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Waiting for fungi: the ectomycorrhizal invasion of lowland heathlands

Fay A. Collier* and Martin I. Bidartondo

Imperial College London and Royal Botanic Gardens, Kew, TW9 3DS, UK

Summary

1. In England, the loss of lowland heathland, a habitat of global conservation importance, is primarily due to the invasion of birch and pine. This encroachment has been researched in depth from a plant perspective but little is known about the role of mycorrhizal fungi. In lowland heathlands the resident dwarf shrubs form ericoid mycorrhizas whereas invading trees form ectomycorrhizas. Therefore, tree encroachment into heathlands can be regarded as the replacement of a resident mycorrhizal community by an invading one.

2. This study examined how fungi form mycorrhizas with *Betula* and *Pinus* in lowland heathlands. We addressed the question of whether there are mycorrhizal fungi that mediate invasion using a molecular ecology approach to compare the mycorrhizal inoculum potential of soil at three levels of invasion (uninvaded heathland, invaded heathland and woodland) and the fungi forming mycorrhizas on tree seedlings and trees across diverse sites.

3. We show that in lowland heathlands: (i) seedlings have severely limited access to ectomycorrhizal fungi relative to woodlands, (ii) there are few keystone spore-dispersed ectomycorrhizal fungi that can mediate tree invasion, (iii) tree seedlings can remain non-mycorrhizal for at least one year when no inoculum is present, even near saplings, and (iv) mycorrhizal seedlings achieve greater biomass than non-mycorrhizal seedlings. Within uninvaded heathland we detected only *Rhizopogon luteolus*, *Suillus variegatus*, *S. bovinus* (*Pinus* symbionts) and *Laccaria proxima* (primarily a *Betula* symbiont).

4. *Synthesis*. Overall, ectomycorrhizal inoculum in lowland heathlands is rare; most tree seedlings growing in heathland soil are not mycorrhizal due to limited spore dispersal, poorly developed spore banks and weak common mycorrhizal networks. These seedlings can persist awaiting mycorrhization to boost their growth.

Key-words: *Betula*, dispersal, ecology, fungi, heathland, invasion, mycorrhizas, *Pinus*

Introduction

Worldwide, native ectomycorrhizal (ECM) trees invade highly valued conservation areas and eventually replace them with woodlands (Crawley 1997; Andersen & Baker 2006; Thiet & Boerner 2007). For example, lowland heathland is a priority habitat of the United Kingdom Biodiversity Action Plan threatened primarily by the native ectomycorrhizal trees *Betula pendula* Roth., *B. pubescens* Ehrh. (birch) and/or *Pinus sylvestris* L. (Scots pine) (Gimingham 1992; Mitchell *et al*. 1997; Richardson & Higgins 1998). Nutrient acquisition for mycorrhizal plants is carried out by horizontally transmitted and obligately biotrophic fungi in exchange for *c*. 15% of the plants' photosynthate (Smith & Read 2008). In the case of *Betula* and *Pinus*, ectomycorrhizas are formed with Basidiomycete and Ascomycete fungi. In contrast, heathlands are dominated by dwarf Ericaceae shrubs associated with ericoid mycorrhizal fungi (predominately Helotiales, Ascomycota) growing in exceptionally nutrient-poor soils (Specht 1981; Rundel 1988; Read 1989). Thus, the widespread tree invasions of heathlands are also ectomycorrhizal invasions of ericoid mycorrhizal communities.

Unfortunately, even simple criteria such as rates and thresholds for fungal invasions of natural environments are largely unknown (Otten, Bailey & Gilligan 2004). Consequently, the potentially key role of ectomycorrhizal fungi in plant invasions remains essentially a matter of speculation (Richardson *et al*. 2000; Simberloff, Relva & Nuñez 2002), even though both the study of positive biotic interactions such as facilitation and mutualism (Bruno, Stachowicz & Bertness 2003) and ectomycorrhizal ecology (Peay, Kennedy & Bruns 2008) are burgeoning fields of research. To date, we lack the most basic information concerning the early symbiotic events involved in the successful establishment of

*Correspondence author. E-mail: f.collier04@imperial.ac.uk

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ectomycorrhizal trees and early plant–fungal symbiotic events leading to successional dynamics (Read 1998; Horton & van der Heijden 2008; Smith & Read 2008), except in the case of primary succession thanks to the landmark work of Nara and colleagues at Mount Fuji in Japan (Nara, Nakaya & Hogetsu 2003a; Nara *et al.* 2003b; Nara & Hogetsu 2004; Nara 2006a,b, 2008) and post-fire succession due to the work by Bruns and colleagues at Point Reyes in California, USA (Gardes & Bruns 1996; Baar *et al.* 1999; Grogan, Baar & Bruns 2000; Bruns *et al.* 2002; Kennedy *et al.* 2007). Ectomycorrhizal tree invasion into arbuscular mycorrhizal habitats dominated by grasses is beginning to be examined (e.g. Dickie & Reich 2005; Thiet & Boerner 2007). However, primary successional habitats bear little similarity to lowland heathlands both in terms of vegetation and geographic proximity (the closest site to the present study area is over 3500 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. Studies have also indicated that ectomycorrhizal inoculum potential (i.e. wind- and/or animal-dispersed ectomycorrhizal fungal propagules) outside, but adjacent to, woodland areas can be restricted both in spatial distribution and species composition (Boerner, DeMars & Leicht 1996; Bidartondo, Baar & Bruns 2001; Dickie & Reich 2005; Haskins & Gehring 2005). As far as we are aware, this is the first study on the role of ectomycorrhizas in any tree invasion in Britain.

Tree encroachment into British heathlands has not escaped the attention of ecologists and consequently it is relatively well characterized from a plant perspective (Gimingham 1978; Miles & Kinnaird 1979). Factors known to determine susceptibility to tree invasion within heathlands include seed availability (Hester, Gimingham & Miles 1991a; Mitchell *et al.* 1997; Manning, Putwain & Webb 2004, 2005), soil nutrients (Hester, Miles & Gimingham 1991b; Mitchell *et al.* 2000; Manning, Putwain & Webb 2004, 2005), disturbance (Manning, Putwain & Webb 2004, 2005), gap formation (Marrs 1987) and heath successional stage (Khoon & Gimingham 1984). Preventing heath-to-woodland succession and encouraging heath 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 densely populated southern England. In fact, controlling invasions of lowland heaths by *Betula* (which re-sprouts from stumps) is rarely cost-effective (Mitchell *et al.* 2000). As a consequence, lowland heathlands provide a uniquely relevant ecosystem to unravel the pathways and mechanisms of a largely unchecked native ectomycorrhizal invasion. As far back as 1979, Miles & Kinnaird hypothesized that a lack of ectomycorrhizal inoculum in heathlands slows invasion by birch and pine and that there are ectomycorrhizal fungal species-specific effects on the establishment of tree seedlings that invade heathlands. Here, we will test four related, but more specific, hypotheses:

1. Ectomycorrhizal inoculum in heathlands is less abundant and diverse than in woodlands.
2. Pioneer, spore-dispersed, ectomycorrhizal fungi are predominant on heathland seedlings.
3. Where saplings or trees are present, seedlings are colonized *via* vegetative fungal growth rather than spore colonisation.
4. Mycorrhizal seedlings achieve greater biomass than non-mycorrhizal seedlings.

So far, the greatest array of studies of any ectomycorrhizal fungal succession (i.e. dispersal, establishment, persistence and replacement) was carried out in the 1980s using a young *B. pendula* plantation at the Bush Estate in Scotland as a model system (Ford, Mason & Pelham 1980; Deacon, Donaldson & Last 1983; Fleming 1983, 1984, 1985; Fox 1983; Last *et al.* 1983, 1984; Mason *et al.* 1983; Dighton & Mason 1984; Mason, Wilson & Last 1984; Fleming, Deacon & Last 1986; Last, Dighton & Mason 1987; Gibson & Deacon 1988; Deacon & Fleming 1992). These studies established the successional dynamics of 'early' through to 'late' stage fungi forming ectomycorrhizas with *Betula*. However, these studies were conducted on agricultural soil and relied mainly on fungal fruiting body 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) and to a lesser extent on morphological identification of fungi from ectomycorrhizal roots, which is a low-resolution and low-throughput approach compared to today's DNA-based identification (Peay, Kennedy & Bruns 2008). In addition, it is possible that other fungi may form mycorrhizas with invading tree seedlings. Some studies have suggested that there are shared ectomycorrhizal-ericoid fungi in *P. sylvestris* forest habitats with an ericaceous understorey, leading Vrålstad, Fossheim & Schumacher (2000) to hypothesize that members of the ericoid mycorrhizal clade *Rhizoscyphus ericae* can form both ecto- and ericoid mycorrhizas. This is yet to be tested under field conditions, where it may explain *P. sylvestris* encroachment into heathlands without the need for, or in addition to, strictly ectomycorrhizal fungi. Similarly, arbuscular mycorrhizas have been reported in *B. pubescens* (Haigh 2001) and in *Pinus muricata* (Horton, Cazares & Bruns 1998), albeit in areas dominated by arbuscular mycorrhizal plants.

In this study, we used seedling bioassays to determine whether the inoculum potential of ectomycorrhizal fungi varies within lowland heathlands with respect to the level of tree invasion within different sites. We complemented our data by sampling tree roots at six sites, naturally established tree seedlings at five sites and outplanted tree seedlings at one site. The sampling of seedlings *in situ* provides us with the opportunity to assess whether bioassays are selective for fungi that colonize seedlings via spores (Newton 1991). Mycorrhizal fungi cannot usually be identified by the morphology of their mycorrhizas alone; therefore, we sequenced fungal DNA from single ectomycorrhizas (i.e. a DNA barcoding approach), which is a more accurate and precise approach than using pooled roots and/or screening with restriction enzymes. The study aims to

determine whether ectomycorrhizal fungal inoculum is homogeneously distributed in heathland soils and to identify which mycorrhizal fungi are involved in tree invasion of heathlands. Specifically, we predicted that ectomycorrhizal diversity differs between uninvaded heathland, invaded heathland and woodland soils (see Materials and methods for definitions).

Materials and methods

STUDY SITES

Seven lowland heathlands in England (abbreviated Cd, Gd, Hw, Kw, Sw, Td, Tw, see Table 1 for details) were surveyed for mycorrhizal fungi. All of these sites are National Nature Reserves (NNR). Five of the sites (Hw, Kw, Sw, Td and Tw) are being invaded by *Betula* spp. and *P. sylvestris*, one (Cd) only by *Betula* spp. and one (Gd) only by *P. sylvestris*. All main types of lowland heathland were surveyed: wet, dry, dune and Breck (a mosaic of grasses and heathers). In field studies, we did not differentiate between *B. pendula* and *B. pubescens* due to their high morphological, ecological and physiological similarities and their overlapping distribution. However, drier areas are generally invaded by *B. pendula* and wetter areas by *B. pubescens* (Atkinson 1992). The sites closest to each other are Tw and Td at *c.* 1.2 km apart and the greatest distance between sites is 250 km between Cd and Sw.

MYCORRHIZAL INOCULUM POTENTIAL BIOASSAYS

To assess mycorrhizal inoculum potential six square 1.5 m^2 plots, two in each of three levels of invasion, were selected at six sites (Cd, Gd, Hw, Kw, Sw and Tw). Levels of invasion were defined as: uninvaded heathland, an area without *Betula* or *Pinus* trees or saplings; invaded heathland, an area with young isolated *Betula* and/or *Pinus* saplings (typically 0.5–1 m tall); and woodland, an area with trees (over 1.5 m tall) and a heath understorey. Plots were set up away from footpaths to avoid disturbance and they were sampled between 29 May and 9 July 2007. Plots were 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 25 m apart (average = 14 m) in woodland. The variation is due to our goal of finding areas with appropriate levels of invasion and similar vegetation across all sites.

From within each plot, five soil cores (*c.* 2.5 cm diameter × 20 cm depth) were removed from arbitrary positions. The soil corer was cleaned with household bleach between sampling each plot. At each site, the uninvaded plots were sampled first to further minimize 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 6 days of soil collection (average of 2 days). Each soil core was manually homogenized in a sterile plastic bag and no material such as roots or small stones was removed. The soil from each core was divided between two bioassay tubes (RLC-3UV Ray Leach Conetainer; Stuewe & Sons Inc., Corvallis, OR, USA), each containing *c.* 45 cm^3 of soil. Control bioassays were set-up using autoclave-sterilized soil originating from a mix of soil cores; the soil was sterilized twice, 48 h apart. A small amount of porous non-organic cloth was placed at 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 *B. pendula* seeds and one with *P. sylvestris* seeds. Seeds were surface-sterilized in diluted bleach (0.25%) and rinsed with deionized water. If seeds did not germinate, additional seeds were added on subsequent days. A cutting of *Calluna vulgaris* was planted in each bioassay tube to more closely reproduce the heathland environment. The cutting used was from the site where the soil was obtained. On occasions when the cutting died, 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 (18 °C for 16 h at 270–290 μmol m^{-2} s^{-1} photon flux density, 16 °C for 8 h in dark, at 60% relative humidity) in ventilated 5 × 23 cm plastic bags to prevent cross-contamination, and they were watered as necessary to retain soil moisture. After 4 months, seedlings were removed from the bioassay tubes, rinsed and their roots were manually disentangled from the soil and ericoid hair root mass and examined using a dissecting microscope. Bioassays were recorded as mycorrhizal if an ectomycorrhiza was present on at least one seedling within the bioassay or as non-mycorrhizal if no ectomycorrhizas were detected on any seedlings in the bioassay. Some seedlings had roots that were not ectomycorrhizal but had morphological features 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 developed 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 ectomycorrhizal morphotype present in each individual bioassay tube was obtained and its DNA was extracted immediately. If other roots of the same morphotype were present, representatives were placed in deionized water, frozen to −80 °C and lyophilized. Seedlings were then lyophilized and weighed. DNA was extracted using Extract-N-Amp (Sigma,

Table 1. Description of the seven lowland heathland sites sampled. Abbr., Abbreviated name. NA indicates that sampling did not take place

Abbr.	Site	County	UK National Grid Reference	Type	Trees	Date*	Date†
Cd	Cavenham Heath	Suffolk	TL 753 724	Breck, dry	*Betula*	NA	18/6/07
Gd	Studland and Godlingston Heath	Dorset	SZ 014 826	Dune, dry	*Pinus*	NA	29/6/07
Hw	Holt Heath	Dorset	SU 065 045	Wet	*Betula*, *Pinus*	13/6/05	14/6/07
Kw	Kingston Great Common	Hampshire	SU 186 035	Wet	*Betula*, *Pinus*	24/5/05	29/5/07
Sw	Stoborough Heath	Dorset	SY 936 849	Wet	*Betula*, *Pinus*	14/7/05	26/6/07
Td	Thursley	Surrey	SU 909 406	Dry	*Betula*, *Pinus*	Various‡	NA
Tw	Thursley	Surrey	SU 907 418	Wet	*Betula*, *Pinus*	7/7/05	9/7/07

*The date of sampling for naturally occurring seedlings is shown as Date.
†The date of sampling for bioassays and tree roots is shown as Date.
‡Naturally occurring seedlings were collected from Td at various dates from May 2005 to October 2006.

Dorset, UK); each root was placed in 10 μL of extraction solution in a 96-well plate, heated to 95 °C for 10 min, allowed to cool to room temperature, and then 10 μL of dilution solution were added and mixed.

IN SITU MYCORRHIZAS

When collecting soil cores for bioassays, an additional five cores were removed per woodland plot to examine ectomycorrhizas. The contents of these cores 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 was extracted immediately using the method described above. The roots of *Betula* sp. and *P. sylvestris* were not distinguished when sampling from mixed woodlands. Remaining samples of each morphotype were stored in deionized water at −80 °C and lyophilized.

Naturally occurring seedlings were sampled from different levels of invasion and from different sites to identify the fungi forming ectomycorrhizas with naturally occurring seedlings and to establish whether mycorrhizal seedlings have a greater biomass than non-mycorrhizal seedlings. From the beginning of germination (18 May 2005) until 27 June 2005 (by which date all seedlings had died or been removed) naturally occurring seedlings in their first year of growth were sampled from six 75 × 75 cm grids (three in woodland and three in invaded heathland) every 10 days at Td. Additional seedlings in their first year of growth were sampled on eleven dates from Td between the 6 June 2005 and 17 October 2005 from outside of the sampling grids at Td, and seedlings estimated to be under 3 years old were opportunistically sampled from four sites (Hw, Kw, Sw and Tw) in 2005 and 2006 and from Td in 2006. The location of these additional seedlings was recorded with regard to level of invasion. Seedlings and surrounding soil were cut out with a soil knife. Then the entire root system of each seedling was thoroughly extricated from the dense mass of ericoid hair roots characteristic of heathland soils using water and tweezers. Samples of mycorrhizas were removed and their DNA was extracted following Gardes & Bruns's (1993) protocol modified for silica binding and purification (Gene-Clean, Q-Biogene). Both the shoot and root of all seedlings sampled from Td in 2005 were then freeze-dried before weighing.

OUTPLANTED SEEDLINGS

In August 2006, two transects were laid out radiating away from each of four *Betula* and ten *P. sylvestris* focal saplings at Tw. Transects were located so that they were at least 4 m away from any other sapling. On 31 August, 15 September and 25 September 2006, five seedlings (either *P. sylvestris* or *B. pendula* to match the sapling) were planted at each of four points along each transect. The outplanted non-mycorrhizal seedlings originated from seeds sown on a pasteurized peat:sand (2 : 1 by volume) mix in 576-cell plug trays (LBS Group, Lancashire, UK) lined with porous non-organic cloth. The trays were placed in a growth chamber for 23 days and then transferred to a cold frame for 5 days. For seedling outplanting, the height of each focal sapling was recorded (between 0.4 and 0.76 m tall) and points 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). Estimates of the rooting zone of *Betula* and *Pinus* were obtained after a wildfire exposed root systems at Td and Tw; post-fire, the length of seven exposed roots from four *Betula* and 14 exposed roots from 13 *Pinus* trees were measured along with the height of the corresponding individual. Using these values, regression analysis indicated that the root systems extended up to at least 1.8 and 1.1 times the height of *Betula* and *Pinus* trees, respectively. Additionally, three control transects were set-up for each species with no focal sapling, the points along these three transects were 0.5 m apart. On 31 October 2006, one or two seedlings were harvested from each point. Seedling survival was recorded at the date of the first harvest in October 2006, after snowfall on 4 February 2007 and on 14 April 2007. After 5 months, on 16 April 2007, all remaining seedlings were harvested.

ENDOMYCORRHIZAL ASSESSMENT

Following a bioassay study conducted in 2006 at six sites (Cd, Gd, Hw, Kw, Sw and Td) at three invasion levels, *B. pendula*, *P. sylvestris* and *Calluna vulgaris* root samples from 169 bioassays (representing all sites and invasion levels) were stained with acid fuchsin following the method in Appendix A of Peterson & Massicotte (2004). Stained roots were viewed under a light microscope to detect endomycorrhizal structures (i.e. arbuscules, coils).

FUNGAL IDENTIFICATION

Polymerase chain reaction (PCR) amplification of the internal transcribed spacer region using primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) was attempted for all samples. An aliquot of 2 μl of extracted DNA was combined with 8 μl of 2x Extract-N-Amp solution or PicoMaxx reaction mix (Stratagene, Cedar Creek, TX, USA). Amplifications were performed with an initial denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 55 s and 72 °C for 50 s, with a final extension of 72 °C for 7 min. PCR products were visualized on a 2% agarose gel with ethidium bromide. If bands of the PCR products were barely visible, a nested PCR of the sample was performed using ITS1 (White *et al.* 1990) and ITS4 from a 1 : 100 H_2O 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, Foster City, CA, USA). The cycle-sequenced products were separated by electrophoresis using an ABI3730 Genetic Analyzer (Applied Biosystems). The DNA sequences were edited in Sequence Navigator (Applied Biosystems) or Sequencher (GeneCodes, Ann Arbor, MI, USA). Preliminary identification was achieved by conducting a BLASTn search on GenBank (http://www.ncbi.nlm.nih.gov/blast). DNA sequences were then aligned using Clustal X (Jeanmougin *et al.* 1998), visually corrected, and a neighbour-joining tree was generated using PAUP* v. 4.0b10 (Swofford 2002). Samples of the same fungal genus or within the same terminal clade of the neighbour-joining tree were then re-aligned with congeners from GenBank using Muscle (Edgar 2004) and visually corrected using MacClade (Maddison & Maddison 2003). Identifications were confirmed using a combination of producing a neighbour-joining tree using PAUP and analysis with DOTUR (Schloss & Handelsman 2005). Representative DNA sequences have been submitted to GenBank under accession numbers FJ876156 to FJ876189.

STATISTICAL ANALYSIS

Unless otherwise stated, all statistical analyses were conducted using R version 2.7.2 (R Development Core Team 2008) and all models reported are the minimum adequate models; models were tested for potential simplification by testing for significant differences between

models with ANOVA. Differences and interactions between sites and invasion level in the proportion of bioassays within a plot that were mycorrhizal versus non-mycorrhizal were tested by applying a generalized linear model (GLM) with binomial errors or quasi-binomial errors if needed, to account for over-dispersion. This analysis was conducted separately for *B. pendula* bioassays and *P. sylvestris* bioassays. Richness and diversity indices calculations omitted the two samples of fungi that could not be unambiguously assigned to a taxon, due to a lack of significant similarity with any samples in GenBank. Fungal richness estimators and diversity indices were calculated using the number of soil cores containing each fungus in order to compare seedling bioassay and mature tree root data. The number of fungi was pooled among sites within the same level of invasion to compare richness and diversity between levels of invasion and mature tree roots. The Shannon index (H') takes into account the number and evenness of species using the equation:

$$H' = -\sum (p_i \log_{10} p_i),$$

where 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 same level of invasion.

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. We report Simpson's reciprocal index ($1/D$). Simpson's Index (D) is calculated as:

$$D = \sum ((n(n-1))/(N(N-1))),$$

where n = the total number of soil cores containing a particular fungus within the same invasion level, and N = the total number of soil cores containing all fungi within the same invasion level. Fungal richness estimates – first-order Chao (Chao1), second-order Chao (Chao2), first-order Jackknife (Jack1) and second-order Jackknife (Jack2) – were calculated using EstimateS (Colwell 2005) with a soil core as sample unit to allow for comparisons between bioassays and mature tree roots. Each estimate was based on 50 randomizations of sample order without replacement. Ectomycorrhizal fungal accumulation curves were calculated in EstimateS using the same data set. Using data describing the presence and absence of ectomycorrhizal fungi at each site where bioassays were conducted, we conducted a non-metric multi-dimensional scaling analysis to assess differences in the ectomycorrhizal community among sites.

To test for an effect of site, level of invasion, mycorrhizal status and any interactions on the number of seedlings in a bioassay, we conducted a GLM with a Poisson error distribution. The effect of site, level of invasion, mycorrhizal status and any interactions on average seedling mass in bioassays was tested with a three-way ANOVA. This analysis was conducted separately for *P. sylvestris* bioassays containing two seedlings, *B. pendula* bioassays containing two seedlings and *B. pendula* bioassays containing three seedlings. The analysis was not conducted for *B. pendula* or *P. sylvestris* bioassays containing any other number of seedlings at harvest. Nearly, all *P. sylvestris* bioassays contained two seedlings (n = 163), and the majority of *B. pendula* bioassays contained two (n = 41) or three seedlings (n = 62). We transformed birch mass data using the natural log to normalize the residuals. Fungal-specific effects on bioassay seedling mass were tested using Minitab (Minitab Inc. 2000) 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, but only when the bioassays contained the same number of mycorrhizal seedlings at harvest and the number of bioassays containing an individual fungus was greater than five. We tested for an effect of mycorrhizal status on the biomass of naturally occurring seedlings at Td using a Kruskal–Wallis test. We also tested for an effect of the level of invasion on the proportion of seedlings which were mycorrhizal versus non-mycorrhizal using a GLM with quasi-binomial errors with data from naturally occurring seedlings under 1 year old sampled from five sites.

Results

MYCORRHIZAL INOCULUM POTENTIAL BIOASSAYS

In total, 360 experimental and 10 control bioassays were set up. Of the 359 experimental bioassays with live seedlings at harvest, 404 root samples were used for DNA sequencing. The proportion of bioassays containing mycorrhizal seedlings was lowest in uninvaded heathland soil bioassays (7.5%, n = 9) and highest in the woodland soil bioassays (48%, n = 58). All control bioassays yielded non-mycorrhizal seedlings. The level of invasion (uninvaded heath, invaded heath or woodland) and site had a highly significant effect on the proportion of *B. pendula* bioassays containing non-mycorrhizal seedlings (GLM with binomial errors; invasion level, $F = 65.3_{2,28}$, $P < 0.001$; site, $F = 27.9_{5,30}$, $P < 0.001$, Fig. 1a). There was

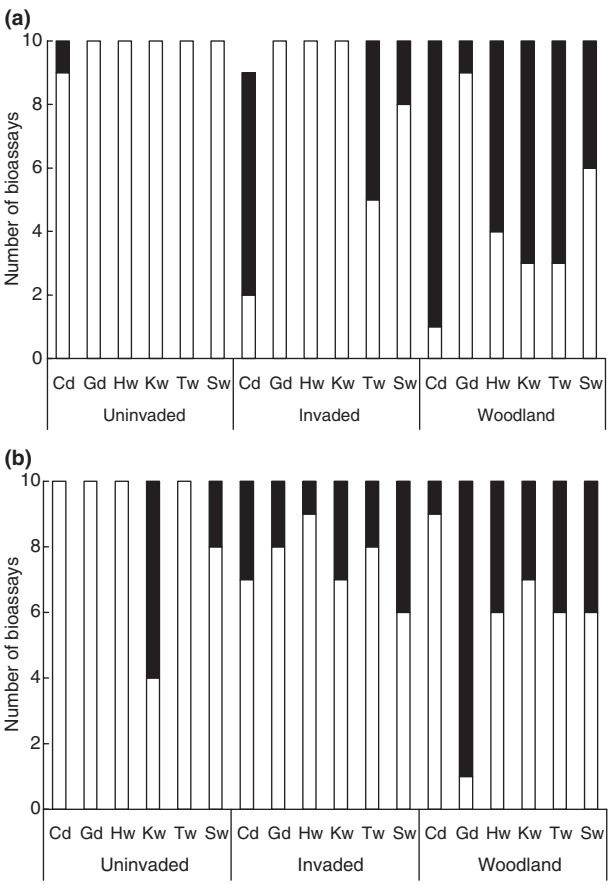


Fig. 1. The number of mycorrhizal (black) and non-mycorrhizal (open) *Betula pendula* bioassays (a) and *Pinus sylvestris* bioassays (b) at each of six lowland heathlands (see Table 1 for site abbreviations).

no significant effect of site on the overall proportion of non-mycorrhizal *Pinus* bioassays, but level of invasion had a weak effect on the overall proportion of non-mycorrhizal bioassays (GLM with quasi-binomial errors, $F = 3.11_{2,33}$, $P = 0.058$, Fig. 1b). There were site-dependent differences in the proportion of *B. pendula* and *P. sylvestris* seedlings becoming mycorrhizal. Cd is invaded solely by *Betula* resulting in nine of the ten *B. pendula* bioassays and one of the ten *P. sylvestris* bioassays grown in woodland soil being mycorrhizal at harvest. The reverse occurred at Gd, which is invaded by *P. sylvestris* only. The *C. vulgaris* cuttings survived in 83% of the experimental bioassays, but only 20% of the control bioassays. Bioassays varied in the number of live seedlings they contained at harvest (between one and three *P. sylvestris* seedlings or between one and eight *B. pendula* seedlings). To eliminate any effects of competition only bioassays containing the same number of seedlings were analysed for effect of mycorrhizal status on biomass (two seedlings for *P. sylvestris* bioassays, *B. pendula* bioassays containing two seedlings and three seedlings were analysed independently). The number of *B. pendula* seedlings in a bioassay was not significantly affected by mycorrhizal status of the bioassay or the level of invasion. Site had a significant effect on the number of *B. pendula* seedlings in a bioassay ($\chi^2 = 14.5_{5,172}$, $P < 0.05$), the number of seedlings per bioassay was lowest at Hw and highest at Tw (average of 2.4 and 3.9 seedlings per bioassay, respectively). The number of seedlings in a *P. sylvestris* bioassay was not affected by site, level of invasion or mycorrhizal status. For *B. pendula* bioassays containing two seedlings, site and mycorrhizal status had significant effects on average seedling biomass (site $F = 8.3_{5,34}$, $P < 0.001$; mycorrhizal status $F = 13.2_{1,34}$, $P < 0.001$). At the four sites where *B. pendula* mycorrhizal and non-mycorrhizal bioassays were present, the average biomass of seedlings in mycorrhizal bioassays was greater than that of seedlings in non-mycorrhizal bioassays. For *B. pendula* bioassays containing three seedlings, the site, level of invasion and mycorrhizal status had significant effects on seedling biomass (site $F = 13.5_{5,53}$, $P < 0.001$; level of invasion $F = 9.5_{2,53}$, $P < 0.001$; mycorrhizal status $F = 5.5_{1,53}$, $P < 0.05$; Fig. 2). On four out of five occasions when mycorrhizal and non-mycorrhizal bioassays were present at the same level of invasion at the same site, the average mass of *B. pendula* seedlings in mycorrhizal bioassays was greater than that in non-mycorrhizal bioassays.

For *P. sylvestris* bioassays, site and mycorrhizal status independently influenced biomass and there were multiple significant interactions. Mycorrhizal status and site showed significant effects ($F = 8.9_{2,131}$, $P < 0.01$, $F = 2.6_{5,131}$, $P < 0.05$, respectively), and there were significant two-way interactions between site and mycorrhizal status, level of invasion and site ($F = 4.3_{5,131}$, $P < 0.01$, $F = 2.4_{10,131}$, $P < 0.05$, respectively) and the three-way interaction between site, level of invasion and mycorrhizal status was also significant ($F = 2.3_{6,131}$, $P < 0.05$). On ten out of fourteen occasions when *P. sylvestris* mycorrhizal bioassays and non-mycorrhizal bioassays co-occurred within the same level of invasion at the same site, the average mass of mycorrhizal bioassay seedlings was heavier than non-mycorrhizal bioassay seedlings. Although the study was not designed to address fungal species-specific effects on seedling biomass, tests indicated there was no significant difference in the mass of *P. sylvestris* seedlings colonized by either *Lactarius hepaticus*, *Rhizopogon luteolus*, *Suillus bovinus* or *S. variegatus* compared to other mycorrhizal bioassays. The mass of *B. pendula* bioassay seedlings mycorrhizal with *Lactarius hepaticus* or *Laccaria proxima* was not significantly different to all other mycorrhizal *B. pendula* bioassay seedlings.

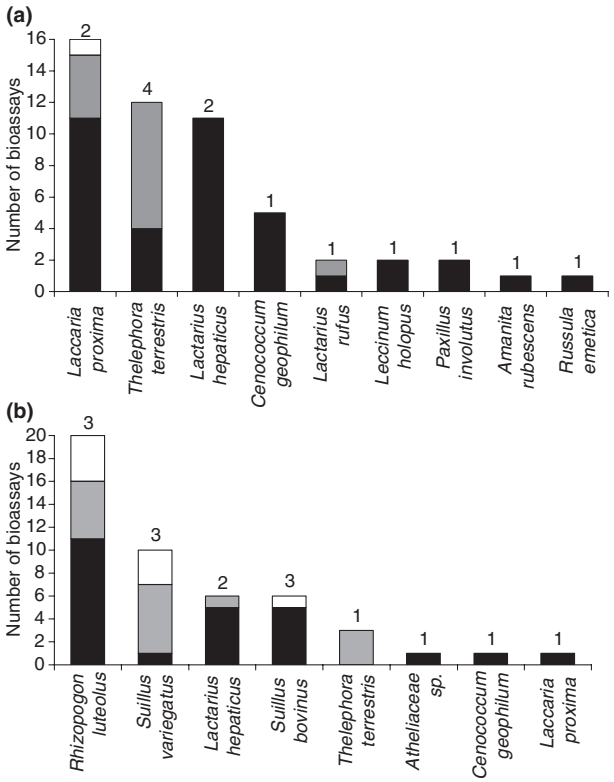


Fig. 2. Cumulative number of (a) *Betula pendula* and (b) *Pinus sylvestris* soil bioassays from each level of lowland heathland tree invasion for each identified ectomycorrhizal fungus across six sites. Black bars indicate woodland, grey bars invaded heath and open bars uninvaded heath. Numbers above bars indicate the number of sites at which the fungus was detected.

Overall, mycorrhizal fungal diversity increased from uninvaded to invaded heathland to woodland (Table 2). Mature trees supported a more diverse mycorrhizal community than that detected on seedlings in bioassays at all sites. Estimated fungal richness also increased with the level of tree invasion. Only *Laccaria proxima*, *Rhizopogon luteolus*, *Suillus bovinus* and *S. variegatus* formed mycorrhizas with seedlings grown on soil from uninvaded heathland (Fig. 2a,b). The only mycorrhizal *B. pendula* bioassay from uninvaded heathland soil was colonized by *Laccaria proxima*, this fungus was prevalent in Cd bioassays (forming mycorrhizas on thirteen of the seventeen mycorrhizal *B. pendula* bioassays). In contrast, *Laccaria proxima* only colonized one of the *P. sylvestris* bioassays at Cd. The proportion of mycorrhizal *P. sylvestris* bioassays grown on uninvaded heathland soil from Kw was high compared to the

Table 2. Ectomycorrhizal fungal richness and diversity in uninvaded and invaded heathland and neighbouring woodland detected from seedling bioassays and *in situ* mature tree root ectomycorrhizas

		Diversity indices		Estimated richness			
Level of invasion	Number of fungi	Shannon (H')	Simpson's reciprocal ($1/D$)	Chao1	Chao2	Jack1	Jack2
Uninvaded	4	0.53	4.00	5.00	4.98	5.97	7.90
Invaded	6	0.66	5.64	7.00	6.98	7.97	9.90
Woodland	12	0.88	8.73	16.33	16.28	17.92	20.85
Tree roots	22	1.23	15.06	23.20	23.18	25.93	26.00

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 closely related fungi are specific to Pinaceae and accordingly, all *B. pendula* bioassays from the same plots were non-mycorrhizal (Fig. 1a,b). The most prevalent fungus forming mycorrhizas in *P. sylvestris* bioassays was *Rhizopogon luteolus* (Fig. 2b). *Laccaria proxima*, *Thelephora terrestris* and *Lactarius hepaticus* were the most prevalent fungi in *B. pendula* bioassays (Fig. 2a). Most fungi identified in the bioassays were only detected at one or two field sites except for three of the more prevalent species, which were at three sites (*Rhizopogon luteolus* and *S. variegatus*) or four sites (*T. terrestris*). *Suillus bovinus* was less abundant but it was also present at three sites. The identity of the fungi forming mycorrhizas on two bioassays remains unconfirmed, e.g. neither they nor their closest relatives are currently represented in GenBank. Non-metric multidimensional scaling generally clusters plots by heathland site rather than by invasion level (Fig. S1).

IN SITU MYCORHIZAS

In total 219 DNA sequences were obtained from tree root samples of 48 soil cores. Some 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 of fungi 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 (40 soil cores) were used for analysis. The data were analysed as the number of fungi within each soil core and duplicates were 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 (total ten cores per site) from Cd, Hw, Kw and Tw.

More fungi were detected on tree roots than on the woodland bioassays (22 and 13, respectively, Fig. 3). *Lactarius hepaticus* was the most prevalent fungus on mature tree roots. Three of the fungi that formed ectomycorrhizas on bioassay seedlings did not occur on any of the mature tree roots sampled (*Laccaria proxima*, *Leccinum holopus* and *Suillus bovinus*). One of these, *Leccinum holopus* only occurred on soil bioassays from Sw where no mature tree ectomycorrhizas were obtained. Additionally, neither of the unidentified fungi from the bioassays was detected on mature tree roots;

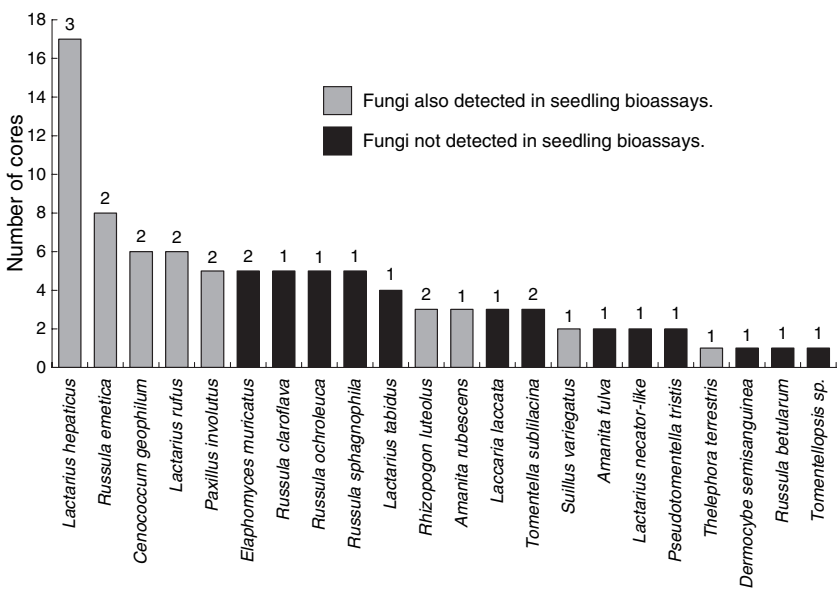


Fig. 3. Cumulative number of soil cores containing each ectomycorrhizal fungus detected on *in situ* tree roots in woodland soil at six lowland heathlands. The number above each bar indicates the number of sites where each fungus was detected.

however, these fungi originated from sites where no tree root ectomycorrhizas could be obtained from soil cores. Nine of the 22 fungi on mature tree roots also formed ectomycorrhizas with bioassay seedlings; these included the five most common tree root symbionts. In addition to well-known ectomycorrhizal fungi, a *Ceratobasidium* sp. was detected forming ectomycorrhizas in two soil cores from Kw. We know of only one previous report of *Ceratobasidium* ectomycorrhizas (Yagame *et al.* 2008). The majority of fungi within each level of invasion were detected as indicated by the near saturation of fungal accumulation curves (Fig. 4).

The number of naturally occurring seedlings less than 1 year old sampled from each site varied from two (Hw) to 1,020 (Td; Table 3). An additional 61 seedlings aged between 1 and 3 years were also sampled from four sites (Hw, Kw, Td and Tw) and 26 of these seedlings were non-ectomycorrhizal. The 1088 naturally occurring seedlings less than 1 year old and analysed for mycorrhizal status show a similar trend as bioassay seedlings in that the proportion of mycorrhizal seedlings increases as the level of invasion increases (Table 3).

In total, 242 seedlings were sampled from invaded heathland grids and no mycorrhizas were detected on any seedling. In contrast, of the seedlings sampled from woodland grids, 275 out of 388 were mycorrhizal. Outside of the grids at Td 49% of seedlings sampled from invaded heathland were mycorrhizal ($n = 83$) and none of the 192 seedlings sampled from otherwise uninvaded heathland were mycorrhizal. We weighed 983 *Betula* seedlings collected from Td in 2005. Naturally occurring mycorrhizal seedlings were significantly heavier than non-mycorrhizal seedlings (median mass $= 8.7 \times 10^{-4}$ g and 1.16×10^{-3} g, respectively, Kruskal–Wallis $H = 6.38$, d.f. $= 1$, $P < 0.05$) and the difference in mass increased over time (Fig. 5).

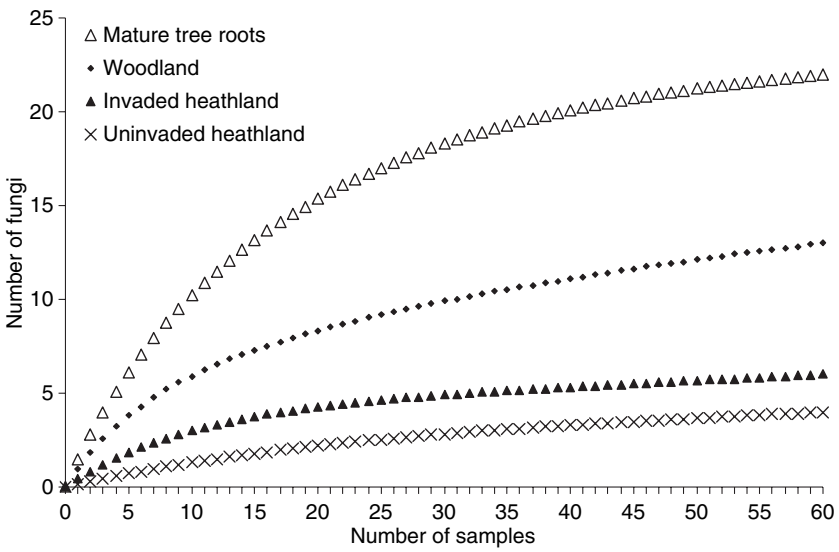


Fig. 4. Fungal accumulation curves for mature tree roots and bioassays from six sites.

Table 3. Number of naturally occurring *Betula* and *Pinus sylvestris* field seedlings less than 1 year old that were mycorrhizal (myc.) versus non-mycorrhizal (non-myc.) at five sites in uninvaded heathland (uninv.), invaded heathland (inv.) and woodland (wood.) and the ectomycorrhizal fungi detected. We did not find seedlings from each level of invasion at all sites. ND, no data

Seedling	Site	Level of invasion	Number myc.	Number non-myc.	Ectomycorrhizal fungi detected
Pinus	Hw	uninv.	0	2	NA
Betula	Td	uninv.	0	192	NA
Pinus	Tw	uninv.	0	6	NA
Pinus	Sw	inv.	5	5	ND
Betula	Td	inv.	83	334	*Laccaria laccata*, *Leccinum holopus*, *Paxillus involutus*, *Scleroderma citrinum*, *Tomentella bryophila*, *Tomentella sublilacina*
Pinus	Td	inv.	1	8	ND
Betula	Kw	wood.	4	12	*Lactarius hepaticus*, *Leccinum holopus*, *Paxillus involutus*
Pinus	Kw	wood.	4	11	*Lactarius tabidus*, *Tomentella sublilacina*, *Tomentella* sp.
Betula	Td	wood.	283	143	*Cenococcum geophilum*, *Laccaria laccata*, *Paxillus involutus*, *Russula ochroleuca*, *Tomentella sublilacina*
Betula	Tw	wood.	4	0	*Amanita fulva*, *Cenococcum geophilum*
Pinus	Tw	wood.	5	0	*Cenococcum geophilum*, *Russula emetica*, *Xerocomus* sp.

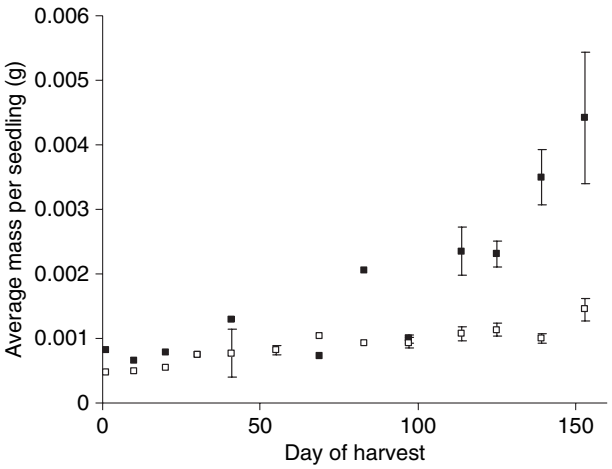
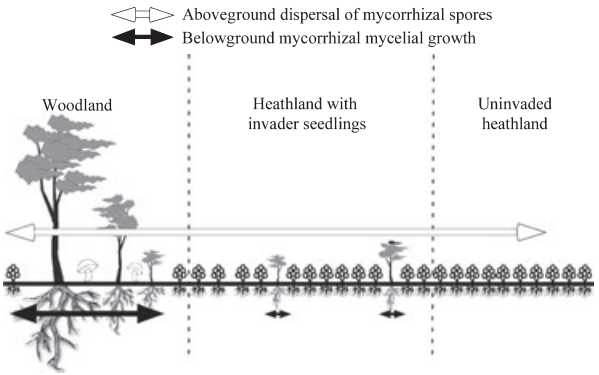


Fig. 5. Average mass of mycorrhizal (closed squares) and non-mycorrhizal (open squares) naturally occurring *Betula* seedlings collected from Thursley Common (Td) between 18 May 2005 (cotyledon stage) and 17 October 2005 (senescence). Error bars correspond to standard errors, error bars are not available for all dates because on some dates seedlings were weighed collectively and the mass averaged.

Based on five sites (Hw, Kw, Sw, Td and Tw) the level of invasion had a significant effect on the proportion of seedlings less than 1 year old which were mycorrhizal versus non-mycorrhizal ($F = 47.2_{2,8}$, $P < 0.001$, Table 3). The list of fungi found on naturally occurring seedlings indicates that richness increases as the level of invasion increases from invaded heathland to woodland (Table 3). Some fungi detected on naturally occurring seedlings were not detected on bioassay seedlings or tree roots. Two of these, *Scleroderma citrinum* and *Tomentella bryophila*, were from a site (Td) that could not be used in the bioassays or mature tree analysis because of a wildfire that occurred in July 2006 devastating our sampling area. Overall, thirty ectomycorrhizal fungi were detected using bioassays and surveys of naturally occurring seedlings and mature tree roots (Fig. 6). Twenty-eight were detected in woodlands, eleven in invaded heathland and five in uninvaded heathland.



Amanita fulva **BP**
Amanita rubescens **BP**
Atheliaceae sp. **P**
Cenococcum geophilum **BP**
Cortinarius semisanguineus P
Elaphomyces muricatus BP
Laccaria laccata BP
Laccaria proxima **BP**
Lactarius hepaticus **BP**
Lactarius necator-like **B**
Lactarius rufus **BP**
Lactarius tabidus **BP**
Leccinum holopus **B**
Paxillus involutus BP
Pseudotomentella tristis BP
Rhizopogon luteolus **P**
Russula betularum B
Russula claroflava **B**
Russula emetica **BP**
Russula ochroleuca BP
Russula sphagnophila **B**
Suillus bovinus **P**
Suillus variegatus **P**
Thelephora terrestris **BP**
Tomentella sp. **P**
Tomentella sublilacina **BP**
Tomentellopsis sp. BP
Xerocomus sp. **P**

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

Laccaria proxima **B**
Rhizopogon luteolus **P**
Suillus bovinus **P**
Suillus variegatus **P**

Fig. 6. The ectomycorrhizal fungi of birch and pine in English lowland heathlands detected across six sites in bioassay and/or *in situ* surveys. B, P or BP (in bold) after a fungus name indicates whether it was detected with birch, pine, or birch and pine. 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. *Lactarius hepaticus* is considered a pine specialist, but it was repeatedly detected in birch bioassays. The proportion of mycorrhizal seedlings is generally > 50% in woodland, *c.* 25% in invaded heathland and < 10% in uninvaded heathland.

OUTPLANTED SEEDLINGS

On 31 October 2006, 144 experimental and 22 control *P. sylvestris*, 54 experimental and 24 control *B. pendula* surviving seedlings were harvested from transects at Tw. Two *P. sylvestris* seedlings were mycorrhizal with *Suillus variegatus* (Table 4). A DNA sequence was not obtained from one mycorrhizal *B. pendula* seedling. After the first harvest, 304 seedlings (225 *P. sylvestris* and 79 *B. pendula*) were left over winter. By February 2007, 113 seedlings were alive (96 *P. sylvestris* and 17 *B. pendula*). On 14 April 2007, the remaining 65 experimental and nine control *P. sylvestris*, and three experimental and two control *B. pendula* surviving seedlings were harvested. All *B. pendula* seedlings and control seedlings of both species were non-mycorrhizal, and four *P. sylvestris* seedlings were mycorrhizal with *S. variegatus* (Table 4). The four mycorrhizal *P. sylvestris* seedlings were located within the estimated rooting zone of their corresponding saplings.

ENDOMYCORRHIZAL ASSESSMENT

On no occasion did any members of the *Rhizoscyphus ericae* aggregate form ectomycorrhizas in our study. In addition, we found no evidence of endomycorrhizal colonisation in *B. pendula* and *P. sylvestris* roots from bioassays. Nonetheless, members of the *R. ericae* clade were detected via PCR from roots of seven naturally occurring seedlings, 13 bioassay seedlings (four *B. pendula*, nine *P. sylvestris*), one tree root and 27 outplanted seedlings. Other clades (predominantly *Meliniomyces variabilis*) within the *R. ericae* aggregates were detected in roots from three woodland cores, 29 bioassays (17 *B. pendula*, 12 *P. sylvestris*), one naturally occurring seedling and two outplanted seedlings. Of 67 *Calluna* hair root samples, nine samples did not contain ericoid mycorrhizal structures, seven samples had ericoid mycorrhizal structures, but the staining

Table 4. Number of outplanted *Pinus sylvestris* and *Betula pendula* seedlings mycorrhizal (myc.) or non-mycorrhizal (non-myc) at harvest in October 2006 and April 2007. Seedlings outplanted along transects radiating away from *Pinus sylvestris* and *Betula* saplings (experimental) and control transects with no focal sapling at Thursley (Tw)

Date of harvest	Transect type	Outplanted seedling	Number myc.	Number non-myc.
October	Experimental	*B. pendula*	1	53
October	Experimental	*P. sylvestris*	2	142
October	Control	*B. pendula*	0	24
October	Control	*P. sylvestris*	0	22
April	Experimental	*B. pendula*	0	3
April	Experimental	*P. sylvestris*	4	61
April	Control	*B. pendula*	0	2
April	Control	*P. sylvestris*	0	9

was too faint to approximate how many cells were colonized, 19 had ≥50% of cells colonized and the remainder had < 50% of cells colonized.

Discussion

Overall, ectomycorrhizal inoculum in lowland heathlands is severely limited so that tree seedlings growing in heathland soil are predominantly non-mycorrhizal. There is an increase in mycorrhization and fungal diversity as the level of tree encroachment into heathlands increases and a higher fungal diversity on tree roots than on seedlings, thus supporting our first hypothesis. Lowland heathland sites differ in their ectomycorrhizal inoculum availability and diversity. Most of the rarely detected fungi were at only one or two sites, but some of the most abundant fungi occurred at three or four sites. Our results are congruent with those from bioassays similarly conducted at a subset of sites in 2005 and 2006 (Collier 2009) and studies demonstrating interannual stability of ectomycorrhizal communities (Izzo, Agbowo & Bruns 2005; Smith, Douhan & Rizzo 2007).

Fungi within the *R. ericae* species aggregate do not form ectomycorrhizas on *Betula* or *P. sylvestris* seedlings invading the lowland heathlands sampled; this is despite the ectomycorrhizal morphotype *Piceirhiza bicolorata* being placed within the same clade as the ericoid mycorrhizal fungus *Rhizoscyphus ericae* (=*Hymenoscyphus ericae*; Vrålstad, Fossheim & Schumacher 2000) and laboratory support for Vrålstad, Fossheim & Schumacher (2000) hypothesis of dual ericoid-ectomycorrhizal fungi (Vrålstad, Myhre & Schumacher 2002; Villarreal-Ruiz, Anderson & Alexander 2004). Arbuscular mycorrhizal fungi were not observed either.

Based on earlier work performed on primary or secondary successional sites (the Bush Estate studies; Newton 1992; Bowen 1994 and references therein; Jumpponen, Trappe & Cazares 2002; Nara *et al.* 2003b) our second hypothesis was that relatively fast-growing and putatively spore-dispersed ectomycorrhizal fungi (pioneer or 'r-selected') such as *Hebeloma, Inocybe, Laccaria, Paxillus* and *Thelephora* should dominate tree seedlings. However, of these fungi only *Laccaria proxima* is present in uninvaded heathland, where it has the potential to colonize primary invader seedlings. Neither *Inocybe lacera*, a common and widespread species fruiting in heathlands in the UK (Legon & Henrici 2005), nor *Hebeloma*, a dominant early-stage genus reported from the Bush Estate, were ever detected on our bioassays seedlings, tree roots, naturally occurring or outplanted seedlings at any of our sites. The lack of congruence with the Bush Estate studies may be due to one of the studies' caveats; the Bush Estate's agricultural soil was an ecologically unrealistic setting (Molina, Massicotte & Trappe 1992) and the rare linked studies that were carried out in native forest settings contradicted them (Fleming, Deacon & Last 1986; unpublished data mentioned in Deacon & Fleming 1992 and Fleming 1983). Additional reports show that *Paxillus* ectomycorrhizas dominate *Betula* seedlings outplanted within *Betula* woodland (Newton 1991; Newton & Pigott 1991); this fungus was rarely detected in bioassays (two bioassays from Tw), and it was present at two sites on tree roots (Hw and Cd) where it was far from dominant. On the other hand, we detected seven fungi not previously reported with *Betula* in Britain (Atkinson 1992) including a new genus (*Elaphomyces*). Taken together, these results give further credit to the idea that the geographic distributions of ectomycorrhizas merit investigation (Lilleskov & Parrent 2007).

One caveat of using bioassays is that taxa which specialize in forming mycorrhizas on established trees such as Russulaceae (*Lactarius* and *Russula*) (Termorshuizen 1991; Ryan & Alexander 1992; Nara, Nakaya & Hogetsu 2003a; Twieg, Durall & Simard 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 the spores that are presumed to constitute the majority of the inoculum. The presence of certain fungal taxa in birch seedling bioassays indicates this study was not completely selective; *Amanita* sp. have been reported to not develop mycorrhizas on seedlings in bioassay conditions (Deacon, Donaldson & Last 1983; Taylor & Bruns 1999) but *Amanita* formed mycorrhizas, although rarely, in this study. Russulaceae were infrequent but not excluded from bioassays; particular exceptions are *Lactarius hepaticus* and *L. rufus*, which were the third and fourth most common fungi detected in *B. pendula* 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 *c.* 5 m^2 large (Nara, Nakaya & Hogetsu 2003a). *Leccinum* is not a common primary colonizing fungus (Nara,

Nakaya & Hogetsu 2003a) and it has been reported not to form mycorrhizas on birch under glasshouse conditions (Deacon, Donaldson & Last 1983) being dependent upon connections to living plants (Fleming 1984); *Leccinum* was detected in only six bioassays in our study. The rarity of *Leccinum* and *Paxillus* in bioassays may be indeed due to their reliance on rhizomorphs (root-like aggregations of hyphae), rather than spores, to colonize new roots (Newton 1992). The presence of these fungi, presumed to colonize vegetatively, indicates at least some viable ectomycorrhizas and/or mycelia, as well as spores, were present in bioassays.

Our Shannon's H' and Simpson's 1/D values were similar to those reported for individual tree species by Ishida, Nara & Hogetsu (2007). Twieg, Durall & Simard (2007) reported a Shannon diversity index near 2 for *Betula papyrifera* that did not increase significantly after trees had reached 5 years of age. The low tree diversity at lowland heathland sites is probably the reason for our overall diversity indices being much lower than in mixed woodlands elsewhere (Richard *et al.* 2005; Walker, Miller & Horton 2005; Tedersoo *et al.* 2006; Ishida, Nara & Hogetsu 2007). As expected, there is a higher mycorrhizal diversity in mature trees compared to seedlings, and the same holds true for invaded heath and woodland soil bioassay seedlings compared to uninvaded heathland soil bioassay seedlings. These findings corroborate those of Nara *et al.* (2003b) who found that the number of ectomycorrhizal fungal species increased as plant size increased at a volcanic desert, and Ashkannejhad & Horton (2006) who reported a higher diversity of fungi in more heavily forested sites on sand dunes. There can be higher fungal richness and diversity on seedlings near trees due to the formation of common mycelial networks (CMNs) in which growing ectomycorrhizal fungi connect the root systems of trees with those of establishing seedlings (Brownlee *et al.* 1983; Cline, Ammirati & Edmonds 2005; Dickie & Reich 2005; Lian *et al.* 2006) and can thereby control seedling survival, growth and nutrient uptake during primary succession (Nara & Hogetsu 2004; Nara 2006a,b).

Fungal life history and mycorrhization strategy largely determines ectomycorrhizal distribution and diversity within lowland heathlands. Common mycorrhizal networks can allow fungi that do not readily colonize seedlings via spores to proliferate and potentially participate in tree invasion secondarily within heathlands. Unexpectedly, CMN formation appeared to be weak for seedlings outplanted near *Betula* and *Pinus* saplings, contrary to our third hypothesis. However, two fungi that form rhizomorphs, *Scleroderma citrinum* and *Paxillus involutus*, were detected in some naturally occurring heathland seedlings. The complete or near absence of these two fungi from bioassays can be assigned to their reliance on CMNs rather than spores to colonize new roots (Newton 1992). In contrast, *Suillus* and *Rhizopogon* use a mixed colonization strategy with both rhizomorphs and spores being effective at colonizing seedling roots. *Rhizopogon* and *Suillus* are the main genera to form ectomycorrhizas on pine seedlings in uninvaded areas (Horton, Cazares & Bruns 1998; Ashkannejhad & Horton 2006) including lowland heathlands. These Pinaceae-specific fungi are also prevalent in deer faeces suggesting a specialized method of dispersal and potential for formation of a spore bank; thus, these fungi are uniquely adapted for dispersal and survival in treeless areas (Ashkannejhad & Horton 2006). In woodland soils, *Rhizopogon* species can dominate the spore bank while being absent on mature tree roots, where they are considered poor competitors (Baar *et al.* 1999; Taylor & Bruns 1999; Twieg, Durall & Simard 2007). Although a generalist, *T. terrestris* is another ruderal fungus that actively colonizes seedlings in tree nurseries and saplings (Colpaert 1999); accordingly, it was common in invaded lowland heathland bioassays, but curiously it was absent from uninvaded heathland. In contrast, *Russula* and *Lactarius* (Russulaceae) were diverse and prevalent on mature tree roots (nine Russulaceae occurred in 37 soil cores) compared to bioassay seedlings (three Russulaceae occurred in 20 bioassays), further supporting evidence that Russulaceae are late-successional (Deacon & Fleming 1992; Ryan & Alexander 1992; Nara, Nakaya & Hogetsu 2003a; Twieg, Durall & Simard 2007).

These colonization strategies agree well with results from a pilot study conducted between April and September 2007 at uninvaded heathland in Tw where we placed woodland-collected fresh sporocarps of *Paxillus*, *Lactarius*, *Amanita*, *Thelephora* and *Suillus* spp. – all abundant fruiters in woodland – next to outplanted *Betula* and *Pinus* seedlings. At harvest, we obtained ectomycorrhizas of only *T. terrestris* and *Suillus bovinus* in 73% of pines and of *T. terrestris* in 36% of birches (Collier 2009). This observation makes widespread ectomycorrhizal suppression (e.g. competitive or allelopathic, Robinson 1972; Genney, Alexander & Hartley 2000) in heathland soil appear unlikely. It is worth stressing that lack of mycorrhization in heathland bioassays and the field is due to a lack of inoculum, rather than to the design of our experiments or to suppression, because: (i) soils from both heathland and woodland in pine and birch bioassays were co-cultivated with *Calluna*, (ii) seedlings in the pilot sporocarp addition study became mycorrhizal within 3 months and (iii) outplanting experiments such as Fleming's (1984) conducted over shorter time periods resulted in all seedlings undergoing mycorrhization. Furthermore, the separation of fungi by their pioneer or non-pioneer abilities agrees with some surveys of naturally occurring and outplanted *Betula* and conifer seedlings in conifer forests and plantations (Thomas, Rogers & Jackson 1983; Blasius & Oberwinkler 1989; Deacon & Fleming 1992).

This study demonstrates that a limited suite of fungi is only rarely present to form mycorrhizas with tree seedlings in lowland heathlands. Under field conditions, the relatively rare mycorrhizal seedlings are significantly heavier than non-mycorrhizal seedlings; this supports our fourth hypothesis and concurs with a meta-analysis of 36 – mostly laboratory – studies (Karst *et al.* 2008). Under laboratory conditions, biomass was also affected by site and level of invasion, but generally, mycorrhizal seedlings were heavier than non-mycorrhizal seedlings. However, we detected weak survival effects of mycorrhization in the field – and no fungal species-specific effects in bioassays – compared to the absolute survival and strongly species-specific effects demonstrated for *Salix* under the harsher abiotic conditions of primary succession (Nara 2008)

and suggested earlier for *Betula* in northern upland heathlands (Miles 1973; Miles & Kinnaird 1979). We observed no growth depression in mycorrhizal seedlings as an initial cost of mycorrhization, even though mycorrhization was observed in some cases as soon as seedlings had emerged above-ground. In most of England, secondary succession from heathland to woodland occurs under relatively, and increasingly, mild abiotic conditions and in the context of a history of soil eutrophication. Both factors may contribute to non-mycorrhizal survival in otherwise extremely nutrient-poor heather-dominated soils and they merit investigation. With limited mycorrhizal spore dispersal from adjacent woodland, poorly developed mycorrhizal spore banks and weak common mycorrhizal networks, a 'sit-and-wait' strategy by tree seedlings is feasible in English lowland heathlands. In conclusion, this study has achieved its main objectives: determining the distribution of ectomycorrhizal fungal inoculum in lowland heathland soils and identifying the ectomycorrhizal fungi involved in tree encroachment upon lowland heathlands. This knowledge framework provides the stepping stones required for future ecologically relevant modelling and targeted experimentation aimed at understanding tree invasions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Non-metric multidimensional scaling (a non-parametric method) was used to test for grouping of sites by level of invasion on the basis of the presence and absence of ectomycorrhizal fungi (binary data) with R version 2.7.2.

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