylor & Bruns 1999, Deacon et al. 1983; Fleming 1985 and see Chapter Four), yet, it was detected on naturally occurring seedlings in woodlands in this study and by Blasius & Oberwinkler (1989) indicating that the genus may rarely colonise new seedlings via CMNs. Seven fungi were detected on naturally-occurring seedlings but not on mature tree roots, two of these fungi were detected with seedlings in an area not surveyed for mature tree roots (*Scleroderma citrinum* and *Tomentella bryophila*). Similarly, *Suillus bovinus* may not be able to compete with other fungi to form mycorrhizas on established trees as a close relative (*Rhizopogon*) has been shown to be a poor competitor (Baar et al. 1999, Taylor & Bruns 1999 and Twieg et al. 2007) and it is less prevalent on older hosts than on younger hosts (Termorshuizen 1991, Visser 1995). Although, *Rhizopogon luteolus* and *Suillus variegatus* were detected on
some mature tree roots they were less prevalent than seedlings in my bioassay studies (Chapter Two). *Leccinum, Clavulina, Xerocomus* and an unknown *Tomentella* sp. were detected on naturally-occurring seedlings at sites surveyed for their ectomycorrhizal community on roots (Kw and Tw) but they were not present on mature tree roots; it is unknown whether this represents differences in host preference between seedlings and mature trees (Deacon *et al.* 1983, Taylor & Bruns 1999), or because root tip sampling did not detect all fungi at the site given that fungal accumulation curves did not saturate. Richard *et al.* (2005) detected a less than 20% overlap in fungal species on the roots of seedlings and mature trees in an oak forest despite the same fungal families dominating both seedlings and trees. Comparisons between the ectomycorrhizal community of mature tree roots and naturally-occurring seedlings concurs with hypothesis three of this chapter; pioneer fungi will be the more prevalent on ectomycorrhizal seedlings than on mature tree roots and “K-selected” fungi will be more dominant on ectomycorrhizal mature tree roots than seedlings

### 3.4.5 Spatial heterogeneity in ectomycorrhizal community

Fungal diversity and community structure differed at a local scale between plots at the same site; approximately a third of fungi were detected in both plots at the same site and at two sites (Cd and Kw) within-core diversity was far lower in one plot than in the other plot at the same site. This previously reported clumped rather than random distribution (Taylor 2002) may be caused by effects of individual trees (Korkama *et al.* 2006). A single soil core typically contains different fungi (Horton & Bruns 2001); I found between one and three ectomycorrhizal fungi per soil core, which is high considering the overall site diversities of between six and eleven fungi, and this suggests that genet overlap is common. Ectomycorrhizal fungal hyphae can exhibit niche differentiation between soil substrate layers (Dickie *et al.* 2002b) which may account for high within core diversity if roots follow a similar pattern. Twenty-three ectomycorrhizal fungi were detected in a Scots pine forest (Jonsson *et al.* 1999) which is similar to the estimated species richness for all sites in this study even though my study sampled more sites and hosts. The low tree diversity at lowland heathland sites is probably the reason for low estimated fungal richness compared to mixed woodlands elsewhere (Cline *et al.* 2005, Richard *et al.* 2005, Walker *et al.* 2005, Nara 2006b,
The lower ectomycorrhizal diversity in woodlands neighbouring heathlands may be due to their small island nature; Peay et al. (2007). Some of the woodlands used (e.g., Hw) were islands of established trees not attached to the nearest woodland. It is difficult to compare Simpson and Shannon indexes with other studies due to differences in recording numbers of individuals; I have conservatively recorded presence of a single fungus in a single core as an individual whereas other studies have recorded each mycorrhiza as an individual despite the fact nearby, and even distant, roots can be colonised by the same fungal genet (Taylor 2002, Lian et al. 2006). Nonetheless, the diversity indices provide useful between site comparisons and richness estimates can be tentatively compared with other studies.

Differences in mycorrhizal communities between sites and between woodland plots in the seedling survey at Thursley (Td2) may be due to the sensitivity of ectomycorrhizal fungi toward different microhabitats (Iwanski & Rudawska 2007, Tedersoo et al. 2008). Environmental factors such as the type of tree species invading and whether the site is wet or dry may mask biome-level geographical distribution patterns.

As in Chapter Two, the ectomycorrhizal genera Hebeloma sp. and Inocybe sp. were not detected on lowland heathlands or in neighbouring woodlands despite their previously reported presence on tree seedlings (Deacon et al. 1983, Fleming 1984, Mason et al. 1984, Fleming 1985, Jumpponen et al. 2002, Nara et al. 2003a,b, Nara & Hogetsu 2004, Ashkannejad & Horton 2006) and in heathlands (Legon & Henrici 2005). I did however, detect eleven fungi including two genera on birch (Elaphomyces and Tomentella) not included on the list of fungi that form ectomycorrhizas with birch in Britain complied by Atkinson (1992). A study on ectomycorrhizas of mature P. sylvestris in Scotland found only seven fungal species (Genney et al. 2006). Three of these fungi were found in this study (Suillus variegatus, Lactarius rufus and Cenococcum geophilum) and another two were in the same genus (Cortinarius) or family (Clavulinaceae) from those found in my study. Unfortunately, a lack of sequence data from the Genney et al. (2006) study prevents further comparisons. As the need for further knowledge on the ecology and distribution of fungi in the UK is being appreciated by major conservation bodies to meet targets on species conservation (Anon 2009), the data presented in this chapter advance this knowledge; providing survey data at a biome scale within
the UK for the belowground presence of fungi which may be of greater ecological significance than aboveground presence.

In conclusion, this chapter has demonstrated that tree seedling recruitment in lowland heathlands is low. If seedlings become mycorrhizal they are heavier than non-mycorrhizal seedlings which may influence their survival. Ectomycorrhizal fungi are only rarely present to form mycorrhizas on seedlings in uninvaded lowland heathland and there is a lower ectomycorrhizal diversity on seedlings in heathlands than neighbouring woodland and on seedlings compared to mature trees potentially slowing tree invasion. My study begins to chart the, to-date unknown, geographic distribution of ectomycorrhizas in Britain.
Chapter Four - The influence of saplings on the mycorrhization of outplanted birch and pine seedlings and the spore dispersal of common ectomycorrhizal fungi in lowland heathland

4.1 Introduction

The density of ectomycorrhizal inoculum and the diversity of ectomycorrhizal fungi is higher where trees are established than in uninvaded heathland (Chapters Two and Three) but how inoculum potential decreases with increasing distance from individual trees is unknown. Birch and pine seedlings have the potential to become mycorrhizal through colonisation by established mycelia and/or germinating spores. Both of these dispersal modes will be dependent on seedling proximity to established trees and/or saplings and the dispersal strategy of the fungi available. To accurately measure the effect of distance from individual birch and pine saplings on the presence and diversity of ectomycorrhizal fungi, transects were laid out centred on saplings along which I planted non-mycorrhizal seedlings. In addition, the ability of common ectomycorrhizal fungi to colonise seedlings via spores was tested in an area of uninvaded heathland.

Seedlings becoming mycorrhizal via hyphae may become part of a common mycelial network (CMN) with the established tree or sapling. CMNs form through hyphal connections between the root systems of individual trees and potentially with establishing seedlings (Brownlee et al. 1983, Simard et al. 2002, Cline et al. 2005, Dickie & Reich 2005, Lian et al. 2006). CMNs can determine seedling survival, growth and nutrient uptake during primary succession, with strong species-specific effects (Nara & Hogetsu 2004, Nara 2006a,b). They may also allow fungi that do not readily colonise seedlings via spores to proliferate and potentially participate in tree invasion secondarily within heathlands. To test the importance of CMN versus spore inoculum, selected saplings were girdled in this study. Girdling involves the stripping of a ring of the bark and phloem of a tree’s trunk below the leaves to prevent photosynthates being transferred from the canopy to the roots and their associated mycorrhizal fungi. Girdling allows the transport of
water and minerals from the mycorrhizal fungi via the roots to the canopy. Girdling of *P. sylvestris* can decrease soil respiration in the field by 37% within five days and by 54% within two months – indicating that ectomycorrhizal fungi are dependent upon recently generated photosynthates – and virtually eliminate ectomycorrhizal fungal fruitbody production (Högberg *et al.* 2001) in agreement with controlled microcosm studies (Bidartondo *et al.* 2001b). The ability of CMNs to form new ectomycorrhizas on seedlings should be hindered by girdling due to the dependence of fungi on current photosynthates. Therefore, seedlings establishing near girdled trees should rely on inoculum from spores only. Girdling was found to be unsuccessful in two studies (Edwards & Ross-Todd 1979, Binkley *et al.* 2006), but this was due to the use of very small forest plots with strong edge effects.

Understanding spore dispersal is an essential part of furthering knowledge on ectomycorrhizal fungal ecology (Ishida *et al.* 2008). Chapter Two demonstrates that away from established trees, seedlings can become mycorrhizal indicating that these seedlings became mycorrhizal via germinating spores. This was prevalent amongst the closely related suillloid fungi *Rhizopogon* and *Suillus*. Therefore, fungi which rely on spore dispersal can have a substantial influence on seedling establishment in lowland heathlands. The importance of both wind- and animal-dispersed spores has been reported previously (e.g., Allen 1987, Ashkannejhad & Horton 2006, Geml *et al.* 2008) but there is still often a low level of inoculum and diversity away from trees (Boerner *et al.* 1996, Bidartondo *et al.* 2001a, Dickie & Reich 2005, Haskins & Gehring 2005, Chapter Two, Chapter Three).

Ectomycorrhizal fungal species vary in their ability to form ectomycorrhizas via spore inocula (Fox 1983, 1986, Ishida *et al.* 2008) and previous spore inoculum experiments have often been glasshouse or laboratory studies (e.g., Fox 1983, 1986, Ishida *et al.* 2008). In this study, I tested the ability of common ectomycorrhizal fungi to form mycorrhizas via spores in an area of uninvaded heathland. Studies of species-specific effects on mycorrhization in natural settings are rare because competition with and replacement of the study-fungus by native fungi can easily occur (Bledsoe *et al.* 1982, Villeneuve *et al.* 1991). Therefore, sites for field-based fungal species-specific experiments must therefore be carefully chosen to ensure the lack of native ectomycorrhizal inoculum (Nara 2006a, Kennedy *et al.* 2007). It has been demonstrated for *Paxillus involutus* that...
overwintering does not affect the spores’ ability to form mycorrhizas and in fact this is the most ecologically relevant strategy for the species in the UK where the spores would need to wait until spring to inoculate new seedlings from their sporocarp production in autumn (Fox 1983); therefore, this study was conducted between autumn and the following summer to comply with this ecological strategy.

Based on the cumulative results of the Bush Estate studies (see sections 1.2 and 2.1), Newton (1992) and Bowen (1994), I would predict that relatively fast-growing and putatively spore-dispersed ectomycorrhizal fungi (pioneer or “r-selected”) such as Hebeloma, Laccaria, Paxillus and Thelephora should dominate tree seedlings. Fleming (1984) found that Leccinum species have a dependence upon CMNs to form mycorrhizas with seedlings; thus I predicted that these species would be active near ungirdled saplings. However, based on results from Chapter Two, I expected that suilloid fungi, Laccaria and Thelephora would be the most common fungi near all saplings and Hebeloma would be absent.

The hypothesis proposed by Vrålstad et al. (2000) that ericoid mycorrhizal fungi in the Rhizoscyphus ericae aggregate can form ectomycorrhizas will again be tested. However, members of the aggregate did not form ectomycorrhizas in the bioassay study with and without Calluna vulgaris cuttings and were not detected forming ectomycorrhizas on naturally occurring seedling or on mature tree roots in heathlands.

As in Chapters Two and Three, mycorrhizal fungi were identified by sequencing fungal DNA from mycorrhizas. This field study had two main objectives: 1) to establish the relative importance of hyphal versus spore inocula and 2) to compare the inoculum potential of the spores of different ectomycorrhizal genera. I hypothesise that: i) “K-selected” fungi will not colonise seedlings via spores, ii) seedlings outplanted near non-girdled trees will be mycorrhizal, iii) seedlings outplanted near girdled trees and away from non-girdled trees will only be mycorrhizal with seedlings that can colonise via spores.

4.2 Methods

4.2.1 Pilot experiment I

Three pine (Pinus sylvestris) saplings (two 1m tall and one 0.88m tall) were arbitrarily selected in an area of invaded heathland at Thursley Common (Tw) in
May 2006. I designated four transects, 3m long, in the four cardinal directions centred on each sapling. Initially no other saplings were noted within a 6m perimeter of the selected sapling, but some pine seedlings (under 15cm tall) that were latterly found along transects were removed. Every 0.5m along the transect, I planted four non-mycorrhizal seedlings. Non-mycorrhizal seedlings were grown from *Pinus sylvestris* seeds (Forestart, Shrewsbury, UK). Seeds were sown 2:1 peat to sand; peat was autoclaved twice, 48 hours apart to kill fungal spores that may germinate after heat exposure and the sand used was acid-washed (Sigma-Aldrich, St. Louis, MO, USA). In 576-cell plug trays (LBS Group, Colne, UK) strips of a porous, non-organic fabric (non-woven polypropylene sold as a protective plant fleece, also used in the bioassay tubes in Chapter Two) were placed weaving in and out of rows of plugs (Figure 4.1). The fabric allowed easy removal of the plug containing the seedling; by pulling the fabric the entire plug was removed in one piece. Plugs were approximately 3cm³. Water, roots and fungal hyphae could penetrate the fabric and if the roots had penetrated the fabric before out-planting the fabric was cut and planted with the seedling to prevent damage to roots by attempting to remove the fabric (it did not degrade during the out-planting period).

![Figure 4.1. Design of seedling plugs for outplanting.](image)

One bleach-rinsed pine seed was placed in each plug. The trays were placed in a growth chamber for 23 days and then transferred to a cold frame for five days. Seedlings were approximately 24 days old when outplanted (on 3/5/06 for saplings 1 and 2 and on 5/5/06 for sapling 3). In total, 288 pine seedlings were outplanted along the transects (Figure 4.2). At each point, seedlings were planted
around the marker at a distance (approximately 8-10 cm) away from neighbouring seedlings to ensure that the harvesting of one seedling would not damage the root system of a neighbouring seedling. Seedlings were watered twice, at outplanting and seven days later.

Figure 4.2. Transects around one of the saplings in pilot experiment I at Thursley (Tw).

Where possible, one seedling per point was harvested after 19 days (17 days for sapling 3, 22nd May 2006) and 28 days (26 days for sapling 3, 31st May 2006). Seedlings and surrounding soil were cut out with a soil knife. To assess mycorrhization, the seedlings and entire root systems were then carefully and thoroughly extricated manually from the dense mass of ericoid hair roots characteristic of heathland soils under a dissecting microscope using tweezers. Potentially mycorrhizal roots were sampled and stored frozen in CTAB buffer until extraction. DNA was extracted from CTAB stored samples using the method in Gardes & Bruns (1993) with modifications (for full details see section 2.2.3). Briefly, after removal from chloroform, 600µl of 6M NaI and 10µl of glassmilk (Qbiogene, Cambridge, UK) were added. This was mixed at room temperature for 5 minutes and centrifuged for 2 minutes. The supernatant was removed and the
pellet washed three times in NEWWash (Qbiogene). The pellet was then dried, resuspended in 45µl of TE buffer and stored at -20°C until use. Seedlings were freeze-dried.

Fortunately, one week before a fire destroyed the area used for this study, on 7th July 2006 (65 days after outplanting for saplings 1 and 2, 63 days after outplanting for sapling 3), all remaining outplanted seedlings were harvested. Seedlings and potentially mycorrhizal root samples were processed in the same manner as in the first and second harvests.

The outplanting of seedlings and initial analyses of the first and second harvest (May 2006) were partly conducted by an undergraduate student, Rose Strickland-Constable for a 3rd year project that I co-supervised with Dr. Martin Bidartondo.

4.2.2 Pilot experiment II

On 23rd May 2006, four transects were laid out centred on each of two pine (P. sylvestris) saplings at Thursley (Td). The saplings were 1.2m tall and 1.4m tall so transects were 3.5m and 4m long respectively (three times height of tree, rounded to nearest 0.5m, for comparison to pilot study). Four transects were set up in the cardinal directions with points every 0.5m. Pine seedlings were grown in a manner similar to pilot experiment I, but they were hardened for outplanting by reducing humidity in the growth chamber for 30 hours and were left for 82 hours at Td in large transparent plastic bags. On 23rd May 2006, five seedlings were planted at each point and five around the base of each sapling. There was difficulty in out-planting seedlings at the exact point on some occasions because of dense heather and/or mosses. Seedlings were not manually watered on the day of outplanting due to heavy rain whilst out-planting.

By 9th June 2006, many of the seedlings had died, probably due to dry weather. The remaining seedlings were watered. On 14th July 2006, the site burnt with no surviving saplings (Figure 4.3).
4.2.3 Root zone dimensions

Exposed root systems after the fire at Thursley (Td and Tw) provided an opportunity to estimate the size of the root zones of saplings for future experiments. Trees with nearly intact carbonised root systems were located in an area where wind had blown away the ash covering them. Roots were measured from the base of the tree until I could no longer follow the root because it either went underground or the rest was burnt away. The tree height was estimated by standing at a point away from the tree at which the top of the tree was at 45° angle to my head. The distance between myself and the tree was then measured and the height of the tree was calculated.

4.2.4 Main experiment

On 16th August 2006, 14 birch (*Betula* spp.) and 18 pine (*P. sylvestris*) saplings were selected in an unburnt area of Tw, half the birch and half the pine saplings were girdled using a penknife. Two transects were centred on each of 14 saplings of each species, the transect direction was chosen to avoid neighbouring saplings. The height of each sapling was measured and recorded. The height of
the sapling was rounded up or down to the nearest 10 cm and points were set up at this distance away from the sapling (point 1, within estimated rooting zone), twice the distance (point 2, edge of estimated rooting zone), three and four times the distance (points 3 and 4, outside estimated rooting zone). For example, a transect around a 0.67m pine was marked at 0.7, 1.4, 2.1 and 2.8m. Additionally, three control transects were set up with no focal sapling, the points along these three control transects were 0.5m apart.

Pine and birch seedlings were grown in growth chambers in the same manner as in pilot experiment I, but the seedlings were not hardened before out-planting to further reduce the possibility of mycorrhizal contamination. When seedlings were approximately 2.5 weeks old, they were outplanted along the transects.

Two “mycorrhizal” seedling transects were also set up. Some seedlings grown in the plug trays described previously were at a size whereby the tap root had penetrated the fabric and drainage hole at the bottom of the plug and some short side roots outside of the plug had started to be produced. A set of such seedlings was placed on top of a pine seedling microcosm containing *Suillus bovinus* growing on twice-pasteurised peat to form ectomycorrhizas. These seedlings were left to grow for as long as possible before out-planting. At the time of out-planting no ectomycorrhizas had formed but some rhizomorphs of *S. bovinus* were visible attached to the seedling roots.

Due to constraints in the number of seedlings I was able to grow and plant out at one time, out-planting occurred on different dates and ultimately not all saplings were used in the experiment (Table 4.1).
Table 4.1. Number of transects along which seedlings were outplanted on each date in 2006 at Tw for the main experiment. Number in parenthesis indicates the number of saplings or focal points for the control transects.

<table>
<thead>
<tr>
<th>Date</th>
<th>Experimental pine</th>
<th>Experimental birch</th>
<th>Control birch</th>
<th>Control pine</th>
<th>“Mycorrhizal” control</th>
</tr>
</thead>
<tbody>
<tr>
<td>31/08/06</td>
<td>4 (2)</td>
<td>12 (6)</td>
<td>2 (1)</td>
<td>2 (1)</td>
<td></td>
</tr>
<tr>
<td>15/09/06</td>
<td>8 (4)</td>
<td>8 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/09/06</td>
<td>12 (6)</td>
<td>3* (2)</td>
<td>3* (2)</td>
<td>2 (1)</td>
<td></td>
</tr>
</tbody>
</table>

* Due to high mortality in seedlings outplanted on 31st August 2006 the control transects were replanted with new seedlings.

Five seedlings (of the same species as the sapling) were planted at each point along the transects on the dates listed in Table 4.1.

Seedling survival was recorded on 11th September, 26th September, and 30th or 31st October 2006, 4th February and 13th April 2007. The first harvest of seedlings was conducted on 30th and 31st October and all remaining seedlings were harvested on 16th April 2007. Seedlings were harvested, and seedlings and roots analysed using the same methods as in pilot experiment I (section 4.2.1).

4.2.5 Sporocarp inoculum

An uninvaded lowland heathland area containing no ectomycorrhizal inoculum as assessed by bioassays at Thursley (Tw) was selected for this experiment. The area is approximately 25m from the uninvaded plots used for a 2007 bioassay study which yielded only non-mycorrhizal seedlings and 15m from the nearest sapling from the main transect experiment described above.

I established five 2m long transects, 0.7m apart. Each transect was designated a fungal taxon and sporocarps of that taxon were placed in a 10cm² block at each of five points 0.5m apart. All fungi are known ectomycorrhizal fungi some of which have been found to form ectomycorrhizas with seedlings in this study and some of which have not despite being abundant as sporocarps in woodlands nearby. The fungi used were determined by presence of sufficient sporocarps at Thursley (Td and Tw) woodlands. Some fungi are only considered at the genus-level or above because of either the difficulty in identifying the taxa to species morphologically in the field or because there were not enough sporocarps
of a single species for all five plots. The transects received: *Amanita* spp., *Paxillus involutus*, *Thelephora terrestris*, suilloid-boletoid and *Lactarius* spp. Sporocarps were replaced 2-3 times with new sporocarps to maximise the chances of dikaryon formation and root colonisation. Sporocarps were left on plots for a minimum of three days before replacement to allow for spore deposition and spore prints were frequently seen underneath sporocarps. The stipe of each sporocarp was removed to allow the cap to be placed directly on the ground and to prevent spores being blown onto other plots. Samples of the stipe of some sporocarps were preserved in CTAB at -80°C. A control row containing no sporocarps was also set up.

Three birch and five pine one-month old non-mycorrhizal seedlings were planted in each plot on 2\textsuperscript{nd} April and seedlings which had died were replaced on 20\textsuperscript{th} April 2007. Non-mycorrhizal seedlings were grown on autoclaved 2:1 peat:sand mix from *Betula pendula* and *Pinus sylvestris* seeds (Forestart, Shrewsbury, UK). The dibber used for planting was cleaned with household bleach between each row and nitrile gloves were worn and changed between each row. Older seedlings (those outplanted on 2\textsuperscript{nd} April) were marked with a small rubber band around the stem. In the absence of rain, seedlings were watered twice a week.

One randomly chosen 10cm\textsuperscript{2} block per row was harvested on 18\textsuperscript{th} May, 14\textsuperscript{th} June, 8\textsuperscript{th} August and 10\textsuperscript{th} September 2007. Seedlings were manually extricated from the surrounding soil and dense ericoid root mass using tweezers under a dissecting microscope. Potentially mycorrhizal root tips were removed and stored in CTAB buffer at -20°C or -80°C for long-term storage or DNA was extracted immediately using Extract-n-Amp (see Chapter Two and Three). DNA was extracted from root tips stored frozen in CTAB using the protocol described above (section 4.2.1) or CTAB buffer was blotted off and DNA was extracted using Extract-N-Amp (Sigma-Aldrich, St Louis, MO, USA). Seedlings harvested in September were freeze-dried and weighed.

### 4.2.6 Fungal identification

An aliquot of 2\mu l of extracted DNA was combined with 8\mu l of Amplitaq Gold reaction mix (Applied Biosystems, Foster City, California) or 2x Extract-N-Amp. Amplifications were performed with an initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 53°C for 55 seconds and
72°C for 50 seconds, with a final extension of 72°C for 7 minutes, using the fungal-specific primer ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). PCR products were visualised on a 2% agarose gel with ethidium bromide. If the gel indicated PCR products had low yield or there was minimal contamination (i.e., a stronger band and weaker band) the extraction was repeated with higher-yielding polymerases using 8µl of PicoMaxx reaction mix (Stratagene, Cedar Creek, TX, USA) or JumpStart (Sigma-Aldrich), except for samples in the first and second harvest of the pilot study. If after amplification with PicoMaxx there was still a low yield, a nested PCR of the sample was performed using the primers ITS1 and ITS4 from a 1:100 H2O dilution including the original negative control. After amplification, the PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA) and cycle sequenced using BigDye v3.1 (Applied Biosystems). The cycle-sequenced products were electrophoresed using an ABI3730 Genetic Analyzer (Applied Biosystems). The DNA sequences were edited in Sequence Navigator (Applied Biosystems) or Sequencher (GeneCodes). Identification was achieved by conducting a BLASTn search on GenBank (www.ncbi.nlm.nih.gov/blast/). Sequence matches of greater than 97% sequence similarity to samples on GenBank were used for identification. To increase the reliability of identification the name obtained by genetic analysis was compared with the descriptions of the ectomycorrhizal morphotype of each fungus whenever these were available (Ingleby et al. 1990, Agerer 1987-2002, Agerer & Rambold 2004–2009).

4.2.7 Statistical analysis

An effect of distance from sapling (based on point along transect) on proportion of seedlings which are mycorrhizal versus non-mycorrhizal in pilot experiment I at Tw was tested for with a GLM with binomial errors. A difference in the number of seedlings surviving between each point along a transect in pilot experiment I by July was tested for with an one-way ANOVA. A difference in the number of seedlings surviving between each point along a transect in the main experiment by April was tested for with an one-way ANOVA. The relationship between tree height and root zone was estimated and tested using a linear model. In the sporocarp experiment I tested for a difference in biomass between mycorrhizal and non-mycorrhizal seedlings using a one-way ANOVA. All
statistical analyses were conducted in R version 2.7.2 (R Development Core Team 2008).

4.3 Results

4.3.1 Pilot experiment I

During the first harvest, one seedling per point was harvested (n = 66, outplanted n = 288). Six points were not harvested; four had only one seedling that was left until the next harvest and two points were inaccessible due to localised flooding following rainfall. Root samples were removed from six seedlings, all other seedlings were non-mycorrhizal. One sample was identified as *Paxillus involutus* (1.5m from sapling) and two were non-mycorrhizal fungi. Three samples were not amplified successfully so remain unidentified yet the morphology of one sample matches that of *P. involutus* (1.5m from sapling).

During the second harvest, 72 seedlings were harvested (one per point) and root samples from ten seedlings were removed for DNA sequencing, all other harvested seedlings were non-mycorrhizal. Four were found to be non-mycorrhizal, one was colonised by *Suillus variegatus* (2m from sapling). Five root samples did not amplify.

On 7th July 2006, the remaining 74 seedlings were harvested from 49 points. Eleven seedlings were mycorrhizal with *Suillus variegatus*, the 63 other seedlings were all non-mycorrhizal (Figure 4.4). There was no significant effect of distance from sapling on survival or proportion of mycorrhizal seedlings.
4.3.2 Root zone dimensions

The length of seven roots from four birch trees and 14 roots from 13 pine trees were measured. The relationship between tree height and root length is;
Birch: \( \text{root length} = 0.24 + (1.44 \times \text{tree height}) \), \( F = 17.33_{1,5}, P < 0.01 \),
Pine: \( \ln(\text{root length}) = 0.22 + (1.12 \times \ln(\text{tree height})) \), \( F = 27.75_{1,12}, P < 0.001 \).

4.3.3 Main experiment

4.3.3.1 Survival

Of the seedlings not harvested in October and therefore remaining outplanted over winter 41% were still alive in February and 29% survived until the 2\textsuperscript{nd} harvest in April (Table 4.2).

4.3.3.2 October harvest

During the first harvest (30\textsuperscript{th} and 31\textsuperscript{st} October 2006) at least one seedling was harvested from each point. In total, 259 seedlings (78 birch and 181 pine) were harvested from 80 pine transect points, 32 birch transect points, 12 points on
control pine transects, 12 points on birch control transects and 8 points on “mycorrhizal” transects. Overall, at harvest 256 seedlings were non-mycorrhizal and three were mycorrhizal (1 birch and 2 pine, Figure 4.5). Both pine seedlings were mycorrhizal with *S. variegatus* and the birch sample was not used for DNA sequencing. One pine seedling and the birch seedling were on transects radiating from girdled saplings and the other pine seedling on a transect from an ungirdled sapling.

**Figure 4.5.** Number of outplanted a) pine seedlings and b) birch seedlings harvested at each point in October 2006. The numbers of mycorrhizal (closed bars) and non-mycorrhizal (open bars) seedlings at harvest are shown. Note the different scales on the y-axes. Point 1 is within the sapling rooting zone, point 2 is at the edge of the rooting zone, points 3 and 4 without.
Table 4.2. Percentage (number) of seedlings outplanted on each date, surviving at 1st harvest, February survey and 2nd harvest and left out over winter. Exp. = experimental transects radiating from a focal sapling, Con. = control transects with no focal sapling, and “Myc” = “mycorrhizal” transects as described previously. unk. = unknown.

<table>
<thead>
<tr>
<th>Date</th>
<th>Species</th>
<th>Transect type</th>
<th>Number planted</th>
<th>1st harvest</th>
<th>Over-wintering</th>
<th>Feb-survey</th>
<th>2nd harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>31/8/06</td>
<td>Pine</td>
<td>Exp.</td>
<td>100 (80)</td>
<td>71 (57)</td>
<td>44 (35)</td>
<td>16 (13)</td>
<td>14 (11)</td>
</tr>
<tr>
<td>31/8/06</td>
<td>Birch</td>
<td>Exp.</td>
<td>100 (240)</td>
<td>0 (0)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>31/8/06</td>
<td>Pine</td>
<td>Con.</td>
<td>100 (80)</td>
<td>64 (51)</td>
<td>45 (36)</td>
<td>9 (7)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>31/8/06</td>
<td>Birch</td>
<td>Con.</td>
<td>100 (40)</td>
<td>0 (0)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>15/9/06</td>
<td>Pine</td>
<td>Exp.</td>
<td>100 (160)</td>
<td>86 (137)</td>
<td>49 (79)</td>
<td>25 (40)</td>
<td>21 (33)</td>
</tr>
<tr>
<td>15/9/06</td>
<td>Birch</td>
<td>Exp.</td>
<td>100 (160)</td>
<td>61 (97)</td>
<td>32 (51)</td>
<td>9 (14)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Pine</td>
<td>Exp.</td>
<td>100 (160)</td>
<td>98 (157)</td>
<td>40 (64)</td>
<td>11 (18)</td>
<td>8 (12)</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Pine</td>
<td>*</td>
<td>100 (40)</td>
<td>unk.</td>
<td>unk.</td>
<td>33 (13)</td>
<td>23 (9)</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Pine</td>
<td>§</td>
<td>100 (40)</td>
<td>unk.</td>
<td>unk.</td>
<td>unk.</td>
<td>unk.</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Birch</td>
<td>‡</td>
<td>100 (60)</td>
<td>85 (51)</td>
<td>47 (28)</td>
<td>5 (3)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Pine</td>
<td></td>
<td>100 (20)</td>
<td>90 (18)</td>
<td>55 (11)</td>
<td>25 (5)</td>
<td>25 (5)</td>
</tr>
<tr>
<td>25/9/06</td>
<td>Pine</td>
<td>“Myc.”</td>
<td>100 (40)</td>
<td>100 (40)</td>
<td>63 (25)</td>
<td>53 (21)</td>
<td>38 (15)</td>
</tr>
<tr>
<td>Total</td>
<td>Birch</td>
<td>Exp.</td>
<td>100 (400)</td>
<td>24 (97)</td>
<td>32 (51)</td>
<td>4 (14)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Total</td>
<td>Pine</td>
<td>¥</td>
<td>100 (480)</td>
<td>73 (351)</td>
<td>37 (178)</td>
<td>18 (84)</td>
<td>14 (65)</td>
</tr>
<tr>
<td>Total</td>
<td>Birch</td>
<td>Con.</td>
<td>100 (100)</td>
<td>51 (51)</td>
<td>28 (28)</td>
<td>3 (3)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>Pine</td>
<td>Con.</td>
<td>100 (100)</td>
<td>69 (69)</td>
<td>47 (47)</td>
<td>12 (12)</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Total</td>
<td>Pine</td>
<td>“Myc.”</td>
<td>100 (40)</td>
<td>100 (40)</td>
<td>63 (25)</td>
<td>53 (21)</td>
<td>38 (15)</td>
</tr>
<tr>
<td>Total</td>
<td>Birch</td>
<td>All</td>
<td>100 (1120)</td>
<td>54 (608)</td>
<td>29 (329)</td>
<td>12</td>
<td>8 (94)</td>
</tr>
<tr>
<td></td>
<td>&amp; Pine</td>
<td>¥</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(134)</td>
</tr>
</tbody>
</table>

^ Seedling survival was low (14 present on 25/9/06); therefore, an additional five seedlings per point were outplanted on 25/9/06. Number planted is the total from both outplantings.

*The survival of seedlings along these transects was not recorded in October and the seedlings were not harvested in October.

§ No data were obtained because the saplings’ location was lost.

‡ Forty of these seedlings were outplanted along the control transects used on 31st August.

¥ Values in this column are based on numbers recorded but should be higher in practise as the number of all experimental pine seedlings was not recorded.
4.3.3.3 April harvest

On 16\textsuperscript{th} April 2007, approximately seven months after outplanting, all remaining seedlings were harvested. In total, 94 seedlings from 65 points were harvested (Table 4.3).

\begin{table}
\caption{The number of mycorrhizal (myc.) and non-mycorrhizal (non-myc.) seedlings and points on transects that were harvested on 16\textsuperscript{th} April 2007.}
\begin{tabular}{llll}
\hline
Type of transect & Number of myc. seedlings & Number of non-myc. seedlings & Number of points \\
\hline
Around pine sapling & 4 & 61 & 45 \\
Around birch sapling & 0 & 3 & 3 \\
Control non-mycorrhizal pine & 0 & 9 & 7 \\
Control non-mycorrhizal birch & 0 & 2 & 2 \\
Control “mycorrhizal” pine & 0 & 15 & 8 \\
\hline
\end{tabular}
\end{table}

Four pine seedlings were mycorrhizal with \textit{S. variegatus}. These seedlings were found on transects radiating from four different saplings (three ungirdled and one girdled), two at point 1 and two at point 2 (Figure 4.6). All birch seedlings on sapling-centred and control transects, and all pine seedlings on control transects were non-mycorrhizal (Table 4.3). There is no significant difference in seedling survival between points. All girdled saplings appeared healthy.
Figure 4.6. Number of surviving outplanted pine seedlings, both mycorrhizal (closed bars) and non-mycorrhizal (open bars), on transects centred on pine saplings at the final harvest seven months after outplanting in the field. Point 1 is within the estimated sapling rooting zone, point 2 is at the edge of the estimated rooting zone, points 3 and 4 without. Seedlings outplanted near girdled and ungirdled saplings have been pooled.

4.3.4 Sporocarp inoculum

A total of 161 (42 birch and 119 pine) seedlings out of 240 outplanted (90 birch, 150 pine) were harvested between 18th May and 10th September, all other seedlings died. On the first two sampling dates (18th May and 14th June 2007) all 78 seedlings (59 pine and 19 birch) harvested were non-mycorrhizal.

From the seven mycorrhizal seedlings sampled on 12th July DNA sequences were obtained from five pine seedlings which were all confirmed as being mycorrhizal with *Suillus bovinus*, the morphotype of another pine seedling matches *S. bovinus* and the seventh seedling (a birch) matches the morphotype for *Thelephora terrestris*. Of the seedlings sampled on 8th August and 10th September, 41 seedlings were mycorrhizal and the fungi were identified using DNA sequencing. The only fungi to form mycorrhizas on the outplanted seedlings were *Thelephora terrestris* and *Suillus bovinus*. Despite my precautions, spores of both
of these species spread from their rows onto other taxon rows. Seedlings mycorrhizal with *S. bovinus* were present on every row. Seedlings mycorrhizal with *T. terrestris* were found on the *Amanita, Thelephora* and suilloid-boletoid rows.

Over the summer, outplanted birch seedling survival was lower than for pine. On 8\textsuperscript{th} August, 27 seedlings (eight birch and 19 pine) were non-mycorrhizal, three birch seedlings were mycorrhizal with *Thelephora terrestris*, the identity of fungi forming an ectomycorrhiza with another birch seedling was undetermined due to a poor DNA sequence, and ten pine seedlings were mycorrhizal with *Suillus bovinus* (Figure 4.7). On 10\textsuperscript{th} September, seven birch seedlings were non-mycorrhizal and four were mycorrhizal with *T. terrestris*, eight pine seedlings were non-mycorrhizal and 23 were mycorrhizal (19 with *S. bovinus*, three with *T. terrestris* and one with both *S. bovinus* and *T. terrestris*). Mycorrhizal pine seedlings from the September harvest were heavier than non-mycorrhizal seedlings but not significantly so (Table 4.4). Only four mycorrhizal birch seedlings were present at harvest in September preventing the use of statistical analysis; however, mycorrhizal seedlings were nearly twice as heavy as non-mycorrhizal seedlings (Table 4.4).
Figure 4.7. Proportion of harvested birch (grey bars) and pine (black bars) outplanted seedlings exposed to fruitbody spores that were mycorrhizal on 8\textsuperscript{th} August and 9\textsuperscript{th} September. The age of seedlings indicates whether the seedlings were outplanted on 2\textsuperscript{nd} April (110 and 143 days old) or 20\textsuperscript{th} April (128 and 161 days old). The number on the bar is the number of mycorrhizal seedlings.

Table 4.4. Average mass at harvest of mycorrhizal and non-mycorrhizal seedlings outplanted in April 2007 and harvested on 10\textsuperscript{th} September 2007. Number in parenthesis is number of seedlings. ± 1 S.E.

<table>
<thead>
<tr>
<th>Seedling</th>
<th>Mass of seedling (mg)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycorrhizal</td>
<td>Non-mycorrhizal</td>
<td></td>
</tr>
<tr>
<td>Birch</td>
<td>14.7 ± 4.8 (4)</td>
<td>8.1 ± 1.9 (7)</td>
<td></td>
</tr>
<tr>
<td>Pine</td>
<td>54.9 ± 3.4 (22)</td>
<td>54.1 ± 5.1 (8)</td>
<td></td>
</tr>
</tbody>
</table>

4.4 Discussion

4.4.1 Limited mycorrhizal influence of heathland saplings

There is little mycorrhizal inoculum for pine and birch seedlings in lowland heathlands even when growing within the rooting zone of saplings. Due to the lack
of mycorrhizal seedlings it is hard to test the second and third hypothesis of this chapter; seedlings outplanted near non-girdled trees will be mycorrhizal and seedlings outplanted near girdled trees and away from non-girdled trees will only be mycorrhizal with seedlings that can colonise via spores. For the few pine seedlings becoming mycorrhizal, *Suillus variegatus* is the dominant mycorrhizal fungus. This concurs with results in Chapter 2 where *S. variegatus* was amongst the most common mycorrhizal fungi colonising pine seedlings in bioassays even outside the woodland habitat (detected at five sites, in uninvaded and invaded heathland and in woodland). *Suillus variegatus* is a Pinaceae-specific fungus (Legon & Henrici 2005) hence its absence on birch seedlings. *Suillus* was found to be one of the main genera to form ectomycorrhizas on pine seedlings in uninvaded sand dune areas by Ashkannejhad & Horton (2006). Horton *et al.* (1998) also found only *Suillus* and *Rhizopogon* in a scrub habitat and a more diverse community in neighbouring woodland. Suillloid fungi have the potential to form a spore bank (Horton *et al.* 1998, Ashkannejhad & Horton 2006, Bruns *et al.* 2009) unlike other ectomycorrhizal fungi (Ishida *et al.* 2008). The lack of mycorrhizal seedlings appears to be due to a lack of inoculum rather than the experiment being conducted over too short a time period as seedlings on the sporocarp addition plots became mycorrhizal within three months, seedlings in the pilot study became mycorrhizal within two months, and rapid field mycorrhization has been reported by Fleming (1984), Newton & Pigott (1991) and Dickie *et al.* (2002a).

The fire at Thursley despite devastating the heathland, and my field study of naturally-occurring seedlings, provided me with a rare opportunity to measure the exposed tree roots. The measurements obtained proved useful enabling me to estimate the rooting zone of saplings used in this study. There is a lack of literature on tree rooting zones due to the difficulty in exposing them.

The low survival of seedlings indicates the severity of the heathland habitat; a third of the seedlings in pilot experiment I died whilst in the field (65 days, n = 76), all of the seedlings in pilot experiment II died (n = 310) and in the main experiment 71% (n=235) seedlings died between October and April. Nonetheless, Miles & Kinnaird (1979) reported that 99% of naturally-occurring birch seedlings did not survive the winter (n = 833). The relatively higher survival in my study may be linked to the milder climatic conditions in Southern England compared to Miles & Kinnaird’s Scottish Highlands; in fact, seedlings in Southern England
could be more threatened by summer drought than winter frosts. An additional study by Miles (1973) estimated that birch in a variety of habitats including heathland suffered 81-94% mortality over winter and Miles (1967) reported that the loss of seedlings was particularly notable during early summer droughts. In addition, a history of high nitrogen deposition in England may also contribute to survival in otherwise nutrient-poor heathland soils. Levels of mycorrhization were too low to detect whether girdling had an effect. The potentially Suillus-inoculated (control “mycorrhizal”) pine seedlings had a higher survival than non-mycorrhizal control and experimental seedlings potentially because they were older than the other seedlings when out-planted. It is unlikely that increased survival was due to mycorrhization because all control “mycorrhizal” seedlings were non-mycorrhizal at harvest.

A higher proportion of seedlings were mycorrhizal in pilot experiment I than in the main experiment. This may be a temporal effect; Koide et al. (2007) found that the relative frequency of hyphae of some pine ectomycorrhizal fungi is temporally partitioned between seasons. Suillus variegatus can form ectomycorrhizas from spores and hyphae; it is possible that during pilot experiment I there was a higher density of S. variegatus hyphae within the soil increasing the chances of contact with seedling roots. The presence of S. variegatus in uninvaded heathland however, and the fact that prevalence of S. variegatus does not increase as level of tree invasion increases (Chapter Two) indicates that this fungus may be better adapted to colonisation via spores than hyphae. Overall, CMNs appear to have little impact on seedling colonisation despite CMNs being a more effective way of colonising seedlings than via spores (Read 1998, Thiet & Boerner 2007). One possibility for poorer CMNs in this habitat compared to others is that the ectomycorrhizal fungi can not compete against the ericoid mycorrhizal fungi which are adapted to this nutrient poor environment (Smith & Read 2008).

4.4.2 Limited spore dispersal of common ectomycorrhizal fungi

Two of the five fungal taxa used in the sporocarp addition study formed mycorrhizas with the outplanted seedlings. Lactarius did not form ectomycorrhizas despite previous studies indicating that Lactarius spores remain in soil over winter under where fruitbodies developed (Miller et al. 1994). However, the spores of most ectomycorrhizal fungi do not germinate or colonise seedlings
beyond 30 days after collection (Ishida et al. 2007). My results from the spore inoculum experiment partly agree with previous pot-based glasshouse experiments indicating that *Amanita* and *Lactarius* species do not form or rarely form mycorrhizas from spores on birch seedlings in pots (Deacon et al. 1983, Fox 1983, 1986) and agree with the first hypothesis of this chapter; “K-selected” fungi will not colonise seedlings via spores. *Paxillus involutus* did not form mycorrhizas with seedlings in my study and *P. involutus* has previously demonstrated a limited ability to form ectomycorrhizas from spores (Fox 1983, 1986). A limited study of mycorrhizas forming on pine seedlings found that *Suillus luteus* would not form mycorrhizas from spores, yet *S. bovinus* did in this study (Fox 1986). However in Fox (1986), *Suillus luteus* may not have been competitive enough against *Hebeloma crustuliniforme*, a contaminant in that study, which formed mycorrhizas on 49% of root tips on the three mycorrhizal seedlings. Another suillloid (*Rhizopogon*) has been reported as a poor competitor (Baar et al 1999, Taylor & Bruns 1999, Twieg et al. 2007). *Suillus* has previously been reported to colonise seedlings via spores in areas of low inoculum potential (Ashkannejhad & Horton 2006, Chapter Two). The other commonly detected species, *Thelephora terrestris*, is known to frequently form mycorrhizas from spores and is commonly found in forestry nurseries and on saplings (Colpaert 1999, Menkis et al. 2005). Cross-contamination between transects (70cm apart from each other) probably occurred through spore transfer (via wind, rain splash, water flows, localised flooding and/or animals) rather than by mycelial growth because hyphae can only grow at 2-3mm day$^{-1}$ (Read 1998); therefore, it would take over 230 days to grow between transects. Unlike results in Chapters Two and Three (see sections 2.3.3.1 and 3.3.1) and conclusions from a meta-analysis by Karst et al. (2008), mycorrhizal pine seedlings harvested in September were not significantly heavier than non-mycorrhizal seedlings. Mycorrhizal birch seedlings were nearly twice as heavy as non-mycorrhizal birch seedlings however insufficient seedling numbers prevent statistical analyses from confirming the significance of these results. The data do however, appear to agree with results reported in Chapters Two and Three that mycorrhizal seedlings are heavier than non-mycorrhizal seedlings.

My seedling outplanting studies indicate that mycorrhizal inoculum from common mycorrhizal networks is limited in lowland heathlands even within the
rooting zone of established saplings and that only a few ectomycorrhizal fungi can form ectomycorrhizas on lowland heathlands via spore inoculum.
Chapter Five - Discussion

5.1 General Discussion

This study has contributed to the growing knowledge on mycorrhizas, a globally important provider of ecosystem services and component of biodiversity (Peay et al. 2008). Most studies on ectomycorrhizas have been based on fewer than 30 soil samples and covered an area less than 1 ha (Horton & Bruns 2001). In this study I have sampled 780 soil cores and harvested over 1,700 seedlings from seven lowland heathlands across England to identify the keystone ectomycorrhizal fungi in the invasion of trees onto lowland heathlands. This biome-level view begins to fill a serious gap in the knowledge of ectomycorrhizal plant-fungal communities at larger spatial scales (Dahlberg 2001) and of the role of mycorrhizas in seedling establishment (Horton & van der Heijden 2008). Major strengths of this study were the use of bioassays, naturally-occurring and outplanted seedlings, and a reliance on GenBank for identifying fungi. This holistic approach is laborious but necessary because, for instance, pot-grown and field-grown seedlings can differ in the presence and abundance of ectomycorrhizal fungi (Newton 1991). Overall, I detected 35 ectomycorrhizal fungi. Of these, I detected 28 ectomycorrhizal fungi on seedlings; 12 on both bioassay seedlings and naturally-occurring seedlings, ten on naturally-occurring seedlings only and six in bioassays only. At sites where both bioassay and tree roots could be sampled, 12 fungi were shared between these methods and ten were not. This represents a dramatically greater overlap in fungi between bioassays and mature roots than in other forest systems (Taylor & Bruns 1999). The potential limitations of GenBank for fungal identification (Brock et al. 2008), did not materialise, except in woodlands; I was able to identify the vast majority of the fungi I detected in this study using data available on GenBank. This improves knowledge on which fungi associate with birch and pine by removing uncertainties from sporocarp surveys in mixed woodlands (Smith & Read 2008) and establishes which fungi are present on roots. Establishing which fungi can colonise seedlings via spores in lowland heathland, which fungi colonise seedlings via mycelia, and uncovering the spatial heterogeneity of mycorrhizal inoculum is important both biologically and ecologically (Peay et al. 2008, Smith & Read 2008). The establishment of
ectomycorrhizas between seedlings and fungi can limit to plant establishment particularly for small seeds with few reserves, such as *Salix* (Nara 2008). Below, I discuss my major findings, with respect to the original aims and I propose future directions for ectomycorrhizal ecology.

### 5.1.1 Sources of uncertainty and critical evaluation of this study

#### 5.1.1.1 Sampling

Conditions at the study sites I used for this thesis could have an effect on the ectomycorrhizal inoculum distribution and fungal diversity I detected. The sites I used varied with respect to their flora, fauna, geology, hydrology and management. To reduce effects of variation between sites I set up the quadrats I used for the bioassay and mature tree root studies in areas that were as similar between sites as possible. This placement of quadrats both in inter-site similar locations and away from footpaths however, meant the location of quadrats was not random. In addition I did not choose the field sites I used randomly for practical reasons; all sites were within two to three hours drive of the laboratories at Kew Gardens (or my parents house with respect to Fd), contained some areas of unmanaged heathlands and were part of National Nature Reserves. The sites however, represent a cross section of lowland heathlands found in England, except for those in the far south-west of England and chalk heath. I visited one chalk heath site (Lullington Heath NNR) however, there was not suitable levels of tree invasion and the ericoid plants were sparse (plants are separated by 2 m of grasses and herbaceous plants) making it very different to other sites I had used for sampling. I did try to sample the site however, when taking soil cores I discovered the soil was only 8-9 cm deep before solid chalk making the sampling different to other sites as well. The sampling within quadrats could also not be random due to natural within plot variation for example I could not take soil cores within the dense centre of *Calluna* plants.

In addition to invasion by birch and pine some field sites in very wet areas are invaded by *Salix* sp. and at two sites (Tw and Hw) single *Quercus* sp. trees are present. I ensured I sampled areas away from these other tree species. Despite the presence of these other tree species I chose to sample only birch and pine roots as these invading species have a greater literature associated with them with regards
to heathland invasion (for example, Gimingham 1978, Miles & Kinnaird 1979, Khoon & Gimingham 1984, Marrs 1987, Hester et al. 1991a,b, Mitchell et al. 1997, Mitchell et al. 2000, Manning et al. 2004, 2005, Mitchell et al. 2007), they are present at all sites I used and are thought of as being the primary threats to lowland heathlands unlike Salix and Quercus.

I did not collect data on the geology, soil chemistry and hydrology of the study sites I used. As the study was conducted at a biome scale I was aware that the above mentioned factors would vary between sites however all sites were within the category of lowland heathland and I felt as though my research would be more productive if I focused solely on variation in ectomycorrhizal community variation between the sites as other aspects were outside of the scope of this study. Literature reveals differences between sites in phosphorus adsorption capacity, with Dorset heaths (Gd and Sw) having a lower capacity than other areas (Chapman et al. 1989). There may also be within site differences; the pH at the NNR around my Sw plots ranges from 4.1 to 7.5 over approximately 6 km however, all my plots within this site are within a few metres rather than km. Mitchell et al. (1997) reported the pH of heathlands in the Poole Basin which includes my sites Gd and Sw as being between 3.4 and 4.4 and also reported that areas invaded by birch tend to be less acidic. Marrs et al. (1992) reported the soil pH at Cd as being 3.6 ± 0.05. A site containing mineral soil and wet heath near Kw has been reported to have pH values of below 4.5 (Newbould 1960). There will also be variation in the management of the lowland heathlands I used. When I sampled the sites however, none were actively managed using grazing domestic animals. The sampled areas were additionally not in or adjacent to areas that had been subject to recent managed burns or recent tree or sapling removal as part of a management plan.

5.1.1.2 Techniques

One potential source of sampling error is the potential removal of additional fungal inoculum and fungal diversity through the homogenisation of soil. Some fungi that are known only to colonise seedlings via vegetative tissue were detected therefore not all inoculum was removed through homogenisation yet there is the potential more would have been present. Homogenisation of soil was necessary though due to the intense stratification of heathland soils and vertical spatial
distribution of ectomycorrhizal roots and mycelia (Dickie et al. 2002b, Genney et al. 2006). The only other option would have been to take two adjacent soil cores one for the pine bioassay and one for the accompanying birch bioassay and use the top 15 cm of soil for the bioassay however ectomycorrhizal inoculum varies over very small spatial scales. Therefore fungal community composition even between adjacent soil cores could have been different.

I identified the fungi present on an ectomycorrhizal root tip using a combination of comparing my sequences to those on GenBank and confirming this with basic morphological information. By using GenBank however, I have to rely on the correct identification of the fungi associated with the DNA sequences being submitted to GenBank by others. I reduced this error by using the most frequent name in the top ten hits.

### 5.1.2 Critical evaluation of other work

#### 5.1.2.1 Bush Estate studies and mycorrhizal succession

Until this study, the most detailed studies of ectomycorrhizas on birch were carried out during the 1980s in Scotland (Ford et al. 1980, Mason et al. 1982, Deacon et al. 1983, Fleming 1983, Fox 1983, Last et al. 1983, Mason et al. 1983, Dighton & Mason 1984, Fleming et al. 1984, Fleming 1984, Last et al. 1984a, b, Mason et al. 1984, Fleming 1985, Fleming et al. 1986, Last et al. 1987, Gibson & Deacon 1988, Gibson et al. 1988, Mason et al. 1988, Deacon & Fleming 1992). These studies suggested a successional dynamic from “early” to “late” stage fungi forming ectomycorrhizas with birch. This study has one key disagreement with the Bush Estate studies; Russula and Amanita are often reported as being late-stage fungi which do not form ectomycorrhizas with seedlings however, I detected ectomycorrhizas formed by both Russula and Amanita on both seedlings in bioassays and on naturally-occurring seedlings under three years old. This is particularly surprising in the case of bioassay seedlings as the majority of inoculum in bioassays will be from spores which late-stage fungi are reported as not being able to form ectomycorrhizas from. My bioassay results indicate that either these “late stage” fungi can form mycorrhizas via spores or that other forms of inoculum such as mycorrhizal root tips or hyphae. As my spore inoculum experiment in Chapter 4 indicated that Amanita and Russula can not form ectomycorrhizas on
seedlings via spores. I can therefore assume that non-spore inocula was available to seedlings in bioassays. The “late-stage” fungi’s lack of ability to form ectomycorrhiza via spores agrees with some of the Bush Estate studies. In partial agreement between my study and the Bush Estate studies is that “late-stage” fungi, in particular, Russulaceae and Amanitaceae are more diverse and frequent on mature tree roots than on seedlings.

Another difference between my study and those based at the Bush Estate was the absence of *Hebeloma* and *Inocybe* in all of my studies. This is despite the fact that *Inocybe lacera* is a common and widespread species fruiting in heathlands in the UK (Legon & Henrici 2005). A cause for this discrepancy and the presumption by researchers of the Bush Estate studies that fungi are separated into early and late stage fungi is that the Bush Estate studies were based on fruitbody surveys which can poorly represent a sites’ ectomycorrhizal diversity (Taylor & Alexander 1990, Gardes & Bruns 1996, Jonsson *et al.* 1999, Horton & Bruns 2001, Taylor 2002, Nara *et al.* 2003b). Morphological identification of fungi from ectomycorrhizal roots was used to a lesser extent in the Bush Estate studies but it is a low-resolution and low-throughput approach compared to today’s DNA-based identification (Peay *et al.* 2008) which I used. This use of DNA-based identification has allowed me to identify fungi which may have been actively forming mycorrhizas during the “early-stages” in the Bush Estate studies but did not form sporocarps and therefore were not detected during the Bush Estate surveys. The basis for this inconsistency between frequency of sporocarps and frequency of mycorrhizas of the same fungal taxa at the same site is unknown (Smith & Read 2008) but may reflect trade-offs between allocation to vegetative versus reproductive growth in fungi.

### 5.1.2.2 Miles & Kinnaird

During my study I tested hypotheses related to Miles & Kinnaird’s 1979 hypotheses which stated that 1) a lack of ectomycorrhizal inoculum in heathlands slows invasion by birch and pine and 2) there are ectomycorrhizal fungal species-specific effects on the establishment of tree seedlings that invade heathlands. Although I did not explicitly test the establishment of seedlings on heathlands my results supported their theories to some extent. There is lower fungal inoculum in uninvaded heathland which may slow tree encroachment. Although seedlings can
survive for up to three years without becoming mycorrhizal I would presume they would not survive much beyond this. In addition, there is not a complete absence of inocula as wind- and deer-dispersed spores are present which may represent the fungal-specific establishment. Mycorrhizal seedlings are larger than non-mycorrhizal seedlings, it could therefore be presumed that larger seedlings would be more likely to survive over winter or through summer droughts. This increased survival could be construed as mycorrhizal inoculum assisting the tree invasion onto heathlands. I did detect fungal-specific effects in that only five fungi were detected in uninvaded heathland, these will therefore affect the invasion of lowland heathlands. Three of the fungi detected in lowland heathlands were pine-specific (Suillus bovinus, Suillus variegatus and Rhizopogon luteolus) and have the potential to form sporebanks; potentially allowing the invasion of pines to be quicker than the invasion of birch. There were however, no fungal-specific affects on the mass of seedlings.

5.1.2.3 Vrålstad et al. (2000) hypothesis

Despite growing evidence for shared ericoid and ecto-mycorrhizas. There is no evidence that fungi within the Rhizoscyphus ericae aggregate form ectomycorrhizas with birch and pine on lowland heathlands.

5.2 Achievement of aims and brief discussion of results

5.2.1 Assess the diversity of ectomycorrhizal fungi on lowland heathlands and in neighbouring woodlands

I detected 35 ectomycorrhizal fungi on lowland heathlands and in neighbouring woodlands; 23 on birch and 19 on pine (Figure 5.1). Of these, ten taxa occurred on both birch and pine, 10 on birch only and 10 on pine only, a further five fungi cannot be attributed to just one tree type because they were detected on mature tree roots and tree was not confirmed when sampling roots. My results confirm the previously reported high degree of overlap in birch and pine ectomycorrhizal fungal communities (Newton & Haigh 1998). In abundance graphs, dominant taxa are followed by a tail of less frequent taxa present at fewer
sites, a common pattern in ectomycorrhizal fungi (Peay et al. 2008) and other diverse taxa.

i) **Key fungi in the invasion are: suillloid fungi** (*Suillus bovinus*, *S. variegatus*, *Rhizopogon luteolus*, *Laccaria proxima* and *Thelephora terrestris*; all occur in uninvaded heathland (Chapters Two, Three and Four). Their adaptations for spore dispersal appear to be the reason for their success (Chapter Four). Suillloid fungi can form spore banks (Ashkannejhad & Horton 2006, Horton et al. 1998, Bruns et al. 2009) unlike many other ectomycorrhizal fungi (Ishida et al. 2008) and *L. proxima* and *T. terrestris* are well-adapted for wind dispersal. The poor competitive abilities of suillloid fungi (Taylor & Bruns 1999, Baar et al 1999; Twieg et al. 2007) means their dominance is superseded by secondary, late-stage fungi such as Russulaceae on mature trees that rely on root-to-root colonization (Ryan & Alexander 1992, Nara et al. 2003a, Twieg et al. 2007, Chapter Four).

ii) **Fungal richness was higher and community composition different on mature tree roots compared to seedlings** (Chapters Two and Three). Twenty two ectomycorrhizal fungi occurred on mature tree roots with estimated richness being between five and 16 fungi per site, generally higher than that found on bioassay seedlings, confirming that mycorrhizal fungal richness increases with host age and stand development (Visser 1995). Fifteen fungi were detected on both seedlings (bioassay and naturally-occurring) and mature trees, similar to Jonsson et al. (1999) who detected ten out of 23 ectomycorrhizal taxa on both seedlings and mature trees. However, mycorrhizal community composition was different with fewer pioneer fungi (those in uninvaded heathland) and more late-stage fungi (Russulaceae) on mature tree roots than on seedlings (Chapter Three).

iii) **Ectomycorrhizal fungal diversity is higher in woodlands than heathlands** (Chapter Two, Figure 5.1). The ectomycorrhizal communities of woodlands and heathlands are distinctly different, and they are stable between years. A higher niche diversity in woodlands (Iwanski & Rudawska 2007, Tedersoo et
al. 2008), compared to the near-monoculture of heathland, a higher number and larger size of plant hosts (Nara et al. 2003b), the ability of fungi to utilise vegetative dispersal in woodlands and increased presence of mycophagous animals to disperse spores (Johnson 1996, Lilleskov & Bruns 2005, Ashkannejhad & Horton 2006) may all account for this increase in diversity from heathland to woodland and prevent woodland bioassay and mature tree root fungal accumulation curves from saturating. Niche partitioning appears to be an important determinant of ectomycorrhizal community structure (Peay et al. 2008).
Amanita fulva B
Amanita muscaria B
Amanita rubescens BP
Atheliaeaceae sp. P
Cenococcum geophilum BP
Clavulina sp. P
Cortinarius sp. P
Cortinarius semisanguineus P
Elaphomyces granulatus P
Elaphomyces muricatus BP
Laccaria laccata BP
Laccaria proxima BP
Lactarius hepaticus BP
Lactarius necator-like BP
Lactarius rufus BP
Lactarius tabidus BP
Leccinum holopus B
Paxillus involutus BP
Pseudotomentella tristis BP
Rhizopogon luteolus P
Russula betularum B
Russula claroflava BP
Russula emetica BP
Russula ochroleuca BP
Russula sphagnophila B
Scleroderma citrinum BP
Suillus bovinus P
Suillus variegatus P
Thelephora terrestris BP
Thelephoraceae sp. B
Tomentella sp. P
Tomentella sublilacina BP
Tomentellopsis sp. BP
Xerocomus sp. P

Laccaria laccata B
Laccaria proxima BP
Lactarius hepaticus P
Lactarius rufus BP
Leccinum holopus B
Paxillus involutus B
Rhizopogon luteolus P
Scleroderma citrinum BP
Suillus bovinus P
Suillus variegatus P
Thelephora terrestris BP
Tomentella bryophila B
Tomentella sublilacina B

Figure 5.1. The ectomycorrhizal fungi of birch and pine in English lowland heathlands detected across six sites, over three years in bioassay and/or in situ surveys. B, P or BP (in bold) after a fungus name indicates whether the fungus was detected on birch, pine or birch and pine roots. B, P or BP (not in bold) after a fungus name indicates uncertainty, these fungi were detected on mature tree roots in mixed woodlands where the identity of the tree was not recorded when roots were sampled. The proportion of mycorrhizal seedlings is generally >50% in woodland, ca. 25% in invaded heathland and <10% in uninvaded heathland.
iv) Common ectomycorrhizal fungal families were present at all sites but community composition differed between sites (Chapters Two and Three). Three fungal families occurred at nearly all sites (i.e. Russulaceae, Suillaceae and Thelephoraceae). The Pinaceae-specific Suillaceae were absent from a birch-only site, and only Suillaceae were present at a pine-only site. Russulaceae and Thelephoraceae have been reported as some of the most abundant taxa in ecosystems sampled for ectomycorrhizas (Horton & Bruns 2001). Sites geographically closer together had a more similar ectomycorrhizal community in the bioassay surveys driven by the dispersible fungi of uninvaded and invaded heathland, but in the mature tree root surveys of woodlands, the geographic association was not detected.

5.2.2 Establish where and when seedlings become mycorrhizal
i) Spatial heterogeneity in mycorrhizal inoculum potential occurs within sites and between lowland heathlands (Chapters Two and Three). The amount of ectomycorrhizal inoculum increases as the level of tree encroachment increases; only fungi that can spread via spore rather than mycelial colonisation can form mycorrhizas in uninvaded heathland whereas woodlands contain higher numbers of mycorrhizal roots, mycelia and fungal spores. There is variation in inoculum potential between heathlands, often due to specificity towards the tree species present.

ii) Seedlings become mycorrhizal very early in the growing season if inoculum is available (Chapter Three). Mycorrhizal fungi critically provide nutrients to seedlings in rhizospheres, deprived of nutrient (Smith & Read 2008) and this will also be applicable within the extremely low nutrient environment of lowland heathlands where fungi may aid seedling survival. In primary invasions, non-mycorrhizal seedlings do not survive (Nara 2008). Non-mycorrhizal seedlings are however, capable of surviving for at least one year in lowland heathlands. If inoculum is present, seedlings can become mycorrhizal very quickly (Chapter 4, Fleming 1985, Newton & Pigott 1991, Dickie et al. 2002a).
5.2.3 Assess whether seedlings become mycorrhizal from spores or mycelia

i) Seedlings in uninvaded heathland can become mycorrhizal via fungal spores present in a poorly-developed spore bank or rarely dispersed by wind (Chapters Two, Three and Four). The fungi present in uninvaded heathland are all adapted for dispersal via spore, typically via mammal vectors and can form long-term spore banks. Seedlings in heathlands appear to use a “sit-and-wait” strategy if they manage to establish and survive without mycorrhization.

ii) The rooting zones of saplings do not contain sufficient spore or mycelium inoculum to cause seedlings to become mycorrhizal (Chapter Four). Very few outplanted seedlings within or at the edge of the rooting zone of saplings became mycorrhizal.

iii) Seedlings in woodlands can become mycorrhizal with fungi adapted to spore and mycelial colonisation strategies (Chapters Two and Three). The fungi adapted for spore-dispersal (those detected in uninvaded heathland) were also present in woodlands along with fungi reported to colonise new seedlings via mycelia or rhizomorphs, e.g., *Paxillus involutus*, *Leccinum* and *Russulaceae*.

iv) Invaded heathland rarely has mycorrhizal fungi that colonise seedlings via CMN (Chapters Two, Three and Four). *Leccinum holopus* and *Paxillus involutus* were both rarely detected in invaded heathland indicating that mycelia from trees can extend out into the heathland but rarely do.

5.2.4 Additional aims and findings

i) Members of the *Rhizoscyphus ericae* aggregate did not form ectomycorrhizas on birch or pine in lowland heathlands (Chapters
Two, Three and Four). In addition, birch and pine did not form arbuscular mycorrhizas on lowland heathlands.

ii) **Mycorrhizal seedlings are heavier than non-mycorrhizal seedlings** (Chapters Two and Three) and become increasingly heavier over the first year of growth.

iii) **Seedling survival is low regardless of mycorrhizal status (Chapters Three and Four).** Birch seedling survival on heathlands has previously been reported to be low but higher for mycorrhizal seedlings (Miles 1967, Miles 1973, Miles & Kinnaird 1979).

iv) **Mycorrhizal fungi prevalent on birch elsewhere, or in other primary invasion areas, are rare or absent on lowland heathlands in southern England (Chapters Two and Three).** *Hebeloma* and *Inocybe* are frequently reported fungi in primary invasion habitats and on birch yet neither were detected in this study. Mapping of ectomycorrhizal fungi in Britain is needed to assess geographic distribution and conservation status of these ecologically important fungi.

### 5.3 Overall conclusion

Tree seedling recruitment onto lowland heathlands, particularly for birch, is restricted due to a lack of inoculum and the harsh environment for seedlings. Invasion occurs mainly from woodland edges where seedlings have the opportunity to join CMNs formed by mature trees and on the rare occasions when seedlings germinate in locations on lowland heathland with spores of the few fungi that can reach and survive in that habitat (e.g., suilloid fungi).

### 5.4 Avenues for future work

The study of ectomycorrhizal fungi is a rapidly growing field particularly as the importance of mycorrhizal fungi in maintaining and developing terrestrial ecosystems is realised (see section 1.1.2.1). This study has generated fundamental ecological knowledge in several areas, primarily ectomycorrhizal inoculum distribution and ectomycorrhizal fungal distribution in England. Below I describe ways in which the results presented may be used in further research on the ectomycorrhizal invasion into lowland heathlands and in nationwide studies of fungi to aid conservation of the “forgotten kingdom”.

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5.4.1 Effects of environmental change on the ectomycorrhizas of lowland heathlands and neighbouring woodlands.

My thesis has produced a baseline dataset, a requirement of most ecological studies (Peay et al. 2008), to provide ecologists with the opportunity to measure for instance, the effects of nitrogen deposition and climate change on the ectomycorrhizal component of lowland heathlands and their neighbouring woodlands in years to come. Several studies have examined the effect of nitrogen deposition on heathlands yet these have focused on grass encroachment, which increases with increased nitrogen deposition (e.g., Barker et al. 2004). Ectomycorrhizal fungi have exhibited a variety of responses to nitrogen deposition in forests from no effect on community diversity or richness (Jonsson et al. 2000) to lower richness and changes in community composition (Parrent et al. 2006, Avis et al. 2008). Elevated CO₂ levels appear not to affect ectomycorrhizal richness but do have species-specific effects on abundance (Parrent et al. 2006) which may be significant but with scant information available on autecology of ectomycorrhizal taxa it is difficult to establish potential significance (Horton & Bruns 2001). I attempted to study the effects of phosphorus - which is known to increase the ability of birch to invade lowland heathlands (Manning et al. 2004, 2005) - and nitrogen addition on seedlings mycorrhizal with specific mycorrhizal fungi, but I was unable to complete the study due to difficulties in producing a sufficient number of seedlings mycorrhizal with the same fungus in the laboratory. The baseline data produced will however, allow future changes to be monitored and provide information for large-scale nutrient-addition experiments that take mycorrhizas into account realistically. The ecologically rare opportunity to grow non-mycorrhizal plants in the field as controls and to avoid mycorrhizal contamination of inoculated plants in the field represents a great advantage for mycorrhizal experiments (Nara 2008), and this situation is unexpectedly common in lowland heathlands.

5.4.2 Avenues for heathland management

The process of ectomycorrhizal invasion into lowland heathlands primarily from forest edges and sometimes from isolated spores in uninvaded heathland is similar to pine invasion in other habitats (Thiet & Boerner 2007). Traditional
methods of heathland management such as managed fires, tree cutting and grazing, do not remove the mycorrhizal community but may alter it. Substantial post-fire ectomycorrhizal inoculum was detected at Thursley NNR a year after an unmanaged fire - which is known to cause more severe damage than a managed fire (see sections 2.3.6 and 2.4.6). Birch can regenerate successfully from stumps suggesting that their ectomycorrhizal partners should still be present on their roots. Grazing controls tree invasion of heathlands and is the traditional, although little used, method for maintaining heathlands. One extreme solution would be the removal of all trees from a heathland site, however, this not ideal as woods with heathlands provide a mosaic of habitats increasing biodiversity. Yet, once a CMN is established, con-specific seedling recruitment is improved, increasing resilience of the new ecosystem and it is difficult to reverse this process which ultimately determines plant succession (Nara 2008). Ectomycorrhizal fungi will always be present near heathlands, but mammal-dispersal of spores, particularly by deer, onto heathland may be prevented by fencing.

5.4.3 Review of distribution and conservation status of ectomycorrhizal fungi

The recently published report on the strategy for fungal conservation in the UK (Anon 2009) stated that an understanding of the geographic distribution of fungi is essential to meet conservation targets. In this study, I established that fungi common to heathlands or to birch (Inocybe and Hebeloma) in other areas of the UK are not found on birch stands on lowland heathlands in southern England indicating that host or habitat presence does not always equate to the presence of fungi. The traditional sporocarp surveys used to assess fungal distribution do not represent the ecologically-relevant belowground community and future mycorrhizal molecular studies will provide the much needed assessment of the geographic distribution of ectomycorrhizal fungi.
References


References


Fleming, L.V. (1985) Experimental study of sequences of ectomycorrhizal fungi on
References

171


References


Lian, C.L., Narimatsu, M., Nara, K., & Hogetsu, T. (2006) *Tricholoma matsutake* in a natural *Pinus densiflora* forest: correspondence between above- and below-ground genets, association with multiple host trees and alteration of


References


Schloss, P.D. & Handelsman, J. (2005) Introducing DOTUR, a computer program
References


References


Appendix A - Examples of DNA techniques

A.1. DNA sequence analysis using Sequencher

To analyse the majority of my DNA sequences I used a computer programme called Sequencher (GeneCodes, Ann Arbor, MI, USA). As can be seen from the image below the programme has a clear user layout indicating the strength of the signal of each base letter and is colour coded (Figure A.1).

![Figure A.1. Example of a DNA sequence analysed using Sequencher.](image)

It is important to note that I cautiously submitted the DNA sequences to GenBank under genus or family names due to a lack of sporocarp morphological information in my study. Fungal names at species can only be undoubtedly given using sporocarp morphology and the species concept within fungi is weak hence my reference to fungi rather than species within my thesis. Throughout my thesis I have used fungal names at the species level in order to easily differentiate between fungi and I have high confidence in the names I have attributed to each fungus based on high levels of support from sequences deposited in GenBank. Two *Laccaria laccata* example sequences were submitted due to the variation between *Laccaria laccata* sequences detected at Tw and Kw. These may represent two
different species or sub-species however insufficient taxonomic studies have been completed on this potential species complex. Table A.1 lists the sequence code and fungus associated with each code as submitted to GenBank.

**Table A.1.** Representative sequences were submitted to GenBank.

<table>
<thead>
<tr>
<th>Sequence code</th>
<th>Fungus</th>
<th>Sequence code</th>
<th>Fungus</th>
</tr>
</thead>
<tbody>
<tr>
<td>FJ876156</td>
<td><em>Amanita fulva</em></td>
<td>FJ876174</td>
<td><em>Rhizopogon luteolus</em></td>
</tr>
<tr>
<td>FJ876157</td>
<td><em>Amanita muscaria</em></td>
<td>FJ876175</td>
<td><em>Suillus bovinus</em></td>
</tr>
<tr>
<td>FJ876158</td>
<td><em>Amanita rubescens</em></td>
<td>FJ876176</td>
<td><em>Suillus variegatus</em></td>
</tr>
<tr>
<td>FJ876160</td>
<td><em>Cortinarius sp.</em></td>
<td>FJ876177</td>
<td><em>Scleroderma citrinum</em></td>
</tr>
<tr>
<td>FJ876161</td>
<td><em>Clavulina sp.</em></td>
<td>FJ876178</td>
<td><em>Leccinum holopus</em></td>
</tr>
<tr>
<td>FJ876162</td>
<td><em>Laccaria laccata</em></td>
<td>FJ876179</td>
<td><em>Paxillus involutus</em></td>
</tr>
<tr>
<td>FJ876163</td>
<td><em>Laccaria laccata</em></td>
<td>FJ876180</td>
<td><em>Pseudotomentella sp.</em></td>
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<tr>
<td>FJ876164</td>
<td><em>Laccaria proxima</em></td>
<td>FJ876181</td>
<td><em>Thelephora terrestris</em></td>
</tr>
<tr>
<td>FJ876165</td>
<td><em>Lactarius hepaticus</em></td>
<td>FJ876182</td>
<td><em>Thelephoraceae</em></td>
</tr>
<tr>
<td>FJ876166</td>
<td><em>Lactarius necator-like</em></td>
<td>FJ876183</td>
<td><em>Tomentella bryophila</em></td>
</tr>
<tr>
<td>FJ876167</td>
<td><em>Lactarius rufus</em></td>
<td>FJ876184</td>
<td><em>Tomentella subilacina</em></td>
</tr>
<tr>
<td>FJ876168</td>
<td><em>Lactarius tabidus</em></td>
<td>FJ876185</td>
<td><em>Tomentellopsis sp.</em></td>
</tr>
<tr>
<td>FJ876169</td>
<td><em>Russula betularum</em></td>
<td>FJ876186</td>
<td><em>Atheliaceae sp.</em></td>
</tr>
<tr>
<td>FJ876170</td>
<td><em>Russula claroflava</em></td>
<td>FJ876187</td>
<td><em>Elaphomyces granulatus</em></td>
</tr>
<tr>
<td>FJ876171</td>
<td><em>Russula emetica</em></td>
<td>FJ876188</td>
<td><em>Elaphomyces muricatus</em></td>
</tr>
<tr>
<td>FJ876172</td>
<td><em>Russula ochroleuca</em></td>
<td>FJ876189</td>
<td><em>Cenococcum geophilum.</em></td>
</tr>
<tr>
<td>FJ876173</td>
<td><em>Russula sphagnophila</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix B – Statistical results

Bioassays in 2005

Birch bioassays – GLM with binomial errors

<table>
<thead>
<tr>
<th>Factor</th>
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<th>P</th>
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</thead>
<tbody>
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<td>Level of invasion</td>
<td>19.2</td>
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<td>&lt;0.001</td>
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<tr>
<td>Error</td>
<td>9.02</td>
<td>15</td>
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</tr>
</tbody>
</table>

An effect of site and an interaction between site and level of invasion were also tested for however these were not significant and removed from the model.

Pine bioassays – GLM with binomial errors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Deviance</th>
<th>DF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of invasion</td>
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<tr>
<td>Error</td>
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</table>

An effect of site and an interaction between site and level of invasion were also tested for however these were not significant and removed from the model.

Bioassays in 2006

Birch bioassays – GLM with binomial errors

<table>
<thead>
<tr>
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<th>Deviance</th>
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<th>P</th>
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</thead>
<tbody>
<tr>
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<td>&lt;0.001</td>
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<tr>
<td>Site</td>
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<td>5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Error</td>
<td>23.9</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

An effect of interaction between site and level of invasion was also tested for however this was not significant and removed from the model.

Pine bioassays – GLM with quasibinomial errors

<table>
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<th>Deviance</th>
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<th>P</th>
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</thead>
<tbody>
<tr>
<td>Level of invasion</td>
<td>43.4</td>
<td>2</td>
<td>&lt;0.001</td>
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</table>

References
An effect of site and an interaction between site and level of invasion were also tested for however these were not significant and removed from the model.

### Chao1 - ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
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<th>P</th>
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</thead>
<tbody>
<tr>
<td>Level of invasion</td>
<td>2</td>
<td>108.5</td>
<td>54.2</td>
<td>4.22</td>
<td>&lt; 0.05</td>
</tr>
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<td>Residuals</td>
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An effect of site was also tested for however this was not significant and removed from the model.

### Chao2 - ANOVA

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<th>P</th>
</tr>
</thead>
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</tr>
<tr>
<td>Residuals</td>
<td>15</td>
<td>164.4</td>
<td>10.9</td>
<td></td>
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</table>

An effect of site was also tested for however this was not significant and removed from the model.

### Jack1 - ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
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<th>P</th>
</tr>
</thead>
<tbody>
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<td>Level of invasion</td>
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<td>&lt; 0.01</td>
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<td>2.8</td>
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An effect of site was also tested for however this was not significant and removed from the model.

### Jack2 - ANOVA

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<tr>
<td>Residuals</td>
<td>15</td>
<td>81.4</td>
<td>5.4</td>
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</table>
An effect of site was also tested for however this was not significant and removed from the model.

Bioassays in 2007

Birch bioassays – GLM with binomial errors

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<tr>
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</thead>
<tbody>
<tr>
<td>Level of invasion</td>
<td>65.3</td>
<td>2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Site</td>
<td>27.9</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>30.4</td>
<td>28</td>
<td></td>
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</table>

An effect of interaction between site and level of invasion was also tested for however this was not significant and removed from the model.

Pine bioassays – GLM with quais-binomial errors

<table>
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</thead>
<tbody>
<tr>
<td>Site</td>
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<tr>
<td>Error</td>
<td>72.0</td>
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An effect of level of invasion and of interaction between site and level of invasion were also tested for however these were not significant and removed from the model.

Chao1 - ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
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<th>P</th>
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</thead>
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<tr>
<td>Level of invasion</td>
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<tr>
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<td>37.2</td>
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An effect of site was also tested for however this was not significant and removed from the model.

Chao2 - ANOVA

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<th>P</th>
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<tbody>
<tr>
<td>Level of invasion</td>
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<td>35.9</td>
<td>17.9</td>
<td>7.32</td>
<td>&lt; 0.01</td>
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An effect of site was also tested for however this was not significant and removed from the model.

Jack1 - ANOVA

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<tr>
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<th>P</th>
</tr>
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<tr>
<td>Site</td>
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<td>30.9</td>
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<td>Residuals</td>
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Jack2 - ANOVA

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</tr>
<tr>
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<tr>
<td>Residuals</td>
<td>10</td>
<td>28.9</td>
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Mass of seedlings

Birch bioassays containing two seedlings – ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
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<th>Mean Sq</th>
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<th>P</th>
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</thead>
<tbody>
<tr>
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<td>16.3</td>
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<tr>
<td>Mycorrhizal status</td>
<td>1</td>
<td>5.2</td>
<td>5.2</td>
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<td>&lt; 0.001</td>
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<tr>
<td>Residuals</td>
<td>34</td>
<td>13.4</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An effect of level of invasion and effects of interactions between all factors were also tested for however these were not significant and removed from the model.

Birch bioassays containing three seedlings - ANOVA

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<th>Mean Sq</th>
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<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
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<td>14.1</td>
<td>2.8</td>
<td>13.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Level of invasion</td>
<td>2</td>
<td>4.0</td>
<td>2.0</td>
<td>9.5</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
An effect of level of invasion and effects of interactions between all factors were also tested for however these were not significant and removed from the model.

**Pine bioassays containing two seedlings - ANOVA**

<table>
<thead>
<tr>
<th>Factor</th>
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<th>P</th>
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</thead>
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<td>0.0000</td>
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<td>Mycorrhizal status</td>
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<td>0.0004</td>
<td>2.44</td>
<td>&lt; 0.05</td>
</tr>
<tr>
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<td>0.0032</td>
<td>0.0006</td>
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<td>&lt; 0.01</td>
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<td>0.0000</td>
<td>0.06</td>
<td>NS</td>
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<tr>
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<td>0.0020</td>
<td>0.0003</td>
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<td>&lt; 0.05</td>
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<td>0.0001</td>
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</tbody>
</table>

**Post fire 06**

**Birch bioassays Td1 – GLM with binomial errors**

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</thead>
<tbody>
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<td>Level of invasion</td>
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</tr>
<tr>
<td>Error</td>
<td>3.84</td>
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</table>

An effect of year (2005 or 2006) and interaction between year and level of invasion were also tested for however these were not significant and removed from the model.
An effect of year (2005 or 2006) and interaction between year and level of invasion were also tested for however these were not significant and removed from the model.

Pine bioassays Td1 – GLM with binomial errors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Deviance</th>
<th>DF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of invasion</td>
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<td>2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Error</td>
<td>12.2</td>
<td>9</td>
<td></td>
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</tbody>
</table>

An effect of year (2005 or 2006) and interaction between year and level of invasion were also tested for however these were not significant and removed from the model.

Pine bioassays Td2 – GLM with binomial errors

An effect of year (2005 or 2006), level of invasion and interaction between year and level of invasion were tested for however these were not significant and removed from the model.

Post fire 07

Birch bioassays 2007 – GLM with binomial errors

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<td>&lt;0.01</td>
</tr>
<tr>
<td>Error</td>
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<td></td>
</tr>
</tbody>
</table>

An effect of site and interaction between site and level of invasion were also tested for however these were not significant and removed from the model.
Site and level of invasion individually had no effect.

**Birch bioassays Td1 2006 and 2007 - GLM with binomial errors**

<table>
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<tr>
<td>Error</td>
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<td>8</td>
<td></td>
</tr>
</tbody>
</table>

An effect of level of invasion was also tested for however this was not significant and removed from the model.

**Pine bioassays Td2 2006 and 2007 – GLM with binomial errors**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Deviance</th>
<th>DF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction between year and level of invasion</td>
<td>5.4</td>
<td>1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Error</td>
<td>14.1</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Level of invasion and year individually had no effect.

**Loss of seedlings - ANOVA**

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat</td>
<td>1</td>
<td>182.5</td>
<td>182.5</td>
<td>3.05</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>4788</td>
<td>4788</td>
<td>80.08</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Interaction between habitat and day</td>
<td>1</td>
<td>308.3</td>
<td>308.3</td>
<td>5.16</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Residuals</td>
<td>26</td>
<td>1554</td>
<td>59.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
There was no significant effect of date or plot on the proportion of seedlings which were mycorrhizal.

Mass of seedlings in heathland and woodland plots - ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycorrhizal status</td>
<td>1</td>
<td>2.3</td>
<td>2.3</td>
<td>9.53</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Residuals</td>
<td>21</td>
<td>5.1</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An effect of date and an interaction between date and mycorrhizal status were also tested for however these were not significant and removed from the model.

Naturally occurring seedlings from all sites – GLM with quais-binomial errors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Deviance</th>
<th>DF</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of invasion</td>
<td>311.89</td>
<td>2</td>
<td>22.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Error</td>
<td>35.23</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>