

Fungal interactions with vascular and non-vascular plants: an investigation of mutualisms and their roles in heathland regeneration

JILL KOWAL

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Abstract

Mycorrhizal mutualisms between aboveground vascular plant communities, which reward their belowground fungal associates with photosynthates in return for growth-limiting nutrients such as phosphate, are widely recognized as stable long-term interactions which helped plants colonize land. *Pezoloma ericae* (D.J. Read) Baral, an ascomycete mycorrhiza-forming fungus present amongst plants in the Ericales, such as heathers, also forms associations in several families of non-vascular leafy liverworts. Whether there is a mutually beneficial functional relationship between these leafy liverworts and the fungus growing in their rhizoids was previously unconfirmed. Furthermore, an ecological role of this 'shared' mycobiont and its link between vascular (Ericaceae) and non-vascular (liverworts) plants was also unknown. Thus the main questions asked in this dissertation are: 1) Is there a measurable mutually beneficial relationship between a liverwort and its fungal partner?; and, 2) Can liverworts harbouring the ericoid mycorrhiza *P. ericae* act as inoculum that facilitates the re-establishment of Ericaceae - and henceforth be proposed as a practical tool in a restoration ecology context.

This is the first time British species of leafy liverworts are conclusively identified to harbour the ericoid mycorrhizal fungus *Pezoloma ericae* using molecular identification. I have demonstrated a mutualism occurring between the leafy liverworts and their fungal symbiont in two independent microcosm growth experiments and confirmatory reciprocal trophic exchanges between phosphorus and carbon and the two organisms.

Glasshouse experiments demonstrated *P. ericae* originating from leafy liverwort rhizoids, can repeatedly colonize Ericaceae plant roots. Under realistic ecological circumstances (further tested at two field sites), liverworts delivered mycorrhizal inoculum and improved the resilience and growth of vascular plants. By providing this novel source of mycorrhizal inoculum, symbiotic non-vascular plants can contribute to the restoration of plant communities dominated by Ericaceous plants. This research leads to broader knowledge about the function of ericoid mycorrhizas in ecosystems with multi-trophic non-vascular – fungi - vascular community interactions, both above- and belowground.

Declaration of Originality

The work presented in this thesis is entirely my own. Collaborative work has been specifically acknowledged, and less formal advice, assistance and support have been listed in the acknowledgements.

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Jill Kowal

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1 Chapter One – Introduction

Belowground interactions, particularly mycorrhizal fungi which are present in most soil communities, affect aboveground plant communities (Wagg *et al.*, 2015). These mycorrhizal mutualisms between aboveground plant communities and their belowground fungal partners are widely recognized as stable long-term interactions, which helped plants colonize land (Bidartondo *et al.*, 2011, Leake, 2015), in addition to absorbing carbon (Hobbie, 2006) and contributing to nutrient recycling. As more sophisticated molecular tools and data sets become available to identify fungal operational taxonomic units the complexity of belowground organismal interactions is beginning to unravel. Less studied is the function of these fungi with respect to the earliest land plants i.e. liverworts, which contribute to the ecological function of plant communities but are often overlooked for their larger more discernable vascular counterparts. Depending on the phase of ecological succession and habitat, their presence could be significant. This research aims to further our understanding about these mutualisms between liverworts and the fungal symbionts inhabiting their rhizoids. Furthermore, I explore whether these liverworts can be used in a practical way, to help create or restore plant communities where the liverworts and vascular plants share the same mycorrhizal fungus.

The model community selected for this research is British lowland heathlands, which are dominated by ericaceous shrubs. In the seasonally wet regions of these heathlands, leafy liverworts can form significant groundcovers with thousands of individuals in a half-metre square. Each individual liverwort has several rhizoids colonized by ericoid mycorrhizal fungi (ErM), the same fungal symbiont present in most ericaceous plants (Duckett and Read, 1995, Chambers *et al.*, 1999, Upson *et al.*, 2007). This research focuses on the function of this ErM symbiont vis-à-vis the liverwort and in turn, whether liverworts may be used practically as tools to deliver mycorrhizal fungal inoculation to stimulate growth and successful establishment of plant communities dominated by vascular plants.

This chapter introduces the project and past research pertinent to the hypotheses described herein. Chapter Two lays the groundwork for the original research developed in my dissertation (Chapters Three, Four and Five) while confirming earlier studies (Duckett and Read, 1995, Chambers *et al.*, 1999, Upson *et al.*, 2007), although this time with British species of leafy liverworts and molecular tests.

1.1 Introduction to this project

This work focuses on ericoid-mycorrhiza forming plants in the Ericaceae family, which dominate heathland communities throughout the world. In this ground-laying research project, leafy liverworts containing the ascomycetous fungus *Pezoloma ericae* (D.J. Read) Baral (syn. *Rhizoscyphus ericae* (D.J. Read) W.Y. Zhuang and Korf, *Hymenoscyphus ericae* (D.J. Read) Korf and Kernan, and *Pezizella ericae* D.J. Read) are tested to assess whether they can deliver mycorrhizal inoculum to heathland habitats or marginal lands targeted for restoration and/or heathland recreation, to facilitate establishment of the dominant heathland plants, *Calluna vulgaris* and *Erica* spp. (Rodwell, 1991). Several British leafy liverworts species (in the families Schistochilaceae, Lepidoziaceae, Cephaloziaceae and Cephaloziellaceae) harbour the same fungus in their rhizoids, considered to be mycorrhizal with ericaceous plants' roots (Duckett and Read, 1995, Read *et al.*, 2000). This is the first time as far as I know that the non-vascular liverworts are examined to determine whether there is a mutually beneficial association between liverworts and *P. ericae* inhabiting its rhizoids; thereby furthering our understanding of the function of the fungal symbiont in nature.

This is also the first time that leafy liverworts are utilized as an inoculum delivery mechanism, introduced in a series of field-simulating glasshouse experiments and further tested in the field. Fungal isolates from liverworts had previously been established to be capable of colonizing a range of Ericaceae plants, fulfilling Koch's postulates (Duckett and Read, 1995, Read *et al.*, 2000, Upson *et al.*, 2007), but only *in vitro*. The experiments designed for this thesis begin to fill the gap between these laboratory based studies and field based observations of colonized heathers (Diaz *et al.*, 2006) and liverworts.

1.2 Aims and objectives

There were two main aims of the research. The first examined the composite organism to determine whether leafy liverworts and the ericoid mycorrhizal fungus (ErM) they host in their rhizoids, *Pezoloma ericae*, are in a mutually beneficial association (Chapter Three). This was assessed by: 1) measuring growth of the liverworts, with and without the ErM fungus; and, 2) establishing whether reciprocal nutritional exchanges occur between the liverworts and *Pezoloma ericae*. The second aim sought to further our knowledge about the functional role and potential uses of this fungal symbiont in nature (Chapters Four and Five). Specifically, could liverworts harbouring ErM act as an inoculum delivery mechanism to facilitate heathland restoration and/or creation. The practical methods of delivery were tested and assessed in a nursery setting. These same

plants were then planted out at two lowland heathland sites. Combined these aims further understanding about mycorrhizal networks (Selosse *et al.*, 2006) while simultaneously providing a practical tool for ecosystem restoration.

1.2.1 Can leafy liverworts provide a practical inoculum delivery device for reintroduction of Ericaceae plants in nature?

This was examined by meeting the following objectives:

- The presence of the ErM fungus *Pezoloma ericae* was established in the wild liverworts through DNA extraction and sequencing.
- Isolated *P. ericae* from both wild liverworts and ericaceous plant roots and maintained a population in culture for experiments.
- Collected wild liverworts and roots, seeds and cuttings from *Erica tetralix* and *Calluna vulgaris* and fertile British native leafy liverworts associated with wet heathland ericaceous plant communities in British wet heathland communities.
 - a. Cultivated and maintained a population of plants from both seed and cuttings.
 - b. Resynthesized populations of axenically-grown leafy liverworts.
- Tested whether the mycobiont isolates from both the liverworts and plant roots are the same fungus. Used DNA barcoding to confirm identity as morphological characters are not definitive, particularly if there are any variations amongst the *P. ericae* in the different cultures from liverworts and from ericoid roots.
- Resynthesized populations of leafy liverwort with *P. ericae* in the laboratory for nursery inoculation experiments and documented colonized rhizoidal apices plus hyphal coils in the liverworts.
- Inoculated nursery plants with resynthesized liverworts and fungal isolates (derived from wild liverworts and ericaceous plants) and established whether *P. ericae* colonizes the plants.
- Determined whether inoculation had an impact on aboveground growth or survival rates in the nursery.
- Planted the populations from nursery experiments in lowland heathland and recreation plots and monitored growth for one year.
- Determined whether the ericaceous plants colonized with fungi from liverworts developed more biomass or had higher establishment success rate.
- Assessed rate of colonization in the field amongst sample control plant groups at two distinct field sites, one an existing heathland site with mycorrhizal fungi in

the topsoil, the other a former agricultural plot with no recorded history of ericaceous plants.

1.2.2 Can the relationship between leafy liverworts and their ericoid fungal symbionts be characterized as mutualistic, whereby there is functional benefit to both plant and fungus, as exhibited in Ericaceae plants?

To test this:

- Liverwort polygon images were traced (using ImageJ software) and measured before and after six weeks to record change in growth. I also measured liverwort dry weight, with and without inoculation, to assess whether inoculation influences nutrient uptake and therefore biomass growth.
- A reciprocal exchange of nutrients between plant and fungus was measured by introducing isotopes to cultures with liverworts (resynthesized with *P. ericae*). Movements of ^{33}P were traced between the fungal hyphae and the liverworts; reciprocity was measured using ^{14}C isotopes, introduced to liverwort microcosms to determine transfer of carbon from the liverwort to the fungus.

1.3 Description of study laboratories, plant nurseries and field sites

The *in vitro* experiments were conducted in three laboratories: Jodrell Laboratory at Kew Gardens, the Natural History Museum (NHM) and Sheffield University. Most of the initial liverwort propagation, fungal isolation and resynthesis culture work occurred at the NHM's Cryptogamic Laboratory facilities under the supervision of Dr. Silvia Pressel and Professor Jeffrey Duckett. The axenic liverwort cultures and growth experiments are maintained in the growth chambers there. The latest round of fungal isolation work occurred in the Jodrell Laboratory and subculturing occurred there and at NHM. The reciprocal nutrient transfer studies occurred at the University of Sheffield, under the supervision of Dr. Katie Field.

The molecular identification work occurred in the Jodrell Laboratory. The vascular plants, both seedlings and cuttings, were propagated and raised in Kew's glasshouses.

The field experiments occurred at two lowland wet heathland sites, Thursley Common, Surrey (OS grid reference SU900416), and The Delft, Norfolk, adjacent to Roydon Common (OS grid reference: TF680229). Thursley Common is a 350-hectare National Nature Reserve, a SSSI, a Special Protection Area and partly recognized as a Ramsar site. Ramsar is an international treaty governing wetlands. It is managed by Natural England. Thursley is still in the recovery phase following a substantial fire in 2006. The Delft, which was purchased in 2010 by the Norfolk Wildlife Trust (NWT) to extend

Roydon Common, is a 20-hectare former agricultural site. Roydon Common was designated a National Nature Reserve in 1995. Lowland heathlands are a top priority habitat under the UK Biodiversity Action Plan (JNCC, 2008) and they are designated as an important European habitat under the EU Habitats Directive (1992).

Ecologists from both Natural England and Norfolk Wildlife Trust provided approval and logistical support for the experimental field designs described herein. Most significantly, the nitrogen-rich upper layer of topsoil was cleared for the experiments using tractors (Fig. 1a). Both organizations are keen to find new ways to improve heathland vegetation establishment as they are currently using techniques that are costly in terms of time and financial resources. Cost estimates to maintain existing and re-establish additional heathlands were last estimated to be approximately £200 and £450 per hectare per year, respectively (Natural England, 2013).

The most common techniques typically employ sulphur enrichment to lower pH or stripping the nutrient rich topsoils to reduce establishment of competitive grasses (as on the Delft). Although not without benefits, there are significant trade-offs which can contribute directly and indirectly to toxicity in the soil and trophic consumers from insects and spiders to birds (Diaz *et al.*, 2011). Establishment can take years or even decades and requires hands-on management to restrain birch and pine trees, the natural ecological successors, from taking hold.



Fig. 1 – Heathland restoration techniques. (a) The Delft, Norfolk, showing topsoil scraped for heathland creation. (b) *Calluna* thatch used for heathland regeneration or creation includes inflorescences with seed but no soil.

Methods for restoration currently employed at the experimental field trial sites have been slow and have mixed success. Thursley Common, ten years post-fire, is recovering its dominant heathers, mainly through regrowth with limited seed recruitment. The Delft, which employed methods involving topsoil scraping and introducing clippings from other heathlands (Fig. 1b), akin to those described by Diaz *et al.* (2006, 2011),

remains thinly populated by heathers, two years after commencing clearing and clipping dispersal activities.

1.3.1 Plant and fungal material

Two vascular plants were used to test the questions asked in this study, *Calluna vulgaris* and *Erica tetralix*. Both plants are members of the family Ericaceae (Kron, 1997, Kron *et al.*, 2002) and occupy a substratum characterized by low pH and low nutrients.

Calluna vulgaris (commonly known as ‘heather’) is the dominant dwarf-shrub in most lowland British heathlands (Rodwell, 1991), as well as moorlands, throughout its range. *Calluna vulgaris* is widely distributed throughout Europe to the Ural Mountains in Russia, Northern Scandinavia, across Iceland, Turkey, the Azores, Madeira and the mountains of Morocco (Featherstone, 2005). It has been introduced to North America, New Zealand and Australia where it is considered an invasive weed (Featherstone, 2005). *Erica tetralix* is a frequently occurring ericaceous dwarf-shrub in wet heathlands in Great Britain (Rodwell, 1991). *Erica tetralix*’s range is more limited on the continent and to wetter slopes in Britain by its inability to withstand summer drought. It is more tolerant than *Calluna vulgaris* of lower winter temperatures and wet habitats such as bogs and moors; its cover increases where the substratum is waterlogged and poorly aerated (Bannister, 1966). As such, its Ellenberg indicator scale value (Hill *et al.*, 1999) for dampness tolerance is a high score of ‘8’ out of ‘11’, compared with 1 for *Calluna*. An Ellenberg indicator of 1 is restricted to soils which often dry out for a time, where 8 indicates dampness to saturation.

The leafy liverworts *Cephalozia connivens* and *C. bicuspidata* were selected as the inoculum delivery vehicle as they are readily available on wet heathlands, have previously been established to contain the ErM fungus *P. ericae* and can be manipulated experimentally *in vitro* (Duckett and Read, 1995). This is the only fungus known to associate with these leafy liverworts and therefore the only fungus tested. Plant materials (including vascular plant cuttings and seeds, and leafy liverworts) were collected from both study sites (with permission of Natural England and Norfolk Wildlife Trust site managers) and have been maintained in controlled environments for isolation of, and cross-colonization by, mycorrhizal fungi. All fungal material has been extracted from this plant material

1.4 Mycorrhizal fungi

Mycorrhizal fungi are the dominant mutualists of plants in all terrestrial ecosystems; they enhance soil nutrient and water uptake in exchange for plant sugars (Smith and

Read, 2008). There are several recognized categories of fungi that have been observed and documented as mycorrhizal, i.e., forming mutually beneficial relationships with their host land plants (Field *et al.*, 2015a).

1.4.1 The role of mycorrhizas in plant establishment

This process of becoming mycorrhizal can be limiting to plant establishment in new habitats (e.g. ex-arable lands). Mycorrhizal fungi are not transmitted directly from parent to offspring plants, so mycorrhizal mutualisms must re-form *de novo* when plants establish from seed or asexually. As the life span of an individual colonized ericaceous plant cell is thought to extend no longer than five or six weeks (Smith and Read, 2008), nutrient transfers must occur in the first few weeks before the breakdown of plant cytoplasm.

The important role of mycorrhizas in ecosystem establishment and recovery has been known since the 1940s. Rayner (1927) review of research on forest trees, orchids and heaths confirmed experimental works (explored earlier by Frank (1885) and Stahl (1900) and subsequently by Melin (1925,1954), per Rayner) indicating that mycorrhizas provide a vital physiological benefit to vascular plant seedlings, helping them establish more successfully when present, depending on edaphic conditions and climate.

Examining evidence on Scots pine (*Pinus sylvestris*) as an example, Rayner (1945) found, whether in the nursery or wild conditions, ‘beyond dispute that the healthier and more vigorous a pine tree under natural conditions, the more profusely is the root system furnished with mycorrhizas.’ Rayner’s further experimental work demonstrated the successful development of the seedling is dependent on specific fungal colonization at germination. This was observed by Rayner to be more acute in peaty heaths than dry sandy soils. This research is particularly applicable to heathland afforestation work, where soils are generally deficient in nitrates. Rayner (1915) had particular difficulty with *Calluna* experimentation in ‘pure culture’ and found the rooting medium of significant importance for the root cell metabolism and growth of fungi. She also noted that Ericaceae species seem to have a more specialized mutualistic relationship with fungi than trees, in general, stating ‘in each species infection is restricted to a specific endophyte showing a relatively high degree of specificity’ (1927).

1.4.1.1 Different types of mycorrhizae

Mycorrhizal fungi are the dominant mutualists of plants in all terrestrial ecosystems; they enhance soil nutrient and water uptake in exchange for plant sugars (Smith and Read, 2008). Generally, there are three recognized categories of fungus that have been

observed and documented as mycorrhizal, i.e., forming mutually beneficial relationships with the associating land plants. In their instructive review, Selosse and Le Tacon (1998) discuss that almost all extant phototrophs which have adapted to land, form these 'mutualisms' with fungi. The below glossary and Fig. 2 is adapted from this review.

Mycorrhiza - are composed of septate or non-septate fungi within a root, and also exploring the surrounding soil.

Ectomycorrhiza or 'sheath-forming mycorrhiza' are comprised of septate fungus which grows between cortical cells forming Hartig nets.

Endomycorrhiza involves non-septate Glomeromycota (also known as arbuscular mycorrhiza, formerly vesicular-arbuscular mycorrhiza) or septate fungi (ericoid and orchid mycorrhizas). In both cases, the fungi penetrate the cortical cells to form arbuscules or coils, respectively. Arbuscular mycorrhizae form the majority of mycorrhiza with vascular land plant roots and are also known to associate with thalloid liverworts.

Ericoid mycorrhizal fungi (ErM) are ascomyceteous and typical of ericaceous plants (Stribley and Read, 1980). They (and some ectomycorrhizal fungi, in particular), play an important role in plant nutrient absorption, by 'attacking' structural polymers, which have nutrients locked up, and releasing N and P from organic polymers (Read and Perez-Moreno, 2003).

Arbutoid Ectendomycorrhiza basidiomycetes in some Ericales (e.g. *Arbutus*) shares features of both ecto- and endo-mycorrhizae, i.e. a sheath and intracellular coils.

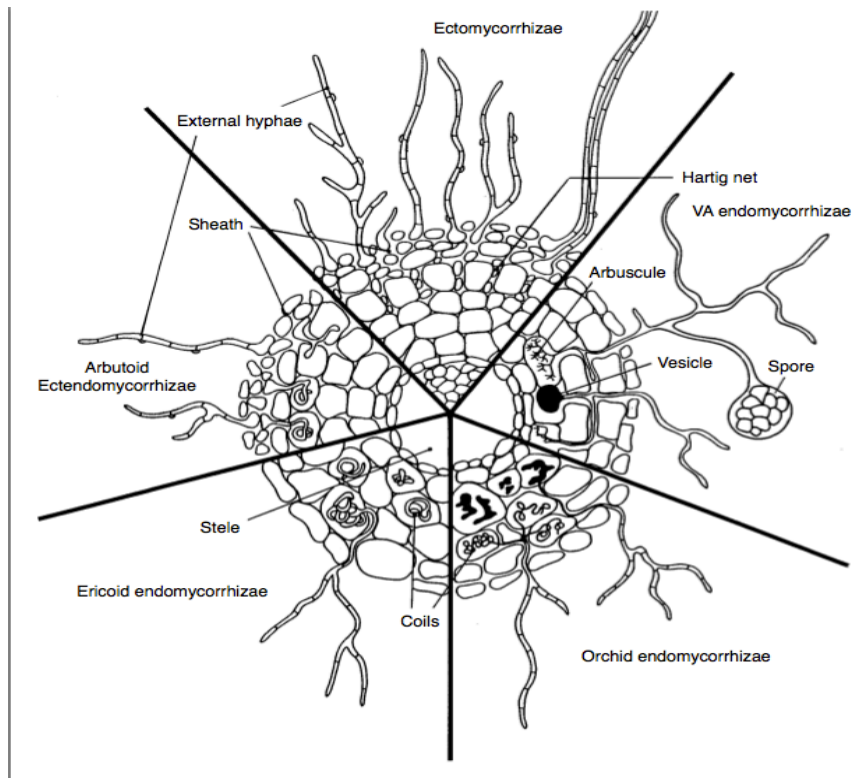


Fig. 2 – Drawing of mycorrhizae types shown colonizing a root cross-section, from Selosse and Le Tacon (1998).

1.4.1.2 Mycorrhizas assist successional processes

Examination of the specificity and complexity of these mycobionts and their role in ecosystem establishment and recovery has continued. Smith and Read (2008) discuss at length how functionally, the presence of mycorrhizas in different biomes assists successional processes, and appreciation of their role as key participants in belowground soil community composition is becoming increasingly recognized in determining ecosystem multi-functionality (Wagg *et al.*, 2015). The direct role of plant root fungal community composition and belowground C and N sequestration capacity is another important factor when studying successional dynamics as ectomycorrhizal and ericoid mycorrhizal fungi may counter one another (Clemmensen *et al.*, 2015). Moreover soil capacity to store carbon and counterbalance climate changes has been linked to the proportion of different mycorrhizal fungal types given their differential nitrogen-degrading enzyme capacity (Averill *et al.*, 2014).

The importance of mycorrhizal relationships in ecological succession is best understood and studied in primary successional environments. Nara (2006a), (Nara, 2006b) looked at the establishment of *Salix reinii* on Mt Fuji. He created networks (common mycorrhizal networks) between seedlings and inoculated mother plants in what was otherwise a 'volcanic desert' and showed substantial increase in fungus species' nutrient

acquisition and growth by seedlings connected to ectomycorrhizal networks. Fukuchi *et al.* (2011) also studied ecological succession on volcanic deserts in Japan. They noted ericaceous plants were colonizing in the most nutrient-deficient environments, always associating with ErM and occasionally with dark septate endophytic fungi. Ectomycorrhizal, arbutoid ectendomycorrhizal and orchid mycorrhizal plants were also present but ericaceous plants (e.g. *Empetrum nigrum* var. *japonicum*, *Ledum palustre* var. *diversipilosum*, *Vaccinium vitis-idaea* var. *minus* and *Loiseleuria procumbens*) were the dominant colonizers on four sites in an acidic area of the Esan volcano in Japan. One Ericaceae genus, *Enkianthus*, associated with arbuscular mycorrhizas, not ErM.

Young *Calluna vulgaris* plants (less than two years old), introduced to ex-arable sites designated for heathland restoration, have less extensive mycorrhizal colonization of their roots than their equivalent age counterparts introduced to cleared plots on established heathlands (Diaz *et al.*, 2006). Moreover, if these mycorrhizal exchanges do not occur from the beginning, reversion to grassland and forbs is more likely owing to their competitive pioneering advantage in tolerating higher soil pH levels and their ability to extract phosphorus (van der Wal *et al.*, 2009). Natural succession processes on heathlands (i.e. towards climactic woodland communities) are also influenced by the array of fungi available to form mycorrhizal relationships with encroaching trees (Collier and Bidartondo, 2009).

Other examples of mycorrhizal effects in primary succession are found in sand dunes and other semi-arid erosion-prone systems (Martínez and Pugnaire, 2009). These show there is some species-specificity in plant–arbuscular mycorrhizal (AM) associations, suggesting a role of AM on plant community dynamics in arid ecosystems (Kikvidze *et al.*, 2010).

1.4.1.3 Mycorrhizas as inoculum

Numerous studies have been published in recent years highlighting both the limitations and prospects of an efficient mycorrhizal inoculum to facilitate establishment and growth on a wide scale (Yang *et al.*, 1998), Vosatka *et al.*, 1999) and for Ericaceae plants with a commercial use, e.g. *Vaccinium macrocarpon* or *V. corymbosum* (Gorman and Starrett, 2003, Eccher *et al.*, 2006, Kosola *et al.*, 2007, Eccher *et al.*, 2009, Eccher and Martinelli, 2010, Zinati *et al.*, 2011). Smith and Read (2008) discuss opportunities and challenges in using fungi as inoculum. The studies they reference, however, mainly focus on arbuscular mycorrhizas, which are obligate symbionts, unculturable without their symbiotic hosts.

Experimental work has demonstrated inoculation with mycorrhizal fungi can enhance growth performance directly or indirectly either via nutrient uptake as in Strandberg and Johansson (1999), or biomass growth and survival rates, or both, following inoculation. In another study, ericoid mycorrhizal fungi (ErM) maintained their ability to form mutualistic associations with *Erica arborea* in a Mediterranean *Quercus* forest, after this initial host was no longer present in the forest (Bergero *et al.*, 2000). Vosatka *et al.* (1999) demonstrated the feasibility of inoculation using both arbuscular and ericoid mycorrhizal fungi, noting some establishment success as well as growth response variable impact with the treatment. Importantly, they found some fungal strains were more successful than others in fostering benefits to the host plants.

Nursery studies focusing on ErM fungal inoculum suggest a positive correlation between treatment and host gain, even if some results are mixed. Establishment of *Vaccinium* (a typical ErM host plant) experienced enhanced growth and nutrient uptake after 167 days by introducing the inoculum to the sterilized peat compost (Litten *et al.*, 1992), while another more recent study with the same host genus has reported mixed results (Villarreal-Ruiz *et al.*, 2012). The fungus *Meliniomyces bicolor* (a close relative to *Pezoloma ericae*) enhanced root length by 2 ½ times but another unknown fungal isolate closely related to *P. ericae* suppressed growth in some *Vaccinium* (*V. myrtillus* and *V. vitis-idaea*), but not *V. macrocarpon*. The later fungal symbiont had presented ErM coils typical of ericaceous host plants. This study might suggest symbiotic specificity between the tested fungus and vascular plants, some other ecological co-factor, or the possible 'costs' associated with early colonization ie. the intricate relationship requires the plant to synthesize photosynthates for the new fungal partner diverting cytoplasmic energy; thus the benefits are masked until the plant matures. Similarly, Gorman and Starrett (2003) examined the impact of *P. ericae* colonization on shoot growth for a range of ericaceous taxa grown axenically in vials for 60 days. Results found the treated groups had statistically significantly more growth, even with little or no evidence of ErM colonization.

While some of the distribution methods of AM propagules may be effective, storage efficacy, economic costs and resilience independent of host is mixed (Gianinazzi and Vosatka, 2004). Importantly, ErM (not AM) are applied to the experimental systems and field sites I have tested breaking this barrier, as ErM are culturable. Moreover the liverworts are promising as a functionally viable and economic delivery mechanism for the ErM fungus.

Nonetheless, contact between ErM fungus and Ericaceae host plants must be renewed regularly because the period of colonization is time limited; epidermal cell breakdown was observed to commence about four weeks after fungal penetration in *Rhododendron ponticum* roots (Duddridge and Read, 1982).

Other studies contributing to this research have focused on Ericaceae horticultural trials measuring colonization and N:P:K concentrations with and without inoculum (Strandberg and Johansson, 1999), restoration ecology experiments documenting a correlative relationship between the level of ericoid mycorrhizal colonization and aboveground biomass (Diaz *et al.*, 2006), and ErM fungal biomass recovery after fire (Green *et al.*, 2013) or in abandoned agricultural plots (van der Wal *et al.*, 2009).

1.4.1.4 Nutrient mobilization

The establishment success of ericaceous plants such as *Calluna vulgaris* and *Erica tetralix* on organic heathland soils with slow rates of decomposition and extremely limited nitrogen (N) and phosphorus (P) is a result of specialized mycorrhizal fungus associations which enable the uptake of both N and P (Leake and Miles, 1996, Read and Stribley, 1973). Availability and mobilization of these macronutrients are the most important constraints on plant fitness in heathlands (Read *et al.*, 2004), which are nutritionally poor and require renewal post-fire, a natural ecological disturbance factor in heathlands. In addition, waterlogged soil which is also common in heathlands, appears to inhibit ammonification and nitrification (Schmidt and Stewart, 1997), further necessitating ErM for plants to obtain nitrogen. The role of mycorrhizas in facilitating access to organic macro- and micro-nutrients has been a subject studied extensively in the last two decades. The following sections highlight additional studies relevant to the nursery and field experiments designed for this thesis.

1.4.1.4.1 Organic and inorganic nitrogen

Stribley and Read (1974), (1976, 1980) established that the ericoid mycorrhiza associating with ericaceous plant roots of *Vaccinium macrocarpon* were utilizing organic compounds to acquire N, as effectively as inorganic ¹⁵N labeled ammonium sulphate, but aseptically-grown, non-mycorrhizal *V. macrocarpon* plants, were not. They highlight that Ericaceous plants in particular grow in peaty humus where mineralization rates of organic N are particularly low. They showed ErM colonization increases biomass production of *V. macrocarpon*, by greatly increasing the plants' ability to access different sources of organic N compounds (in HCl-hydrolysable + insoluble humin). In particular, the ericoid fungi, like the fungi of ectomycorrhizas (Lundeberg, 1970), are unable to utilize the complex humic N compounds directly and therefore their

importance must lie in the capacity to utilize the simpler organic compounds or their breakdown products (Stribley and Read, 1980). Chitin has been shown to comprise a substantial proportion of total N in the F horizon of *Calluna vulgaris* heathland (Kerley and Read, 1997), demonstrating the importance of fungi in nutrient recycling. The importance of mycorrhiza fungi in unlocking N, may be a greater limiting factor than P in some ericaceous plants (Read *et al.*, 2004). In their study, Read *et al.* (2004) showed positive growth responses in *V. macrocarpon* with simple organic N added but addition of P led to little growth response. This study also showed *V. macrocarpon* prefers organic N to byproducts of N through nitrification. Kosola *et al.* (2007) demonstrated inoculation with *P. ericae* increased the capacity of cranberry to utilize nitrate. Interestingly, the longer the plant was in culture was found to negatively correlate in the ability of the ErM to use N and probably other nutrients as well (Grelet *et al.*, 2005).

1.4.1.4.2 Organic and inorganic phosphorus

Phosphorus may be the most limiting nutrient in ericaceous habitats (Mitchell and Read, 1981, Bolan, 1991, Myers and Leake, 1996). Mitchell and Read (1981) tested the utilization of organic P (e.g. sodium inositol hexaphosphate) and inorganic orthophosphate, by ErM associated ericaceous plants *Vaccinium macrocarpon* and *Rhododendron ponticum*. The ErM fungus decreased the pH of the medium and caused enhanced growth of mycorrhizal seedlings. The ErM's ability to utilize organic P, where DNA was the only source, was demonstrated by Leake and Miles (1996) who found the mycelial dry weight was considerably more on medium with DNA than the alternative with phosphoric acid. Furthermore, Myers and Leake (1996) introduced P in the form of salmon sperm nuclei to *Vaccinium* plants and compared it to plants grown with orthophosphate, mycorrhizal (M) and non-mycorrhizal (NM). The M plants significantly increased the efficacy of usage of both organic and inorganic forms of P, which was inaccessible to the NM plants.

1.4.1.4.3 Other nutrient uptake considerations

Importantly the level of atmospheric CO₂ has also been shown to impact mycorrhiza affecting vascular plant growth (by enhancing nitrogen and/or phosphorus use efficiency), as seen with dark septate fungus in partnership with Scots pines (Alberton and Kuyper, 2009, Alberton *et al.*, 2010) and thalloid liverworts' associations with Mucoromycotina and Glomeromycota fungi (Field *et al.*, 2015b, Field *et al.*, 2015c).

Another consideration is the presence of micronutrients, which are affected by microbial interactions, but rarely studied in this context. One notable study found *Calluna* roots colonized by mycorrhizal fungi had a significant increase of Fe

concentration in their shoots (Leake *et al.*, 1990a). This would be particularly relevant where soils are high in calcium and Fe is sparsely available.

Climate also influences microbial interactions between mycorrhizal fungi (and/or bacteria) and plants (Compant *et al.*, 2013) thereby affecting nutrient availability and uptake by plants. Compant *et al.* (2013) contextualize this with respect to climate change in their meta-analysis which incorporates studies of arbuscular mycorrhizal fungi (AM), ectomycorrhizal fungi and 'plant growth-promoting' bacteria. Although they exclude ericoid mycorrhizal examples, their findings inform experimental design and analyses in terms of temporal considerations. In a more recent series of experiments manipulating soil temperature, AM mycelium colonization of host plants' roots and external growth were variably responsive to changes in root zone (Carvalho *et al.*, 2015) and ambient (Barrett *et al.*, 2014) temperatures. Barrett *et al.* (2014) also showed this can influence nutrient uptake and host plant growth.

1.4.1.5 Buffering plant communities from toxicity

Evidence from environments that are contaminated by toxic metals indicates that ErM, and in some instances their plant hosts, have evolved resistance to these metals. Experimental results suggest a vital role of ErM in nature is detoxification of the soil (Leake and Read, 1991, Cairney and Ashford, 2002), allowing the delicate hair roots to develop in otherwise stressful chemical environments. Shaw *et al.* (1990) showed the presence of *Pezoloma ericae* can be a stabilizing organism during seasonal fluctuations in Fe (which are typical of heathlands), in addition to mobilizing organic forms of N and P. Furthermore, the phosphatase enzymes known to be generated by *P. ericae* (Pearson and Read 1975) provide resistance to inhibition by metal ions Al^{3+} and Fe^{2+} (Shaw and Read 1989).

The presence of mycorrhizas has also been shown to reduce vascular plants' exposure to arsenate on toxic sites by enhancing P supply (Cairney *et al.*, 2001), allowing ericoid mycorrhizal plants to establish and colonize formerly polluted sites (Cairney and Meharg, 2003). It has also been shown that *P. ericae* isolated from plants growing on mine sites are able to resist a much higher concentration of toxic contaminants (both arsenate and copper) than heathland-sourced isolates, at least *in vitro*.

The apparent ability of ErM fungi to develop resistance to metal toxicity is thought to enable ericaceous plants to colonize polluted soil. Initially demonstrated by Bradley *et al.* (1981) with *Calluna vulgaris* seedlings, ErM are now recognized to be a major factor in the establishment success of ericaceous taxa in polluted environments (Cairney and

Meharg, 2003, Perotto *et al.*, 2012) and mechanisms employed by the fungus to detoxify the roots intracellularly are becoming better understood (Perotto *et al.*, 2012).

1.4.2 Vascular and non-vascular plants share the same mycobiont

Leafy liverworts, amongst the earliest branching lineages of land plants (Kenrick and Crane, 1997) and their fungal symbionts, may play an important role in restoration or expansion of wet heathland environments. It is widely assumed that the earliest plants colonized the land by forming symbiotic associations with soil dwelling fungi (Pirozynski and Malloch, 1975, Bidartondo *et al.*, 2011) and continuing the alga: oomycete fungal union recorded in the fossil record (Kenrick and Crane, 1997), paving the way for the subsequent establishment of pioneering vascular plant communities. Liverworts, modern analogues of the first land colonizers, and their mycobionts may still provide a valuable role in successional ecological dynamics in some habitats today, for example in wet heathland systems, where leafy liverworts and Ericales plants co-exist and share the same ascomycete fungal symbiont *Pezoloma ericae* (Duckett and Read, 1995, Chambers *et al.*, 1999, Upson *et al.*, 2007).

Based on fossil records, ericoid mycorrhizal fungi (ErM) originated approximately 140 million years ago or during the early Cretaceous period (Cullings, 1996), predating the earliest Ericales, which radiated from Gondwana during the mid- to late-Cretaceous period (Dettman, 1992), Smith and Read, 2008). The Ericaceae family, which is thought to be monophyletic (Kron *et al.*, 1999, Cairney, 2002), and whose members often dwell in inhospitable nutrient-poor environments, may have originally benefitted from mutualisms between ascomycete fungi and leafy liverworts which predate the vascular plants (Field *et al.*, 2015a) - although a different origin of the fungal symbiont in the Ericales or the possibility of a reversal (or several switches) of host, from vascular plant to liverwort, cannot be excluded (Kottke and Nevel, 2005).

Regardless of its genesis, the ascomycetous fungal symbiont *P. ericae* is shared between major ericaceous genera such as *Calluna*, *Erica* and *Vaccinium* and the leafy liverwort families Cephaloziaceae, Cephaloziellaceae, Lepidoziaceae and Calypogeiaceae (Pressel *et al.*, 2010), and it can be isolated from both the vascular and liverwort hosts and resynthesized in cross-inoculation experiments *in vitro*. Because these ericaceous plants and liverworts occur in the same wet heathland systems a major hypothesis is that the presence of liverwort-associated mycorrhizal fungi in the substrate may provide an inoculum and therefore an advantage for ericoid plant establishment, as first proposed by Duckett and Read (1995). Testing this hypothesis will help determine how

liverworts pioneer colonization in wet heath communities and their potential influence on vascular plant establishment in the field, the focus of this study.

The widespread association between the ericoid symbiont *P. ericae* and the leafy liverwort *Cephaloziella varians* in undisturbed maritime and subarctic conditions (Upson *et al.* 2007) across a 1,875 km transect, points to the potential pioneering or facilitative nature of these fungus-liverwort relationships, in laying the groundwork for succession of heathland communities. Upson *et al.* (2007) fulfilled Koch's postulates for *Cephalozia varians* and *P. ericae* by inoculating axenically-grown liverwort with a fungal isolate from the plant, resynthesizing the isolates and documenting the resulting coils in the rhizoid cells.

As Kottke and Nebel (2005) are careful to point out, physiological experiments demonstrating nutrient exchange had yet to be carried out between liverworts and fungi (written before Field *et al.* 2015; Kowal *et al.* in draft), and therefore they deem these relationships 'mycorrhiza-like'. Also, liverworts do not form mycorrhizal fungal relationships *sensu stricto* as they do not have roots, but many liverwort species exhibit intimate relationships with fungi in their rhizoids, thalli and stems - or "mycothalli" (Davis and Shaw, 2008). Regardless of the extent of the mutualism between liverworts and their fungal partners, they seem to have developed a mutualistic mycorrhiza-like relationship with fungi before vascular root-fungi relationships evolved (Kottke and Nebel, 2005) as suggested by the presence of genes (DMI1, DMI3 and IPD3) for mycorrhizal function (in legumes and rice) that have been conserved across all land plants (Wang *et al.*, 2010). Wang *et al.* (2010) reported that three mycorrhizal genes in the common ancestor of all land plants were vertically inherited supporting the idea that mycorrhizas played a major role in the colonization of land by plants. My research aims to further the understanding and characterize this mutualism amongst extant leafy liverworts and the ericoid mycorrhizal fungus occupying its rhizoids.

Symbiotic fungal links between non-vascular and vascular plants have also been demonstrated in other plant and liverwort families; *Plantago lanceolata* and the thalloid liverwort *Pellia epiphylla* were colonized by the same fungus *in vitro* (Read *et al.* 2000) and Bidartondo *et al.* (2003) showed in microcosm experiments that the fungi of *Aneura mirabilis* (a non-photosynthetic underground liverwort) were able to form ectomycorrhizas with *Betula* spp.; the liverwort was also shown to benefit by obtaining carbon via shared basidiomycete fungi from *Betula*.

1.4.3 Diversity of ericoid mycorrhizal hosts

Recent data and molecular studies have expanded knowledge of the range of ericoid mycorrhizal fungi (ErM) host plants. Taking plant samples from a range of plant families occupying the same sclerophyllous forest, Chambers *et al.* (2008) found that fungal isolates from 17 plant species representing a wide range of families - Apiaceae, Cunoniaceae, Cyperaceae, Droseraceae, Fabaceae-Mimosoideae, Lomandraceae, Myrtaceae, Pittosporaceae, Proteaceae and Stylidiaceae - were able to form ErM coils in the epidermal cells of an Ericaceae plant, *Woolfsia pungens*. Three of the isolates were also able to grow intercellularly, intracellularly and/or form microsclerotia in roots of another plant family, Stylidiaceae, demonstrating adaptability of ErM forming in other vascular plant families, not closely related to Ericaceae. This may be relevant for further applications of leafy liverworts as inoculant vehicles.

Pressel *et al.* (2010) reviewed how recent molecular and cytological work has advanced our knowledge about bryophyte-fungal symbiosis, host ranges, and function. With respect to leafy liverworts, they conclude the only putative mycorrhizal fungi occupying the rhizoids are ascomycetes; to date all have been identified as *Pezoloma ericae*, the fungal taxon isolated in this study.

1.4.4 Genetic diversity of ericoid mycorrhizal fungi strains

Ericaceae plants are also more promiscuous than previously thought (Perotto *et al.*, 2002, Zhang *et al.*, 2009, Tian *et al.*, 2011), with multiple fungal associates, both ascomycetes and basidiomycetes, found in a single plant. Early DNA fingerprinting of mycorrhizal fungi using ITS PCR-RFLP, supported by morphological analyses, established that hair roots of *Calluna vulgaris* contained greater mycorrhizal diversity than previously reported (Perotto *et al.*, 1996). Using the ITS 2 region, Monreal *et al.* (1999) detected even greater diversity of fungal isolates, all of which were able to form ericoid mycorrhizas with *Gaultheria shallon*. Perotto *et al.* (2002) later showed three taxa dominating ErM relationships in ericaceous host plants, *Pezoloma (Rhizoscyphus) ericae*, *Scytalidium vaccinii* and *Oidiodendron* spp. This was further extended by Hambleton and Sigler (2005) who identified a new genus, *Meliniomyces*, its DNA fingerprints closely related to *P. ericae*, but morphologically distinct. More recent molecular analyses of *Rhododendron decorum* by Tian *et al.* (2011) yielded 12 taxa, several similar to *Oidiodendron maius*. Thus, the diversity of ErM fungi occupying hair roots of *Rhododendron* species is complex, probably reflecting similar diversity in other ericaceous species. Koch's postulates remain to be tested for most putative ericoid mycorrhizal fungi.

1.4.5 Mycorrhizal fungi isolation

Although *Pezoloma ericae* is not an obligate fungus, isolating the fungus from host plants -either roots of vascular plants or rhizoids of liverworts plants - can be challenging. The next two sections reference studies and describe methods relevant to techniques later described in this dissertation.

1.4.5.1 Isolating fungi from liverworts

A literature review focusing on the isolation of *Pezoloma ericae* from host plants resulted in three studies focusing on liverworts. The studies below established isolation of this ericoid mycorrhizal fungus (ErM) from leafy liverworts is possible and also demonstrated reciprocal colonization of the fungal isolate to a vascular plant, albeit, all *in vitro*.

Duckett and Read (1995) extended the range of *P. ericae* hosts and demonstrated typical ericoid mycorrhizal coils could be formed in Ericaceous vascular plants (*Calluna*, *Rhododendron*, *Erica* and *Vaccinium*) from leafy liverwort symbionts sources. This work also mentioned, for the first time, the possibility of using liverworts as inoculum in an ecological setting. Following brief immersion (5s) in hypochlorite and several rinses in sterile distilled water for surface sterilization, the stem axes were planted in either 2% malt or nutrient free agar. Both media were successful.

Chambers *et al.* (1999) identified *P. ericae* in Australian and Antarctic *Cephaloziella exiflora* using ITS DNA sequences with primers ITS1 and ITS4. Previous determinations of *P. ericae* were based on morphological examinations and interaction behaviour (e.g. Duckett and Read 1995). This work established a more global distribution of *P. ericae* as a mycobiont of these cosmopolitan leafy liverworts. Its presence in the harsh Antarctic environs provides further support that fungal symbionts may have assisted land plants to evolve in adverse edaphic conditions. These isolates were developed and maintained on modified Melin-Norkrans agar and 25% potato dextrose agar. Surface sterilization techniques were not mentioned.

Upton *et al.* (2007) isolated *P. ericae* from *Cephaloziella varians* (a sub-Antarctic species) and observed ErM coils in the epidermal cells of *Vaccinium macrocarpon* hair roots *in vitro*. As above, they also used ITS region DNA sequences to identify the fungi with primers ITS1 and ITS4 (as well as partial large subunit ribosomal DNA). Surface sterilization was achieved by serial washing in sterilized distilled water; 20 changes, five minutes per wash using a shaker and then blotted on sterile filtered paper before planting in media. Culturing was executed on either 10% modified Melin-Norkrans agar (pH 4.5) or 1% malt extract agar stored in the dark at 18°C.

1.4.5.2 Isolating fungi from ericaceous plant 'hair' roots

A review of studies isolating fungi from Ericaceous hair roots found similar media in use for the vascular plants as for liverworts (section 1.4.5.1). There is no single medium demonstrating a higher success rate in terms of isolating ErM fungi *in vitro*. The array of surface-sterilization techniques employed is of varying complexity. As the epidermal cells of vascular plants are thicker than in liverworts, they can withstand longer exposure to cleansers and higher concentrations of chemical sterilants without compromising the vitality of the cells. Below is a summary of studies referenced for this research (summarized in Table 1); all reported success without noting a high contamination rate.

Table 1 - Methods and outcomes for isolating ericoid mycorrhizal fungi

Plant	Fungi	Method	Success	Reference
<i>Calluna vulgaris</i> and <i>Vaccinium myrtillus</i>		Two methods: direct plating method: hair root placed directly on to 2% malt agar plates containing streptomycin as a bacteriostatic agent; incubation at 20°C. The maceration method: Serially washed roots macerated with a pestle in an autoclaved vial containing 3 ml distilled water for a period sufficient to detach most of the cortical cells from the central stele and to disperse them singly or in groups as a cell suspension using a Pasteur pipette a small volume of suspension spread thinly over 0.5 distilled water agar in a Petri dish.	<i>Calluna</i> : direct plating (DP) – 7 slow growing isolated from 100 root tips and macerated (M) – 191 from 100 root tips. <i>Vaccinium</i> : DP- 5 of 100 and MM, 174 for 100 roots.	Pearson and Read (1973)
Ericaceous plants	ErM fungi	Roots submerged in 5% bleaching solution for 3 minutes, washed in sterilized distilled water and transferred to 5% hydrogen peroxide and another 5 minutes in 5% bleaching solution. Both 1% malt agar and water agar (pH 4.5) culture plates with streptomycin. Incubated	200 isolates	Jansa and Vosatka (2000)

		for three weeks, 25°C in the dark.		
<i>Quercus ilex</i> and <i>Erica arborea</i>	<i>Oidiodendron maius</i> , <i>O. griseum</i> , <i>O. tenuissimum</i> , <i>Paecilomyces carneus</i> , <i>Penicillium</i> sp. and <i>Torulomyces lagena</i>	Roots sterilized in 20% household bleach (1 min) and rinsed twice. Gently homogenize with a glass pestle. Samples plated on 2% malt agar supplemented with streptomycin (20mgL).	Approx. 50 root tips were plated for both. <i>E. arborea</i> produced 150 fungal isolates and <i>Q. ilex</i> produced 70.	Bergero <i>et al.</i> (2000)
<i>Epicaris pulchella</i>	<i>Oidiodendron</i> , <i>Myxotrichaceae</i> and Ericoid mycorrhizal endophyte.	Roots sterilized in 100% commercial bleach solution (4.5% available chlorine) containing 100 mgL ⁻¹ of Tween 20™ (30s), followed 70% ethanol solution (30s) and rinsing in sterile distilled water (1min x3). Roots plated on 2% malt extract agar with streptomycin (15 mgL ⁻¹), gentamycin (15 mgL ⁻¹) and tetracycline (12mg L ⁻¹) (Pearson and Read,1973); incubation in dark at 23°C. slower-growing fungi were subcultured onto 2% PDA; (Oxoid).	124 slow-growing isolates were cultured from 135 hair root pieces.	Bougoure and Cairney (2005)
<i>Apiaceae</i> , <i>Cunoniaceae</i> , <i>Ceratopetalum</i> , <i>Cyperaceae</i> , <i>Droseraceae</i> , <i>Fabaceae</i> - <i>Mimosoideae</i> , <i>Lomandraceae</i> , <i>Myrtaceae</i> , <i>Pittosporaceae</i> , <i>Proteaceae</i> and <i>Stylidiaceae</i>	<i>Epacris pulchella</i> root-associated fungus EP50, 8 <i>Woollsia</i> mycorrhizal fungus, <i>E. microphylla</i> root-associated fungus, <i>Woollsia pungens</i> ericoid mycorrhizal fungus	Surface-sterilized roots in 20% bleach (30s), followed by a solution containing 70% ethanol (30s) and finally rinsed three times with 1-minute rinses in sterilized distilled water. Sterile roots were cut into 0.5 cm pieces and plated on 2% potato dextrose agar (PDA, Oxoid) with streptomycin (15 mgL ⁻¹), gentamycin (15mgL ⁻¹) and tetracycline (12 mg L ⁻¹) (Pearson and Read, 1973) in Petri dishes; incubation at 21C in the dark.	Isolates from all 17 plant species formed ErM coils in Ericaceae plant; 3 intercellularly, intracellularly and/or microsclerotia form.	Chambers <i>et al.</i> (2008)
<i>Rhododendron fortunei</i>	<i>Oidiodendron maius</i> , <i>Epacris microphylla</i> , <i>E. pulchella</i> , Epacrid root endophyte	Roots sterilized in 72% ethanol solution (30s), 10% sodium hypochlorite (10–15 min) and rinsed in	220 isolates from 100 root samples.	Zhang <i>et al.</i> (2009)

	sp., <i>Cryptosporiopsis ericae</i> , <i>Basidiomycete spp.</i> + various non-mycorrhizal fungi.	sterile distilled (4x) water. roots were cut into 0.3 to 0.5 cm segments, plated on modified Melin–Norkans agar (Xiao and Birch 1992), and incubated in the dark at 25°C. slower growing fungi were subcultured on 2% malt extract agar.		
<i>Rhododendron decorum</i>	<i>Neonectria</i> , <i>Radicalia</i> , <i>Bonectria ochroleuca</i> , <i>Plectosphaerella cucumerina</i> , <i>Oidiodendron maius</i> , <i>O.citrinuman</i> , <i>Gliocladium cibotii</i> , <i>Chaetomium sp.</i> and <i>Chalara sp.</i>	Roots surface sterilized with 0.1% mercuric chloride solution for 3–5 min, rinsed in sterile distilled water and dried with sterilized gauze. Roots plated on PDA medium mixed with streptomycin (30 mg mL ⁻¹). Plates sealed and incubated in the dark at 22–25°C.	218 isolated from 300 root samples.	Tian <i>et al.</i> (2011)

Pearson and Read (1973) surface-sterilized using either calcium hypochlorite (concentration 10:140 w/v solution) or serial washes. They experimented with both direct plating (1-cm strips of root and planting on 2% malt agar containing streptomycin) and macerated roots to release the cortical cells for a suspension. The maceration technique was much more effective in yielding the usually sterile ‘slow-growing dark mycelia with segmented hyphae’ and the direct plating method yielded more contaminating fast-growing organisms found in the rhizosphere, e.g. *Penicillium expansum* and *Trichoderma* spp. All of the later studies used a modified version of Pearson and Read (1973) with more extensive surface-sterilization and sterilized tap water, rather than distilled, to prevent plasmolysis of the fungal hyphae.

1.5 Heathlands and wider application of research

Worldwide, ericaceous plant zones are widespread, occurring across latitudinal gradients and climatic zones (Read, 1993); their common constraint being a low availability of N and/or P, amongst other nutrients.

Most lowland heathland (defined as 300 m or less) has been lost over the last 100 years due to land use changes, including both urban and agricultural development. Much of the UK’s historic heathland commons have already become woodlands or neglected bracken-dominated fields. As plagioclimatic communities, heathlands require active management (e.g. grazing) to keep competition from pioneering trees, invasive scrub

grasses and bracken at bay. Consequently, lowland heathlands now stand as one of the most threatened habitats in Europe. As the UK has 20% of the world total (SEEBF, 2008) this habitat has been given priority status under the UK Biodiversity Action Plan (BAP) (Maddock, 2008 (updated 2011)) and heathlands are designated as important European Union habitats, codes 4010, 4020 and 4030 (Northern Atlantic Wet Heaths with *Erica tetralix*, Temperate Atlantic wet heaths and European dry heaths, respectively) under the EU Habitat Directive 1992 (JNCC, 2008).

Heathland landscapes are generally defined as having at least 25% cover by dwarf heather and associated higher plants in the Ericaceae family, which have adapted to nutrient-poor acidic soils in cool wet temperate climates (Rodwell, 1991). These landscapes host a unique and rich wildlife population and contain an assembly of associated trees, scrub, bracken, bare ground, acid grassland, and cryptogamic organisms, often with bog systems. The landscape is dynamic, experiencing successional changes from bare ground to mature heather scrub. Variation in height and structure encourages biodiversity.

Liverworts are pioneers and might help nutrient-poor wet heath soils become more hospitable for seed recruitment and re-sprouting of ericaceous scrub, following fire or other disturbances. Demonstration of an ecological link between liverworts and ericaceous mycorrhizal fungi in the glasshouse and/or field has major implications for understanding the requirements for early establishment of mycorrhizas in vascular woody plants and thus for the recovery of neglected or degraded landscapes due to either anthropogenic or ordinary ecological successional movement.

Further, preservation and reestablishment of wet heathlands have increasingly wider implications than the maintenance of their inherent fauna and flora biodiversity. Because heathlands are important in sequestering carbon (Longton, 1992), they function as climate regulators and a reduction in heathlands and therefore peat decomposition, is expected to accentuate climate change by releasing C. Heathlands containing peatlands and *Sphagnum* bogs are becoming increasingly under threat and higher nitrogen levels in lowlands further increase decomposition rates (Jones and Power, 2011) making lowland heathlands particularly vulnerable.

Although the focus herein is Ericaceae plants dominating heathlands, plants that interact with ErM fungi occur in many other habitats and some are very important economically. Most genera in the order Ericales are colonized by ErM; notable examples include *Gaultheria*, *Vaccinium* and *Rhododendron*; but ericoid mycorrhizal fungi are also

found in other vascular plant families not closely related to Ericaceae e.g. Stylidiaceae (Chambers *et al.*, 2008).

In addition to habitats dominated by Ericaceae plants there are many other ecosystems dominated by other plant families where liverworts (containing fungal symbionts) contribute to the vegetation ecology. As liverworts are known to associate with all four mycorrhizal fungal groups (Field *et al.*, 2015a), restoration of a wide range of habitats, may be aided using this triptych strategy: bryophyte (non-vascular) - mycorrhizal fungus – tracheophyte (vascular). Restoration projects in cloud and rainforests may benefit from identifying a mycorrhizal fungus present in both vascular plant roots and liverworts (leafy or thalloid) found in the same habitat. For example, perhaps an important understory plant has been removed from an area and reestablishment of that plant has been deemed important for the local fauna or vegetation composition. If reestablishment through ordinary channels proves difficult, perhaps the mycorrhizal partner can be delivered to the forest floor by introducing liverworts, which share the same fungal symbiont as that understory plant. This same approach can be used in wadis or other areas prone to landslides, where the fungal symbionts present in the topsoil have been washed down through seepage, or away altogether. Liverwort-dominated slurries might also be tested in cloud forests or rainforests. The two prerequisites: the liverwort must harbour a symbiotic fungus, which is shared by a dominating vascular plant(s) and an environment moist enough to keep the liverworts alive.

Demonstrating the unique resilience of liverworts, several studies have shown liverworts in bogs, particularly associated with ericoid plants (Longton, 1992), where they appear immune to attacks by bacteria and fungi (Ando and Matsuo, 1984, Asakawa, 1990) or withstanding harsh Antarctic environments (Upton *et al.*, 2007). The role of the fungal symbiont and the liverwort's poikilohydric physiology in assisting their resilience, has not been tested yet, but these previous studies point to their strength as an inoculum delivery mechanism.

1.6 Nomenclature and definitions

1.6.1 Taxonomic nomenclature

Most of the work referenced herein, including the key reference, Mycorrhizal Symbiosis (Smith and Read, 2008), refer to *Pezoloma ericae* by the previous nomenclature *Rhizoscyphus ericae* W.Y. Zhuang and Korf or previous to that, *Hymenoscyphus ericae* (Read) Korf and Kernan. As in Smith and Read (2008), the latter is used when referring to the broader related endophytes not known to form typical ericoid mycorrhizas (ErM),

i.e. endophytes *sensu* Davis and Shaw (2008). This work follows the latest nomenclature based on Baral and Krieglsteiner (2006). Nomenclature for liverworts and fungi follows (Hill *et al.*, 2008) and www.speciesfungorum.org, respectively.

1.6.2 Definitions

'Gain' herein is measured either via macronutrient uptake as in Strandberg and Johansson (1999), or biomass growth and survival rates or both, following the application of ErM fungal inoculation, either via the leafy liverwort or as an isolate.

In referring to the fungus, it is '*Pezoloma ericae*' when it is isolated from its hosts, and 'ErM fungus' when it is a functioning mycorrhizal symbiont without molecular verification.

2 Chapter Two – Isolation and resynthesis of fungus from wild liverworts and ericaceous plants

2.1 Introduction

A widespread mycorrhiza-forming fungus (*Pezoloma ericae* (D.J. Read) Baral) with plants in the order Ericales also forms mutualistic associations with several families of liverworts (Duckett and Read, 1995, Read *et al.*, 2000). To determine whether leafy liverworts (colonized by *P. ericae*) are practical as an inoculum delivery device, the first step required isolating the ericoid mycorrhizal fungus *in vitro*, verifying its molecular identity, and resynthesizing the association *in vitro* *P. ericae* and axenically-grown liverworts.

2.2 Methods

2.2.1 Collection technique and storage of plant materials

2.2.1.1 Liverworts

Several liverwort species, from three families known to be colonized by *Pezoloma ericae* (Duckett and Read, 1995) - Lepidoziaceae, Cephaloziaceae and Cephaloziellaceae - grow on Thursley Common and were considered for this study. The two species *Cephalozia connivens* and *C. cuspidata* were selected because they are the most abundant. Plants were collected during the winter of 2012 and the spring of 2013. For each species, plants were selected from three colonies (at least 10m apart), removed from peaty topsoil with a knife, examined with a hand lens for genus identification and sexual stage and placed in the same plastic bag. To prolong the useful life of the sporophytes for *in vitro* cultivation, material was selected with sporophytes still housed in their perianths rather than on extended setae, and stored in a 'humid chamber' (Duckett *et al.*, 2004) occasionally removed for daylight.

2.2.1.2 Vascular ericaceous plants

Erica tetralix and *Calluna vulgaris* cuttings were originally collected from Thursley Common in late autumn 2011, following cutting propagation protocols by the Heather Society (Heather Society website, 2013). Stems for cuttings were selected with at least 10 cm of new growth. Plants were selected for tender shoots, which were beginning to firm but not yet woody. Cuts were made with secateurs in the field at least two cm below this zone. An additional collection of cuttings was repeated in spring 2012.

Inflorescences were selected using a hand lens to confirm presence of ripe seed heads. They were cut and placed in bags; care was taken to keep the two taxa separate. Samples were taken from over a dozen arbitrarily spaced plants. A number between 1

and 15 was selected to determine the number of paces between plant samples, each time walking in a different direction. All cuttings and seeds were collected from plants located in wet heath areas of both sites, but away from the common walking tracks to avoid public view and dog urine impact. Upon returning to the laboratory, the dried inflorescences were stored in paper envelopes in the refrigerator until sowing.

The *Calluna vulgaris* and *Erica tetralix* root material for ErM fungal isolation was collected from a wet heath area in Thursley Common in March 2012. Special care was taken to collect plants at least two years old as younger plants tend to be less colonized (Diaz *et al.*, 2006). Older mature plants are more difficult to remove. Five plants were removed from patches separated by at least 10m. Care was taken to remove the young hair roots close to the surface where most of the ErM activity is expected to occur (Smith and Read, 2008). These were placed in bags in the field with a little moisture and stored in a refrigerator at approximately 8°C for three days prior to commencing fungus isolation cultures.

2.2.2 Cultivating liverworts axenically

The liverwort cultures were developed using fresh liverwort material collected from Thursley Common (March 2012). A few days after collecting the material, the sporophytes were freshly above the perianths, thus easy to remove. The sporophytes were carefully removed by holding the seta with forceps and tugging gently from the plant body, followed by brief surface-sterilization (Duckett and Read, 1995). The spores were released by gently gliding the sporophytes across Parker medium (Appendix 3). Phytigel™ (Sigma-Aldrich) base medium is an alternative to agar, especially for liverworts and hornworts, as agar, even the most purified, has residual quantities of cations (particularly Zn²⁺) which may be toxic to some bryophytes (Duckett *et al.*, 2004). The apical side of the liverwort was used, away from the rhizoids, where in British species of *Cephalozia*, the fungal symbiont is contained. Upson *et al.* (2007) documented colonization by *P. ericae* of the entire liverwort in the sub-arctic species of *Cephaloziella* but this has not been observed in British species of liverworts in the same family; only the rhizoids are known to be colonized.

As the leafy liverworts grow slowly (Duckett *et al.*, 2004), at least six-months lead time was needed to build a population for experimental work.

2.2.3 Sampling of wild liverwort rhizoids for colonization by typical ErM fungal hyphae

Prior to molecular detection and identification of the fungi, the presence or absence of swollen rhizoids was established in the wild liverwort material (*Cephalozia connivens*

and *C. bicuspidata*) from the above-described collection. Forceps were used to arbitrarily select 20-25 individual stems from the collection bag. These were placed in separate pre-numbered Petri dishes. Four sample groups were selected in this way and examined under a dissecting microscope to document the percentage with swollen rhizoids and obvious hyphae emanating from the rhizoids (Fig. 3a). Presence or absence counts were documented. Samples of the rhizoids were examined under 20 and 60x magnification (Fig. 3b) to observe the fungal coils at various stages.

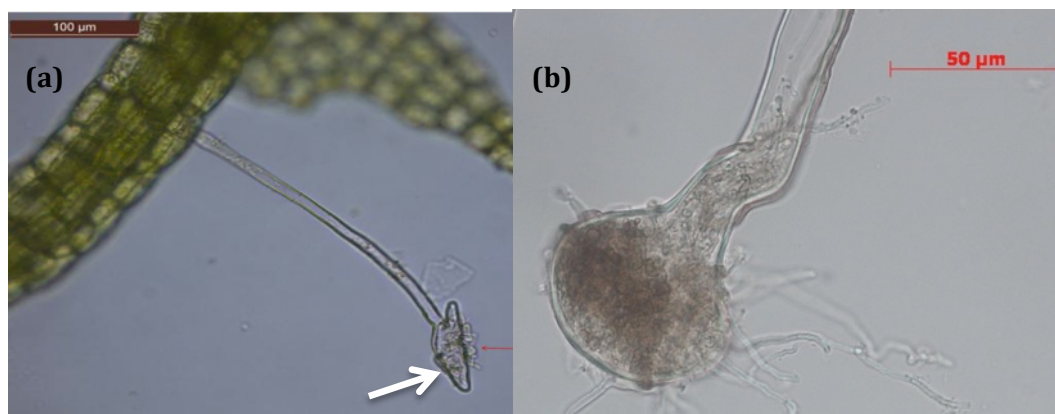


Fig. 3 - Swollen tip of *Cephalaria bicuspidata* rhizoid (a) and at higher magnification (b), both filled with hyphae of *Pezoloma ericae*.

2.2.3.1 DNA extraction, purification and sequencing analysis

Before commencing the nursery experiments with liverwort treatments, DNA from a sampling of the *Cephalaria connivens* and *C. bicuspidata* plant material was extracted to verify the presence of *P. ericae*.

The rhizoids were removed under sterile conditions and placed in a 1.5 ml Eppendorf tube containing 300 µl of cetyltrimethyl ammonium bromide (CTAB) lysis buffer; between six and ten rhizoids per tube (occasionally liverwort fragments were included).

The DNA was extracted using CTAB mini-preparation protocols (Gardes and Bruns, 1993) and were purified using QBioGene Gene-Clean kit (Fisher Scientific). Fungal ITS was amplified by PCR using PicoMaxx high fidelity (Agilent Technologies) with the primer combination ITS 1 and ITS 4 (White *et al.*, 1990) using the following temperature regime: 95°C for 2 min; 32 cycles each of 95°C for 40 s, 53°C for 30 s, 72°C for 1 minute; and, 72°C for 5 minutes. Post PCR, 2% agarose gels were prepared with ethidium bromide to check results. There were 44 reactions with a volume of 10 µl each (9.5 cocktail + 0.5 template). Prior to sequencing, PCR products were purified and cleaned using Affymetrix ExoSAP-IT, incubated at 37°C for 15 minutes and 80°C for 15 minutes. PCR products were cycle sequenced bi-directionally using BigDye v.3.1, precipitated using EDTA/ethanol and run on an ABI3730 Genetic Analyser (Applied Biosystems).

The ITS region sequence results were edited using Applied Biosystems DNA Sequencing Analysis Software Version 5.1, and compared using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) against reference DNA sequences on GenBank.

2.2.4 Isolation and *in vitro* cultivation of liverwort-associated fungi

Prior to isolating and culturing the fungi, a literature review was conducted to gather information about fungal isolation and culture techniques, including culture medium and expected success rates (Table 1). Summarized below are the techniques applied to the liverwort rhizoids and the ericoid roots.

2.2.4.1 Liverwort mycobiont isolation

Fungal cultures were initiated using fresh liverwort plant material collected from the field sites (described in section 5.2) and confirmed under a dissecting microscope to contain swollen rhizoids. Both isolated rhizoids and entire stem axes were used in culture. Prior to isolating the fungus, the cleaning regime described below was carried out.

The cleaning regime followed a combination of modified protocols from Pearson and Read (1973) and Upson *et al.* (2007). The liverworts were placed under tap water for two hours in sterile muslin and then serially washed in sterilized distilled water 20 times. In between each rinse the liverworts were placed in 50ml tubes and agitated in an orbital shaker for five to six minutes at 250 revolutions per minute. The liverworts were placed in 50ml tubes to enhance agitation. The transfer in between rinses was executed under a laminar flow hood to avoid contamination.

The growing medium used to isolate the target ErM in culture involved over 20 variations of surface sterilization protocols varying time and concentration of sterilization agents, with and without antibiotics (detailed in Appendix 1). Most either 'over sterilized' the tender leafy liverwort killing not only the surface pathogens but also the liverwort, or failed to inhibit faster growing contaminants. Dr. K. Newsham suggested using Czapek dox medium; he found MMN too strong for the fungi (conversation January 2013). He observed my cultures and suggested success may be enhanced by gently pressing the liverworts further into the culture medium.

The most successful combinations were on 1.5% and 2.0% water agarose (with antibiotics) and included two additional combinations: plates with 10% modified Melin-Norkrans agar (acidified to pH 4.5 with 10% HCl) (Upson *et al.*, 2007) and, plates with 2% Potato Dextrose Agar (PDA). Two antibacterial agents, streptomycin (50mg L⁻¹) and ampicillin (50mg L⁻¹), were added to half of the plates.

One to three liverwort stems were placed in each 9cm plate, sealed with Parafilm and stored in a cupboard, in the dark at room temperature. The isolated rhizoids were similarly plated, under sterile conditions, after carefully removing the rhizoids with fine forceps and a blade. A total of 83 plates were prepared, 41 with MMN and 42 with PDA. The 'promising' cultures, based on qualitative examination of morphological features (described in section 2.2.5.1), were used to analyse DNA.

2.2.4.2 Ericaceae hair root mycobiont isolation

Clumps around the base of the plant were gently broken off and placed first in a container with water to soak and loosen the topsoil and then placed under tap water for one hour in a sieve to remove soil particles. Care was taken to keep delicate hair roots intact and they were selected arbitrarily for observation. Slides were made to examine them under the compound microscope to confirm presence of ErM colonization in the cells. Once confirmed, a selection of these roots was used for isolation.

Surface-sterilization followed Bergero *et al.* (2000) using 1% bleach for 30s, rinsed three times with distilled water and serially rinsed 11 times in sterilized distilled water tubes. Pre-prepared 50ml tubes were autoclaved with distilled water and used for rinsing according to Pearson and Read (1973). These were stored for 24 hours in the refrigerator at 8°C.

According to protocols established by Pearson and Read (1973), half of these were homogenized (macerated) for lysing the tissue using a sterile glass homogenizer pestle; an amount equivalent to a match-head was added to each plate. The other half were plated directly using small 5 - 7 mm segments of hair root, three segments per plate. A third treatment application was tested by preparing a suspension in sterilized water with the homogenized roots (4:1), and distributed using a Pasteur pipette, roughly 1ml drop/plate. The total number of culture plates created, by method, were: (1) direct plating – 33; (2) macerated – 33; and, (3) suspension – 14; PDA medium cultures were used for all plates.

2.2.5 Molecular identification of dominant fungal morphotype

The identity of the fungi isolated in cultures was confirmed by analysing ITS ribosomal DNA. The plates selected for testing presented the dominant morphotype (described below). In total, there were six hair root source plates and 81 liverwort source plates.

2.2.5.1 Morphological characteristics of fungal isolate

The fungus is relatively slow growing, waxy, starting out white-pale grey and darkening to tawny brown-black, with age showing light grey margins or rings (Williams, 1994,

Chambers *et al.*, 1999, Smith and Read, 2008). The type specimen for *Pezizella ericae* (Read, 1974), housed at Kew Gardens, and photographs of *P. ericae* cultures isolated by Dr. Rebecca Upson (2013) (unpublished), were also examined for comparison.

As the hyphae grow, the mass develops concentric circular rings of black with shades of grey-brown, with white in the middle as described by (Upson, 2013). One of the key characteristics is the central white hyphae grow vertically and spiral with age. The shading and rings can be better viewed in photographs taken of successful isolates from this study (Fig. 5). Cultures for DNA extraction were selected according to these features. Although the colours, texture and thickness varied across the different culture medium, they generally shared the same appearance.

2.2.5.2 DNA extraction and sequencing

A ca. 2 mm scraping was removed from each culture dish with a sterilized scalpel. The blade was either changed or sterilized with ethanol and fire (10 s) in between each scraping, to avoid cross-contamination. Scrapings were placed in a 1.5 ml Eppendorf tube containing 300 µl of CTAB lysis buffer following CTAB mini-preparation methods using the fungal primer set ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990), by PCR. Care was taken to avoid the growth medium and all transfers were done under a laminar flow hood. DNA was extracted according to the methods described above (section 2.2.3.1). Two blank negative controls were introduced to the 96-well plates to test for airborne contamination.

Pezoloma ericae DNA sequences were downloaded from GenBank and were aligned with culture sequences using MUSCLE algorithms (Edgar, 2004). Neighbor-joining phylogenies were produced using a Tamura-Nei evolutionary model. Reliability was estimated using 1,000 bootstrap replicates.

2.2.6 Resynthesis *in vitro* of liverwort-fungus association

2.2.6.1 Inoculation of *Cephalozia connivens* and *C. bicuspidata*

Petri dishes were prepared for resynthesis between the axenically-grown liverworts and the fungal strains isolated from both wild leafy liverworts and Ericaceae plant roots. Phytigel (1.5%) was autoclaved and poured between eight and ten mm high into Petri dishes under a laminar flow hood. Using a sterilized blade, a small scraping of *Pezoloma ericae*, isolated from either the wild liverworts (JK38.12 or JK38.37) or *Erica* and *Calluna* hair roots (PDA1), was removed from subculture plates and transferred to a new plate, gently inserted beneath the surface of the medium using a sterilized metal pointer, ensuring that the fungus was covered with Phytigel. The fungal scrapes measured 4-5 mm in diameter, less than 0.5 mm thick, and weighed approximately one mg. A single

stem of axenically-grown *C. connivens* or *C. bicuspidata* was placed centrally, on top of the media surface, gently pressing down to secure the stem above the fungus 'plug'.

2.2.6.2 Microscopy analysis

After approximately six weeks, the liverworts were harvested from all fungal strain cultures and their rhizoids examined under the microscope (40x-100x) for evidence of colonization. Several rhizoids were selected and stored in glutaraldehyde (2.5%) should later analysis and photographs using a scanning electron microscope (SEM) be required.

2.3 Results

2.3.1 Isolating and culturing the fungi

Isolating fungi from the liverworts was significantly more successful than the Ericaceae plant roots. Over half of the initial 83 fungal isolation plates from liverworts were without contamination compared with only six of the 80 ericaceous root plates, which were typically contaminated with bacteria or fast-growing fungi.

While there was no statistically significant difference in fungal isolation from either the liverworts or Ericaceae roots' culture success due to medium (MMN and PDA), all of the above viable plates were a result of the direct plating method. A contributing factor to success, was pressing the leafy liverwort and plant material gently into the culture surface, rather than placing it on the surface.

2.3.2 Colonization and molecular verification of ErM in wild liverwort rhizoids

In all four of the sample groups, between 85% and 90% of the rhizoids displayed swollen rhizoids with fungal hyphae (Fig. 4).

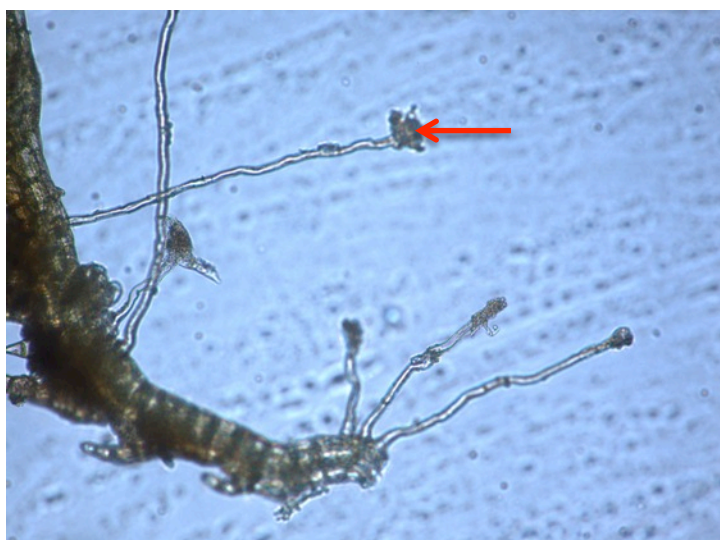


Fig. 4 – Wild collected liverworts showing swollen rhizoids (x20).

The presence of fungi in the *Pezoloma ericae* group was confirmed using PCR (methods as above in 2.2.5.2) in ten of 23 sample specimens of isolated rhizoids from wild, naturally-sourced liverworts. One sample was clean and showed *P. ericae* with over 98% identity with strain UAMH 6735 for British *Calluna vulgaris* (Hambleton and Sigler, 2005); ten failed to sequence, suggesting the amount of fungal DNA may have been too low, two matched sequences of *Chlorophyllum brunneum* and one to *Galerina* sp., both basidiomycete fungi not reported to form ErM. The two control wells were blank.

2.3.3 Molecular identification of the cultured fungi

Pezoloma ericae was successfully isolated in culture from both hair roots of naturally sourced ericaceous plant material and leafy liverworts (Fig. 5).

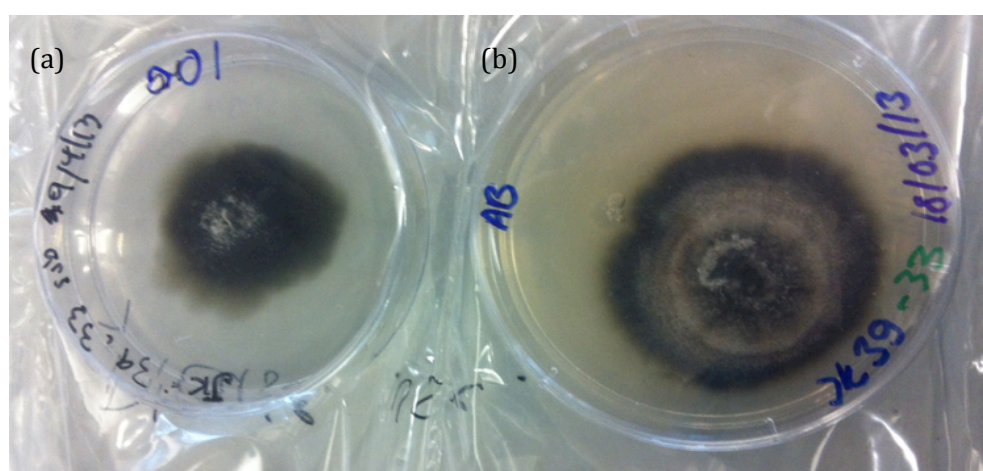


Fig. 5 - Cultures of *Pezoloma ericae*, in 9 cm Petri plates. (a) after three weeks (b) and two months.

Twenty-eight of the fungal isolates were found to be 98% similar in the ITS to *P. ericae* strain UAMH 6735 from British *Calluna vulgaris* (Hambleton and Sigler, 2005). Representative DNA sequences have been accessioned in GenBank (KT827742, KT835080).

Several ericoid mycorrhizal fungi strains were identified in the neighbor-joining phylogenies (Fig. 6). The tree showed a distinct clustering between sequences originating from the ericaceous hair roots (blue outline) and those from the liverwort rhizoids (red-outline), with the exception of one liverwort-derived isolate which aligned more closely with the Ericaceae clade.

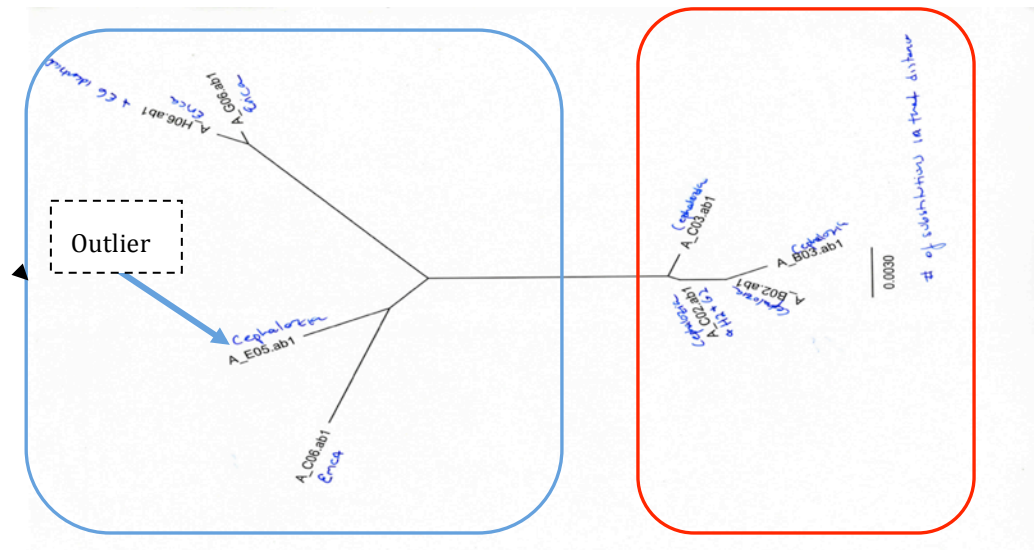


Fig. 6 – Neighbor-joining phylogeny of *Pezizoma ericae* strains derived from *Cephalozia* spp. rhizoids and Ericaceae roots.

Still given the extremely close identity amongst all of the specimens, a much larger sample group would need to be analysed to determine whether there is a sub-population of *Cephalozia* spp. harbouring a *P. ericae* strain significantly more similar to Ericaceae ErM than the leafy liverwort ErM.

Prior to this successful extraction and isolation of mycorrhizal fungi, there were several previous unsuccessful attempts using either PicoMaxx (as above) or Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich).

2.3.4 Resynthesis of fungal isolate with axenically-grown liverworts

Within six to eight weeks, the fungal isolates derived from both liverwort and ericaceous hair roots colonized the rhizoids of the leafy liverworts. The presence of ErM colonization was confirmed by both Prof. Jeff Duckett (in person) and Sir David Read (Fig. 7). Prof. Duckett further confirmed the presence of typical ErM coils and hyphal extensions and Prof. Read noted the rhizoids do not always swell when colonized despite morphological representations pictured in Duckett and Read (1995). In their observations, *C. bicuspidata* was less likely to swell than *C. connivens*.

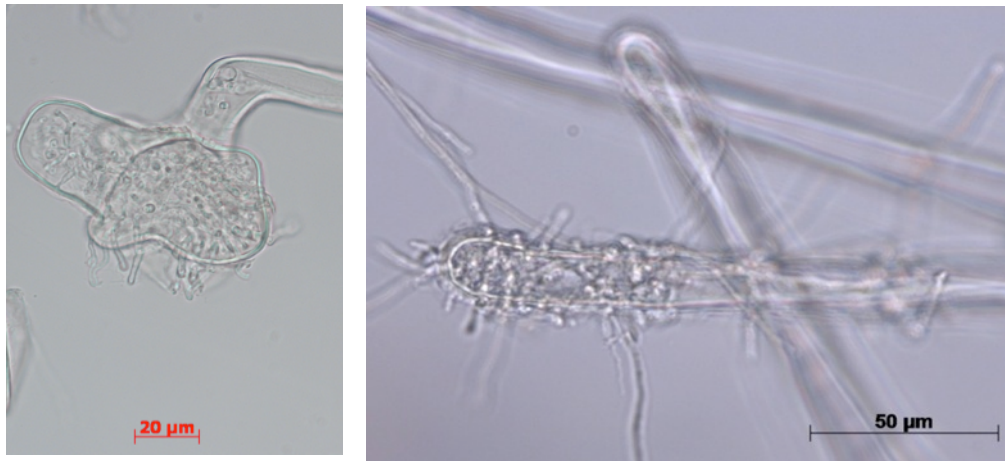


Fig. 7 - Reference pictures of an axenically cultured *Cephalozia* rhizoids resynthesized with *Pezoloma ericae*. The higher magnification shows a swollen rhizoid.

2.4 Discussion

Molecular identification of the British species tested herein, *Cephalozia bicuspidata* and *C. connivens*, and the heathers *Calluna vulgaris* and *Erica tetralix*, confirmed previous research demonstrating a shared mycorrhizal fungus between an Antarctic species of *Cephalozia* (Upton *et al.*, 2007) and *Vaccinium*. This initial isolation, identification and resynthesis work laid the groundwork enabling the mutualism experiments conducted in Chapter 3.

The foundation of this research relies on the assumption that UK native leafy liverworts (in the family Cephaloziaceae), commonly found in UK wet heathlands, share the same fungal mycobionts as Ericaceae plants in nature. Through molecular identification, this was confirmed unequivocally. This was the first step to validate the potential use of the liverwort in a restoration scheme involving Ericaceae plants. Following molecular support for this assumption, the fungal isolates were resynthesized with axenically-grown liverworts. All samples selected for examination were successfully colonized, as found in nature, whether the fungus was originally derived from an Ericaceae plant root or a leafy liverwort, thus a population of ErM inoculated liverworts was in place to commence the planned nursery-to-field trials. Although the association between the ErM *P. ericae* and leafy liverworts closely related to Cephaloziaceae had been established by Upton *et al.* (2007), they tested only sub-Antarctic species. Thus the work done here was the first time UK leafy liverworts were evaluated for this association, thereby furthering molecular evidence for this association outside of Antarctica, further supporting these associations are widespread i.e. present in temperate climate British species of *Cephalozia* (plus two further plants in the Ericaceae). Duckett and

Read (1995) observed these associations microscopically, based on the presence of ErM-like coils in the epidermal cells of Ericaceae plant roots. In their work, the ErM isolate successfully resynthesized with 11 species of leafy liverworts in four families, including Cephaloziaceae, but at that time, molecular analyses were not employed.

As the Ericales number approximately 8,000 species and include familiar plants such as heathers, blueberries and cranberries, this research could potentially impact the economic success of these horticultural plants and agricultural crops.

2.4.1 Techniques for isolating and culturing fungi

The techniques used here, to both isolate the fungus in culture, and resynthesize with axenically-grown leafy liverworts, both reinforce and clarify previously published methods. Most notably, the fungus appears to be non-selective to growth medium type; however, the method by which the roots were placed i.e. direct or macerated, had an impact. All of the viable plates were a result of the direct plating method in contrast to Pearson and Read (1973), who had a much higher success rate using the maceration method. While my maceration method plates appeared to cultivate the fungus, there were visible signs of bacterial contamination. There are considerable more contamination possibilities using the maceration method (e.g. the distilled water tap or tubes used for the suspension), but the output could be significantly higher and worth pursuing if large quantities of fungi are sought.

The isolation experiments were trialed in PDA and modified MMN cultures, with and without antibiotics. There was no statistically significant difference in culture success due to medium. Success was defined as cultivating fungal hyphae without (or minimal) contamination. In both culture media, the fungi grew out of the surface-sterilized rhizoids in 7-10 days.

2.4.2 Molecular findings

The one liverwort-derived strain (labeled E05, Fig. 6) which associated more closely with the Ericaceae roots, was not employed in the nursery trials. Given time constraints imposed by the PhD and limited plant sample sizes, a representative from the liverwort-derived group (C03) was used for resynthesis with axenically-grown liverworts, as well as treatments for the nursery trials. In a follow-up study, it may be useful to test the E05 strain against the more common C03 liverwort fungus strain to see if there is a stronger response to this inoculum.

3 Chapter Three – Are *Pezoloma ericae* and leafy liverworts mutualistic?

3.1 Introduction

Leafy liverworts are amongst the latest diverging non-vascular plants (Shaw *et al.*, 2015) and unlike thalloid liverworts which have demonstrated mutualisms with ancient fungal groups (Humphreys *et al.*, 2010, Field *et al.*, 2015a), it remained unknown whether the leafy liverworts benefit from symbiosis with fungi. I hypothesized the leafy liverworts would receive a nutritional benefit, *i.e.* phosphorus, as a result of their association with the ericoid mycorrhizal fungus occupying their rhizoids. Like their earlier thalloid relatives, it is likely leafy liverworts would acquire smaller concentrations of P under current levels of atmospheric CO₂ compared with Palaeozoic CO₂ (Field *et al.*, 2012) present when the earlier thalloid liverworts began to develop land adaptation strategies. I also hypothesized the fungus would benefit from reciprocal transfers of plant carbon.

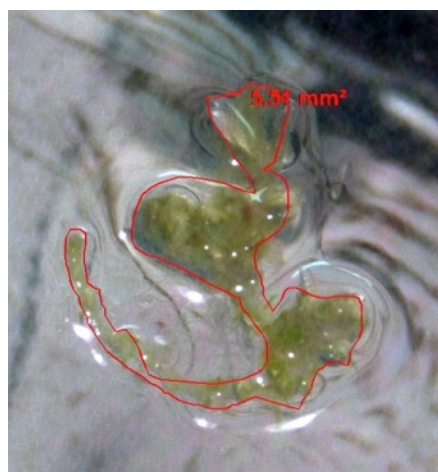
Recent studies have demonstrated a functional mutualism between thalloid liverworts and fungi (AM) by measuring nutrients-for-carbon (³³P labeled orthophosphate and ¹⁵N-amonium chloride from the liverworts to the fungus and ¹⁴C to the liverworts)(Field *et al.*, 2015b, Field *et al.*, 2015c). To test whether later-derived leafy liverworts also share these nutritional benefits with their ascomycete associates, hitherto unknown, I designed a new system, based on these previous experiments but suitable for the smaller more delicate leafy liverwort tissue. The following interaction experiments aimed to establish whether *Pezoloma ericae* is playing a functional role in the leafy liverwort *Cephalozia* spp. (which it inhabits in nature), and there is a nutritional benefit to the fungus. Two types of experiments were initiated. The first measured biomass growth in the leafy liverworts *Cephalozia connivens* and *C. bicuspidata*, with and without the presence of the fungus *P. ericae*. The second group of experiments determined whether reciprocal nutrient exchanges can occur between the same *Cephalozia* spp. and *P. ericae*, thereby confirming *P. ericae* forms mycorrhiza-like relationships with liverworts, similar to ErM associations in vascular ericaceous plants.

3.2 Biomass growth experiments

Two *in vitro* interaction experiments examined and measured whether the presence of *Pezoloma ericae* boosts tissue growth by helping the liverwort access trace nutrients from the growth medium (Phytigel) or added nutrients.

3.2.1 Methods

In the first growth experiment, fragments from the ericoid mycorrhizal fungus isolate JK38.12 (confirmed as *Pezoloma ericae* using molecular methods, section 2.2.4.1, sequences deposited in GenBank (KT827742 and KT835080)), were introduced to 9 cm Petri plates (filled half of the depth with 1.5% Phytigel) with subcultures of the axenically-grown leafy liverworts (section 2.2.2). After fungal colonization of the liverwort rhizoids was established microscopically (about three weeks later), individual liverworts were re-plated. Photographs and measurements of polygons, using ImageJ free software (Rasband, 1997-2012), were taken of each liverwort plant the day of re-plating and six weeks later (Fig. 8) to estimate change in growth. There were two treatment groups with inoculated liverworts and a control with no fungus ($n = 16$).



Treatment groups were either rinsed (in sterilized deionized water) and agitated to remove the growth medium prior to re-plating, or not rinsed ($n = 14$ and 22, respectively).

Fig. 8 – Photo of traced leafy liverwort (in red) used to measure change in growth area.

For the second growth experiment, the liverworts and fungal isolates were derived from the same stock as in the above growth experiment and grown in Sigma Magenta vessel (GA-7-3) systems (Fig. 9). In a laminar flow cabinet, 25 Sigma vessels were filled with growth medium (1.5% Phytigel) to a depth of 30 mm. Initially 15 were prepared as complete systems (containing an individual liverwort plant stem with fungal isolates) and 10 as controls, either plant or fungus only (see Fig. 9a-c). A fragment of the *P. ericae* isolate (approximately 7 mm²) was inserted beneath the surface of the Phytigel for systems a and c; for system c, an axenically-grown leafy liverwort stem was gently pressed in to the surface, on top of the fungus fragment. The vessels were stored in controlled environment growth cabinets (BDR16, Conviron, Winnipeg, MB, Canada) with temperature constant at 16°C, 80% relative humidity and 20 µmol/m²/s light.

After approximately six months, liverworts were randomly harvested (six from control microcosms and six from complete systems), freeze-dried and weighed to assess the impact of *P. ericae* inoculation on biomass.

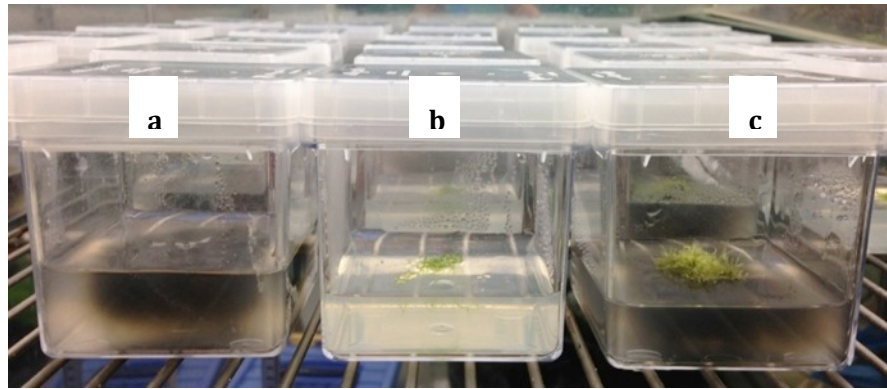


Fig. 9 – Sigma vessel systems. (a) *Pezoloma ericae*, only; (b) *Cephalozia* spp., only; and, (c) *Cephalozia* spp. growing with *P. ericae* isolate.

3.2.2 Results

Both independent growth experiments demonstrated liverworts growing with fungi yield greater growth than liverworts without. In the polygon growth experiment, the inoculated liverworts (not rinsed) demonstrated a statistically significant difference in growth compared with the control; 195% compared with 99%, respectively (P -value < 0.05, using one-way ANOVA)(Fig. 10a) (data in Appendix 2a).

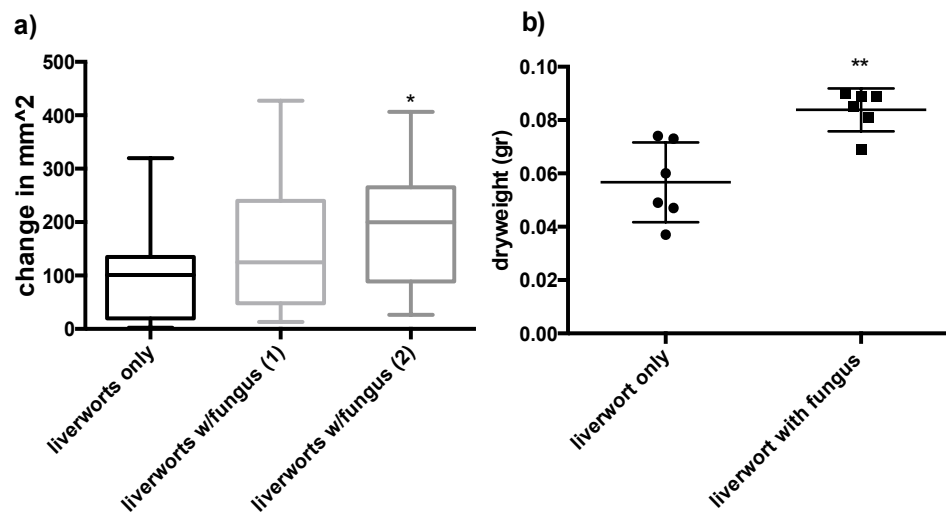


Fig. 10 – Liverwort growth with and without *Pezoloma ericae*. (a) change in growth measuring polygon area (six weeks), treatments: 'liverworts only' control, (1) liverworts rinsed of fungus before re-plating, (2) not rinsed ($n = 16, 14, 22$); (b) dry weight (biomass) after six months ($n = 6$).

The growth of the other liverwort group with fungus (1) which were rinsed in sterilized deionized water to remove the Phytigel before re-plating, was not significantly greater than the control, even though the average increase in growth from the start of the experiment is higher, 158% compared with 99% in the control liverworts (Appendix 2a).

Biomass in the second growth experiment (as measured by dry weight)(Fig. 10b) also indicates the inoculated liverworts grew significantly more (48%) than the control group (no fungus) (Appendix 2b)(P -value < 0.005, using Mann-Whitney test).

3.3 Transfer of nutrients experiments

These experiments were designed to determine whether the tested leafy liverworts absorb phosphorus (P) as a direct result of the ErM fungus *Pezoloma ericae* in the culture medium. The reciprocal flow of photosynthates to the fungus was also tested. The ErM fungal isolate used was isolated from the corresponding leafy liverwort taxa and molecularly confirmed as *P. ericae* (section 2.3.3.).

3.3.1 Methods

3.3.1.1 Growth medium trials

Prior to commencing the phosphorus (P) transfer experiment, three different concentrations of nutrients were tested in 100 mm split Petri plates (Fig. 11) to gauge both the leafy liverwort's and the ErM fungus's growth performance and susceptibility to contamination. Nutrient concentration variations added to the Phytigel were either: none ('0') (n=13), 0.01% concentration (n=14), or 0.1% concentration (n=14).

Nutrients (see Parker medium, Appendix 3) were added to the Phytigel in the top half of the split-plates with the exception of phosphorus (potassium phosphate). Instead, phosphorus, which may be the most limiting nutrient in ericaceous habitats (Mitchell and Read, 1981, Bolan, 1991, Myers and Leake, 1996), was added to the medium poured into the bottom half of the split-plates (potassium phosphate 0.2 mg/L). A total of 41 Petri plates were prepared under a laminar flow hood with Phytigel poured to a depth just below the plate barrier. Plates contained two single axenically-grown liverwort stems planted approximately 1.5 cm from the plate divide. The fungal plugs were inserted beneath the surface before placing the liverworts on the surface on top of the fungus (see diagram, Fig. 11).

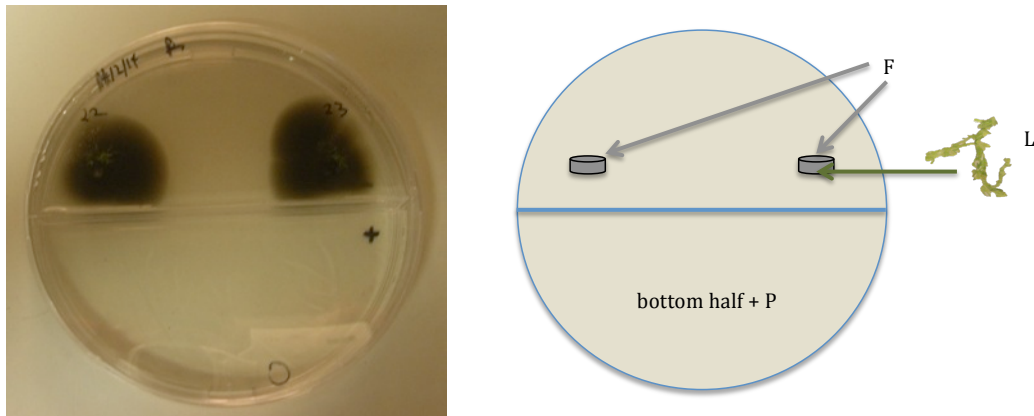


Fig. 11 - Picture (four weeks) and diagram of split-plate design for P transfer experimental trials. The top hemisphere contains two leafy liverworts (L) placed on the surface of the Phytigel with dark *Pezoloma ericae* plugs inserted beneath (F). Phosphorus was added to the Phytigel in the bottom hemisphere '+' with '0' additional nutrients.

Also tested was the distance the liverworts and fungal plugs should be placed from the Petri plate divider to allow mycelial networks to grow over the barrier (which is shorter than the lid). The plates were stored in controlled environmental conditions (irradiance at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, 18°C constant temperature, with 14-hour day length). Although less light than experienced in nature in full sun ($500\text{-}700 \mu\text{mol m}^{-2} \text{s}^{-1}$), this light and temperature regime is ample to sustain growth for the liverworts and fungi *in vitro* (Duckett *et al.*, 2004). After six weeks, the performance was assessed and liverwort samples were harvested from two plates to confirm the rhizoids were colonized.

3.3.1.2 Phosphorus transfer experiment

3.3.1.2.1 Design of phosphorus transfer experiment

In total, the experiment consisted of 18 split plates (two liverworts each, inoculated with *Pezoloma ericae*, Fig. 12b), and eight control plates (one liverwort each, no fungus, no barrier, Fig. 12a). Based on the results from the above trials, Phytigel without added nutrients was deemed adequate for the liverwort and fungal growth and the plates with zero nutrients from the above trial were used if the fungus had not crossed the barrier. Phytigel was removed from the bottom half of the plate if phosphorus had been added and re-poured (using the same concentration (1.5%) and lot number of Phytigel (Sigma)). To trace the P, ^{33}P -labelled isotope Phytigel plugs were inserted in small wells carved out of the original Phytigel.

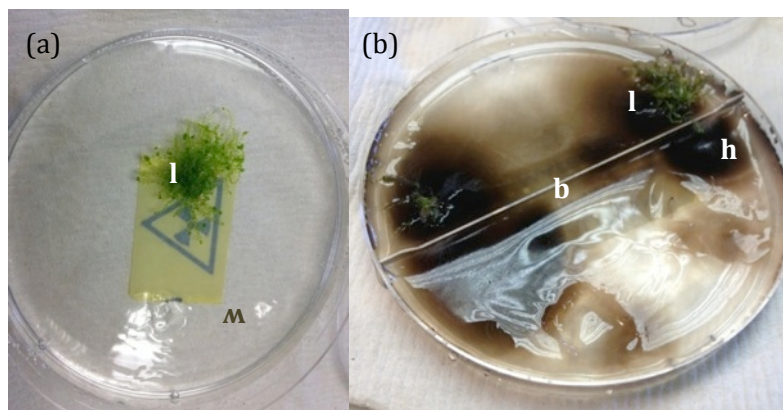


Fig. 12 – N and P transfer experiment plates. (a) control; (b) inoculated split plate. l – liverwort; h – hyphae; w – well, and b – barrier.

For the split plates, the wells were located 1-2 mm from the margin of the hyphae, which had successfully crossed the barrier separating the liverwort from the well. Each well received 0.1 MBq of ^{33}P -labelled ($2.04\text{E}+9$ Bq/mol) orthophosphate (and 1.25mg of ^{15}N as ammonium chloride) in 10 μl of micro-filtered sterile deionized water. The wells were then backfilled and sealed with warm Phytigel, using a pipette. Quantities for ^{33}P labeling were based on previous inorganic P absorption research (Cameron *et al.*, 2007), which had targeted 0.2MBq using orchids; liverworts are a much smaller plant, therefore a smaller amount was applied here. The control plate wells were placed 2.5 cm away from the liverwort. The plates were resealed with Parafilm and stored in an environmentally controlled growth unit. Light and temperature regimes were set to irradiance of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, $16^\circ\text{C} : 14^\circ\text{C}$ day : night temperatures, a 12-hr day length, 80% relative humidity and 440 ppm CO_2 .

3.3.1.2.2 Assessment of plates

After eight weeks, the plates were examined for growth patterns and contamination. Plates deemed contaminated by bacteria or fast-growing fungi were discarded. Plates with steady and healthy patterns of growth were opened and monitored for radiation using a Geiger mini monitor, and counts per second (CPS) were recorded (Appendix 11). Qualitative assessments were made as to whether the hyphae network was continuous between the radioactive wells and the original fungal fragment (inserted on the opposite side of the Petri dish barrier i.e. the fungi had grown across).

3.3.1.2.3 Plant and fungus harvest for ^{33}P acid digestion, liquid scintillation and nutrient budgeting

Fragments of the freeze-dried Phytigel from both plate hemispheres (plant half and fungus half), and the entire liverwort, were removed and weighed directly into clean

and dry tubes. The target weight for each sample was between 50 and 100 mg. The plates containing the fungus and plant material were freeze-dried in preparation for the ^{33}P acid digestion, methods were modified from Leake *et al.* (1990b). All materials were homogenized into a fine powder using an analytical grinder (Yellowline A10, IKA, UK). Between 10 and 250 mg of material was weighed into acid-washed boiling tubes and each tube (including four control blanks) received 1ml of concentrated sulphuric acid. Tubes were agitated and while digesting the organic matter at room temperature for one hour, the digestion block was pre-heated to 365°C. After the fragments were digested they were placed in the digestion block and boiled for 15 minutes. After this time samples were cooled and 100µl hydrogen peroxide (H_2O_2) were added to each tube which were then re-heated to 365°C for a further five minutes allowing the H_2O_2 to clear the digested sample. Cleared samples were diluted up to 10 ml using distilled water. One ml of this diluted digest was then added to 10 ml Emulsify Safe (Perkin Elmer, UK) in a scintillation vial and shaken until the solution cleared.

Total ^{33}P content for each sample was quantified using a liquid scintillation analyser (TRI-CARB 3100TR, Isotech, UK) following Hendry and Grime (1993), Cameron *et al.* (2006, 2007) and Field *et al.* (2012, 2015b, c). The vials were then placed in the liquid scintillation counter (Packard Tri-carb 3100TR; Isotech, UK). A total of 110 samples were analysed.

To determine ^{33}P transferred from the fungus to the plant, the data output was recorded in a budget analysis matrix using equations (Eqn 1) previously published by Cameron *et al.* (2007):

$$M^{33}\text{P} = \left[\left((c\text{DPM}/60) / S_{\text{Act}} \right) M_{\text{wt}} \right] Df \quad \text{Eqn 1}$$

($M^{33}\text{P}$, mass of ^{33}P (mg); $c\text{DPM}$, counts as disintegrations per minute; S_{act} , specific activity of the source (Bq mmol^{-1}); Df , dilution factor; M_{wt} , molecular mass of P.)

3.3.1.3 Carbon (C) transfer experiment

Transfer of ^{14}C to the plant and fungus were measured through sample oxidation and liquid scintillation counting (Cameron *et al.*, 2006, 2007).

3.3.1.3.1 Design of systems for the C exchange experiment

The microcosms for this experiment are shown below in Fig. 13 (adapted from Fig. 9); the preparation of these vessels is described in section 3.2.1. The liverworts and *Pezoloma ericae* isolate were derived from the same sources as applied in the ^{33}P transfer experiment (section 3.3.1.2). In total, twelve sample microcosms were labeled with ^{14}C ; six complete, three fungus-only and three plant-only systems; Fig. 9c shows the

complete set-up. Polythene squares (the same area as the vessel) were placed on top of the Phytigel to separate the fungal growth (below) from the $^{14}\text{CO}_2$ headspace (above); a 25 mm diameter circle hole was cut to expose the liverworts, the circumference of which was sealed with anhydrous lanolin.

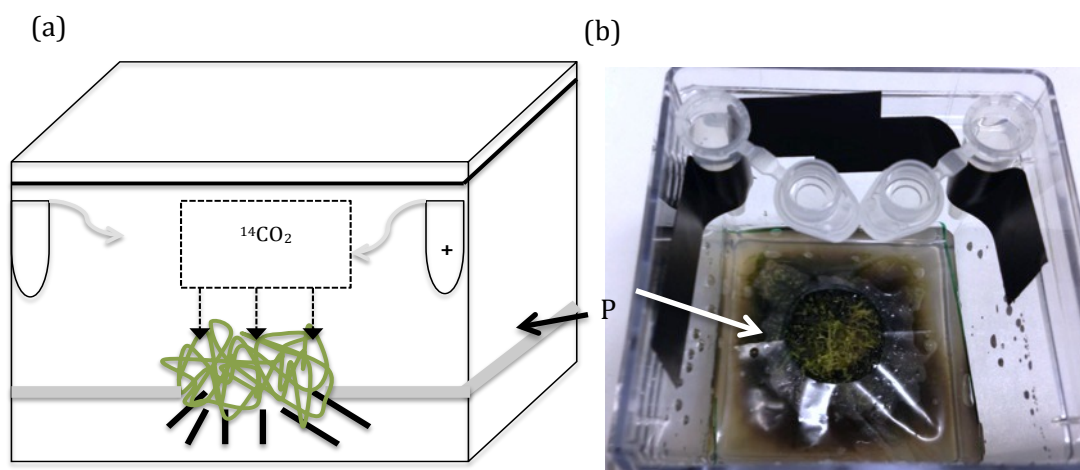


Fig. 13 – Microcosm set-up for carbon transfer experiment. a) longitudinal drawing. b) photo, bird's-eye view. P – polythene square; '+' indicates Eppendorf tube.

Three Eppendorf tubes (1.5ml) were installed into the corners of each vessel (using adhesive tape) above the Phytigel, and added 6.8 μl ^{14}C -sodium bicarbonate (0.5 MBq) to one of the Eppendorf tubes; the others were initially left empty. We then added 500 μl 25% lactic acid to liberate $^{14}\text{CO}_2$. Next we removed 1 ml headspace gas from each chamber and added this to 10 ml Carbo-sorb (to trap the CO_2) (Perkin Elmer, Beaconsfield, UK) in gas-evacuated scintillation vials. This was mixed with 10 ml Permafluor E⁺ (Perkin Elmer, UK). The vessels were incubated for four hours (to allow $^{14}\text{CO}_2$ to be fixed and transferred) and then 1 ml 2 M KOH was introduced into each of the empty Eppendorf tubes (1-2) to trap and removes excess $^{14}\text{CO}_2$ from the headspace of each vessel. These vessels were sealed with and remained *in situ* for 30 minutes before opening them to remove the KOH traps and leftover isotope. After homogenizing, the liverwort tissue and Phytigel were then placed in individual polythene bags and freeze-dried. The freeze-dried material was weighed and samples were oxidized to quantify the ^{14}C in each sample. The activity of ^{14}C passed from the plant to the fungus was determined using a liquid scintillation counter. Total carbon (^{12}C plus ^{14}C) fixed by the plant and transferred to the fungus was calculated using Eqn 2 and is a function of the total volume and CO_2 content of the vessel headspace and the proportion of the

supplied $^{14}\text{CO}_2$ label fixed by the plants. Cameron *et al.* (2006) express the equation as follows:

$$T_{\text{pf}} = \left((A/A_{\text{sp}})m_a \right) + (P_r \times m_c) \quad \text{Eqn 2}$$

The T_{pf} is the transfer of carbon from the plant to the fungus; A is the radioactivity of the tissue sample (Bq); A_{sp} is the specific activity of the source (BqMol^{-1}); m_a is the atomic mass of ^{14}C ; P_r is the proportion of the total ^{14}C label supplied present in the tissue; and m_c is the mass of the carbon in the CO_2 present in the labeling vessel (g) (from the ideal gas law; Eqn 3):

$$m_{\text{cd}} = M_{\text{cd}} \left(PV_{\text{cd}}/8.3 \text{ J/K/mol}(T) \right) : m_c = m_{\text{cd}} \times 0.27292 \quad \text{Eqn 3}$$

The m_{cd} is the mass of CO_2 (g); M_{cd} , molecular mass of CO_2 (44.01 g mol^{-1}); P , total pressure (kPa); V_{cd} , volume of CO_2 in the chamber (0.0001 m^3); m_c = mass of unlabeled C in the labeling chamber (g); M , molar mass (M of C = 12.011 g); R , universal gas constant ($\text{J K}^{-1} \text{ mol}^{-1}$); T , absolute temperature (K); m_c , mass of carbon in the CO_2 present in the labeling vessel (g), where 0.27292 is the proportion of carbon in CO_2 on a mass fraction basis (Cameron *et al.*, 2008).

3.3.2 Results from nutrient transfer experiments

Radioactivity measured using a Geiger counter eight weeks after applying the ^{33}P isotope showed minimal trace isotope movement via the fungal hyphal networks across the split-plate barrier (section 3.3.1.2.2). By 18 weeks however, we recorded a small but significant transfer of ^{33}P to the plant and C to the fungus, each in separate microcosm systems. The resulting C for P calculation shown below (and detailed in Appendix 6) is discussed later in relation to other recent studies on earlier diverging liverworts.

3.3.2.1 Growth medium trials for the phosphorus transfer experiment

These trials sought to determine which medium nutrient mix was optimal for both liverwort and fungus. Nutrient addition might enhance fungal growth but also poses a risk of higher bacterial contaminants and may suppress the nutritional function of the fungus. At six weeks, most of the plates continued to foster growth of both liverworts and fungi. Six were contaminated and discarded. There was no visual difference in performance of either the liverwort or fungal growth or contamination as a result of the media treatment, with 0 or 0.01% added nutrients. As such, nutrients were not added which was a better proxy for nutrient-poor soils of heathlands. If nutrients were added, this also may have discouraged the liverwort's use of the ErM fungus to access and

unlock trace nutrients in the Phytigel. Given the growth rate observed under the environmental conditions herein, it was also determined that for the hyphae to cross the barrier within six weeks, the liverwort and fungal plug beneath should be placed closer to the barrier (approximately 3 mm).

3.3.2.2 Readings of ^{33}P radioactivity

3.3.2.2.1 Radioactivity assessment of plates, eight weeks after set-up

Eight weeks after the ^{33}P isotope was applied, Geiger readings were taken (Appendix 5). Summarizing the results, the Geiger registered counts per second (CPS) between 3 and 10 (indicating more than background noise) when held over the seven positive controls (without barriers). This is contrasted by negligible CPS readings from the treated plates (with barriers), with one exception explained by a design error.

3.3.2.2.2 Harvesting liverworts for ^{33}P transfer analysis

Upon harvesting the plant and fungal material 18 weeks later, most split plates were observed to have strong mycelial growth over the barriers connecting the fungal hyphae to the liverwort on the other side. Radioactivity, measured in disintegrations per minute (DPM, Fig. 14) remained at negligible background levels on material digested from both the plant half of the growth medium and the liverwort itself, yet showed significant radioactivity on fungal half samples (where the ^{33}P wells were placed) as well

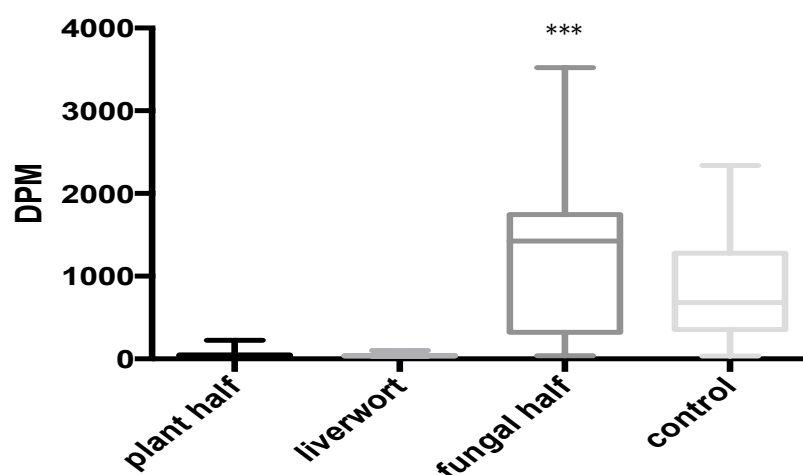


Fig. 14 – Comparison of radioactivity. X-axis: Phytigel from the plant compartment, the liverwort, and Phytigel from fungal compartment, P -value < 0.0001 ($n = 35, 33, 22$; using non-parametric tests, both Kruskal Wallance and Kolmogorov-Smirnov).

as the control liverworts. The control plants' DPMs, where no barriers or fungus were present, registered significant radioactivity, suggesting the plants were able to access P without *Pezoloma ericae*.

3.3.2.3 Transfer of phosphorus (P) and carbon (C)

We recorded a small but significant transfer of ^{33}P in 23 of 33 samples, and C in 5 of 6 samples (see *Cephalozia*, Fig. 15, Fig. 16a). The ^{15}N analysis was deferred due to time constraints and later digests proved unfeasible. It is useful to compare the findings of leafy liverwort tested in this dissertation with recent studies on earlier-branching liverwort and fungal lineages (Field *et al.*, 2015b).

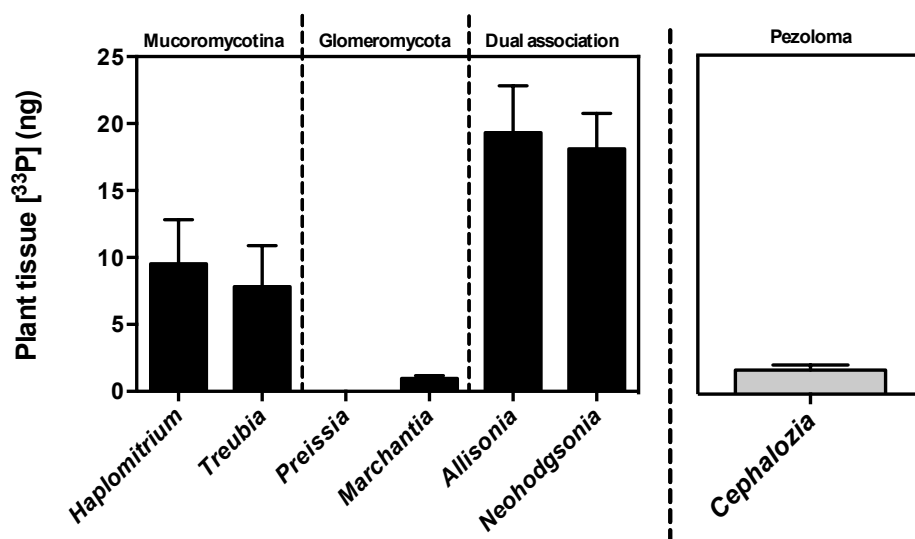
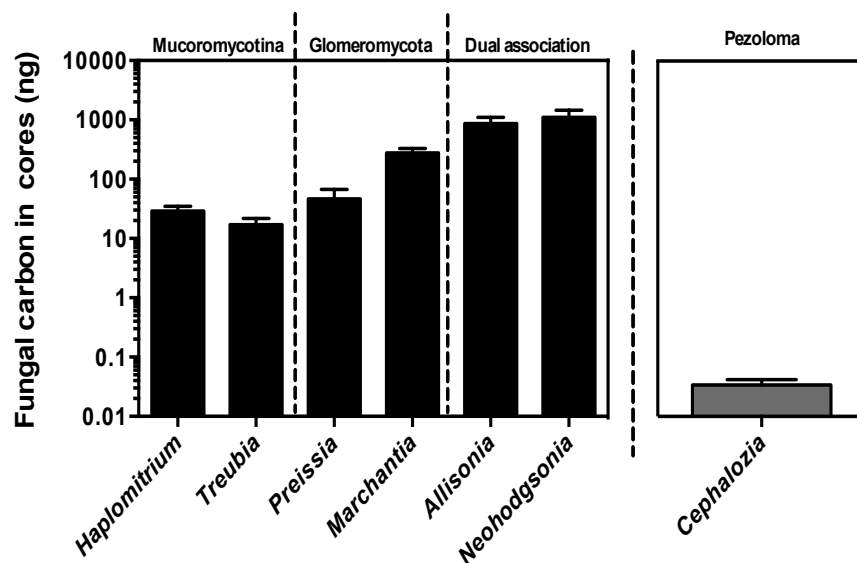


Fig. 15 - Fungal acquired ^{33}P concentration in *Cephalozia* tissue compared with a range of earlier-branching liverworts (Field *et al.*, 2015b).

a)



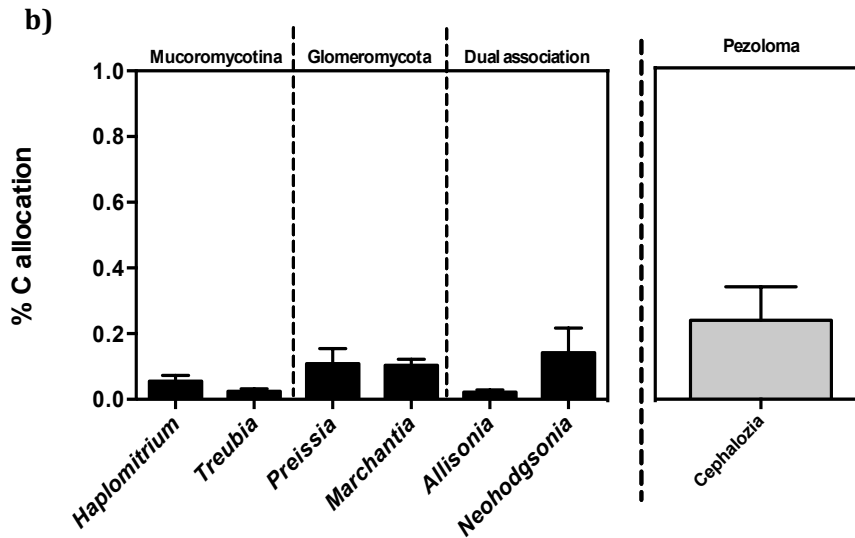


Fig. 16 - Liverwort transfer of C (a) and % C allocation (to fungus from plant) (b), comparing earlier-branching liverworts' fungal symbionts to *Pezoloma ericae* (Field *et al.*, 2015b).

The ratio of P-for-C, 0.17, was derived by taking the average fungal-acquired ^{33}P (ng) (by the liverworts) divided by the average transfer of C to fungus (ng) (Table 2). This value is three times greater than that calculated in previous experiments on thalloid liverworts forming nutritional symbioses with Glomeromycota and/or Mucoromycotina fungi. These averages were calculated using P and C budgets (Appendix 6), respectively) from separate microcosm experiments.

Table 2 – Nutrient transfer calculations: average liverwort content (ng) and tissue concentration ^{33}P (ng g), plant transfer of C to fungus (ng) and % C allocation to fungus.

	fungal acquired ^{33}P (ng) ⁽¹⁾	fungal acquired ^{33}P ng g (ng/mass) ⁽²⁾	plant transfer of C to fungus (ng) ⁽³⁾	% C allocation to fungus (from plant)
Average	0.00524	1.78675	0.03159	23.8%
SE	0.00095	0.37192	0.00826	10.1%
P for C ⁽⁴⁾	0.17			
n	23	23	5	5

(1) represents total content; (2) P concentration of plant tissues; (3) without microcosm; and (4) (1)/(3); n = number of samples.

Comparisons between the leafy liverwort and thalloid liverworts in Field *et al.* (2015b) suggest that the P gained by the *Cephalozia* liverwort may ‘cost’ little in terms of C to the fungus compared with other mutualisms (Fig. 17). However, while instructive, a direct comparison of nutrient exchanges is inappropriate as the transfer of ^{33}P and C experiments herein were tested in two different microcosms, Petri plates and Sigma vessels, respectively (as previously described) whereas Field *et al.* (2015b) used cylindrical core systems in pots filled with sand, soil and basalt.

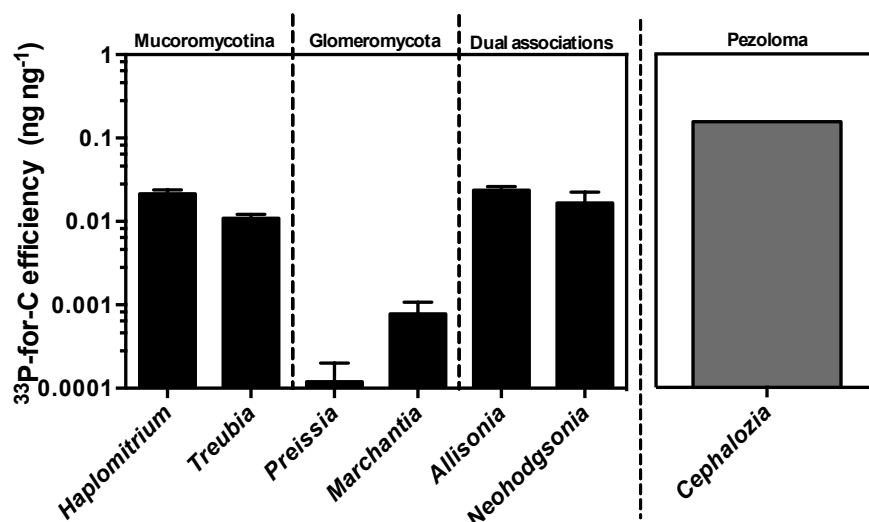


Fig. 17 - Comparison of *Cephalozia* and *Pezoloma ericae* ^{33}P -for-C efficiency with mutualisms of earlier-branching liverworts interacting with non-ericoid mycorrhizal fungi) (Field *et al.*, 2015b). There is no error bar for *Cephalozia* as P and C were not compared from the same microcosm systems thus the ratio is based on the average for each.

3.3.3 Discussion

3.3.3.1 Determination of a mutualism

Both groups of experiments – biomass growth and transfer of nutrients – unequivocally confirm there is a nutritional mutualism between the leafy liverworts *Cephalozia bicuspidata* and *C. connivens* spp. and their fungal symbiont *Pezoloma ericae*. As far as I know, this is the first demonstration of a mutualism between these organisms and a new example of a mycorrhizal fungus of a vascular plant forming a mutualism with a non-vascular plant.

It is evident from the two independent microcosm growth experiments, that the liverworts inoculated with the ericoid mycorrhizal fungus (ErM) gained more biomass than the control liverwort microcosms (with no fungus). When the fungus is added, the liverworts grow larger (e.g. compare Fig. 9c, showing a microcosm containing the

liverwort and fungus with Fig. 9b the liverwort without fungus). The measurements in both independent growth experiments verify this empirically.

Although both treatment groups' liverworts in the polygon growth experiment were colonized before re-plating, only the unrinsed liverworts encased in the dark hyphal network showed significant change in growth after six weeks. The rinsed group had a relatively broad distribution in growth (change since plating) for its sample size, which explains the insignificant *P*-value when compared with the controls even though this group averaged a total growth of 158% compared with 99% in the control liverworts. Rinsing the liverwort of the dark Phytigel either slowed down or hindered further growth; the disturbance alone potentially being the reason. Perhaps the rhizoids need to draw from external ErM reserves to continue stimulating liverwort growth, or with more time, they would have done so. Still this group was growing more than the control group, possibly from the initial colonization, but not at the same statistically significant level as the unrinsed group.

Still unclear from these experiments is which nutrients, other than P, are being transferred and what other functional traits the ErM fungi may contribute. The nutrient transfer experiments aimed to identify whether P and N, often limiting in these ecosystems where ericaceous plants and liverworts harbouring ErM fungi dwell, are influential. At the onset of the experiment, ^{15}N was delivered into the wells but this was not measured however due to time constraints and inadequate plant materials to run the CS-IRMS. Thus there is a possibility that the ^{15}N was also transferred with the ^{33}P contributing to the liverwort's growth in the second biomass experiment.

The possibility that the Phytigel, which contains trace levels of nutrients, including phosphorus, might be a disincentive for the liverwort to rely on the fungal hyphae for P absorption – *in vitro* – was tested. As the no-fungus control plates' liverworts successfully absorbed significant quantities of ^{33}P (as measured by DPMs), despite the presence of un-labeled P in the Phytigel – it was confirmed that the growth media did not totally impede the transfer of ^{33}P (and this was later confirmed with the isotope), although it is reasonable to expect the absorption rate and concentration would be different with other growth media. It could be argued that the ErM fungus is redundant in the tested microcosms as the liverwort grew well without its symbiont. Importantly however, these experiments established that the later-derived leafy-liverworts still maintain nutritional mycorrhiza-like traits and possibly provide another link to the function of mycorrhizal genes *sensu* Wang *et al.* (2010). Regular microscopic

examination of the re-synthesized liverworts and their fungal symbiont *in vitro* for over three years confirms there was no parasitic behavior by the ErM fungus.

If tested in soil core systems similar to those used by Field *et al.* (2015b), there may have been greater benefit to the liverwort without supplementary nutrients from the growth medium. However, due to the significantly smaller size of the leafy liverworts, this set-up was impractical. Nonetheless, it is instructive to compare the nutritional exchange results from this study with Field *et al.* (2015) to gauge the efficiency of the leafy liverworts compared to other organisms, particularly with respect to P-for-C, *i.e.* plant P gain per unit of C invested on the fungus.

The P-for-C ratio is 0.17 *i.e.* carbon provided by the liverwort in exchange for P, is similar to liverwort-fungus mutualisms between *Marchantia* and Glomeromycota (Fig. 15) reflecting a relatively low 'cost' again compared with other liverwort-fungus mutualisms (Field *et al.* 2015) (Fig. 16). Viewed from the fungal perspective, similarly the cost of the mutualism is cheap, absorbing photosynthates from the plant despite trace sugars being present in the culture medium.

In a different experimental system more closely reflecting nature - *i.e.* more realistic mineral content and edaphic constraints - the liverwort may require more P although atmospheric content may be sufficient given current day CO₂ (pers. comm. David Read). It is possible that the fungal hyphae played a much larger role delivering nutrients in a Palaeozoic atmosphere of 1500 p.p.m. a[CO₂] compared with current day 440 p.p.m. a[CO₂], as seen with the ³³P content for *Pressia* with *Glomeromycota* (Field *et al.*, 2015b).

Although the nutritional mutualism experiments were not paired, *i.e.* ³³P and C were measured independently from different experiments, these findings are an important link to understanding mutualisms in later-diverging liverworts. Other studies concerning plant responses to inoculation with mycorrhizal fungi (Cameron *et al.*, 2007) including a meta-analysis by Hoeksema *et al.* (2010) suggest plants are more responsive to mycorrhizal inoculation when P-limited, rather than N-limited. However, studies focusing on ericoid mycorrhizal fungi, have shown either a transfer of N to the host plant, or N to be the limiting nutrient. Cairney and Ashford (2002) using ¹⁵N-labelled organic N substrates, found mycorrhizal fungi transferred N to epacrid plants in some habitats; epacrids are closely related to Ericaceae and are colonized by ericoid mycorrhizal fungi. Grelet *et al.* (2009) found that genetic differences among ericoid mycorrhizal fungi influence N transfer to plant shoots, and it is also understood N efficiency is linked to C availability (Grelet *et al.*, 2005). Significantly greater yields and N contents were found in ericaceous plants colonized by *P. ericae* and grown in

nitrogenous substrates (Kerley and Read, 1997). This is consistent with previous studies (Stribley and Read, 1980, Bajwa and Read, 1985, Bajwa and Read, 1986), that have shown N assimilated from organic sources by ErM fungi transferred to the plant. Furthermore, positive growth responses were measured in *Vaccinium* with N added but addition of P led to little growth response; that study also showed plants prefer organic N over N resulting from nitrification (Read *et al.*, 2004).

The nutritional needs of leafy liverworts, especially in an *in vitro* environment lacking the usual competitive stresses, are undoubtedly different from those of the vascular ericaceous plants tested in the studies mentioned above. Recent liverwort symbiosis studies, albeit testing the functionality of more ancient lineages of thalloid liverworts and their fungal symbiont counterparts find that N plays a significant role (Field 2015). Moreover, as mycorrhizal studies of Ericaceae and ErM have mainly focused on N and P, there is a further possibility that there are other nutrients critical to liverwort growth which have not been previously studied and identified as limiting conditions.

4 Chapter Four – Nursery inoculation experiments using leafy liverworts (resynthesized with *Pezoloma ericae*) to inoculate Ericaceae plants

4.1 Introduction

Duckett and Read (1995) were the first to show (through Koch's postulates) that leafy liverworts harbour the same ericoid mycorrhizal fungus (ErM) which is mycorrhizal in Ericaceae plants. They were also the first to suggest a potential ecological application. Following Duckett and Read (1995), several other authors developed this idea further from a molecular perspective (Chambers *et al.*, 1999, Upson *et al.*, 2007), but their studies were all conducted *in vitro*. Observations in nature have demonstrated the function of ErM showing a link between ErM colonization of roots and heather growth (Diaz *et al.*, 2006) suggesting restoration may be improved by finding ways to accelerate colonization. Numerous studies provide insights to the functional role of the ericoid mycorrhizal fungus (Perotto *et al.*, 2012) demonstrating either a nutritional mutualism or other benefit to the host plants, but none of these studies tested liverworts as a partner for the vascular plant community. My research aimed to fill in this gap between laboratory-based microcosms and practical methods to deliver fungal inoculum to the field.

Between November 2011 and August 2014, there were three cuttings (Experiments 1A, 1B and 1C) and three seedlings (Experiments 2, 2A and 3) experiments conducted (see Table 3), plus pre-trial experiments. Liverworts resynthesized with *Pezoloma ericae* (as detailed in Chapter Two), were co-planted with *Erica tetralix* and *Calluna vulgaris* cuttings and seedlings, to determine whether leafy liverworts are practical as an inoculum delivery device, either facilitating plant establishment and/or promoting growth of ericaceous plants.

None of the experiments were identical replicates; either the scale or timings varied due to seasonal differences or availability of plant material (heathers and liverworts), or growth conditions. This included numerous design challenges due to growth chamber malfunctions, weak glasshouse climate controls and pest disruptions. The later experiments were refined with knowledge assembled from early trials and published in Kowal *et al.* (2015) (Appendix 10).

4.2 Methods

Plant and liverwort material (previously inoculated with *Pezoloma ericae in vitro*) were collected (using same plant material and sites described in section 1.3.1), propagated

and bulked up for the nursery trials. Methods for collecting both vascular and non-vascular plant materials have been described previously in section 2.2.1.

4.2.1 Propagation techniques for *Calluna vulgaris* and *Erica tetralix* cuttings and seedlings

4.2.1.1 Compost and pasteurization treatment

The same compost was used for growing the cuttings and seed sowing. The compost mixture (roughly two parts peat to one part sand), approximating the particle sizes found at Thursley Common, was irradiated to a temperature of 100° C, two times, using a microwave and left to cool down for one day in between the two heating periods. Although this may have released nutrients (Read and Stribley, 1975), and hindered the development/need for mycorrhizal root formation, it was nonetheless the most practical way to inactivate mycorrhizal fungi before carrying out experiments. Soil was stored in zip lock bags in between pasteurization treatments.

4.2.1.2 Preparing and growing the cuttings

Before planting, the field cuttings were spliced with a sharp blade to remove the hardened tip (between 1-2mm) and stimulate rooting. The knife was cleaned with ethanol before use, stems were kept moist during planting preparation, and only a few at a time were handled. The bottom two cm of the cutting was stripped of leaves and the cutting was inserted directly in their containers (prewashed at 50°C) up to the leaves, filled with the compost mix, watered, and placed in sealed bags. Attention was given to filling the containers to avoid air gaps by tapping the compost several times and watering/draining before introducing the plant material. The compost was firmed around the cutting and a gentle tug was applied to test resistance, ensuring secure cuttings. Tubes were topped up, around the cutting base to a similar height. Plants were periodically watered and bags opened to release condensation and allow for air circulation. Holes for drainage were also added once germination commenced. Once the majority of plants established roots (by eight weeks), the bags were removed and the containers covered in cling film, leaving the plant open to airflow. This helped to maintain moisture around the liverwort (treatment explained below) and suppress potential movement of spores, thereby reducing the risk of the control plant compost becoming a repository for ErM propagules. No rooting hormones or fertilizers were added but on a few occasions it was necessary to apply DiPel DF (1gr/litre), a biological insecticide, to control for moth caterpillars.

For the pre-trials, cuttings were initially planted in 50ml tubes (Ray Leach Cone-tainers RLC3 - 2.5 cm diameter, 12 cm long). The tubes were placed in 250ml bags, sealed and

maintained in a controlled growth chamber. The light and temperature settings were: 14 hours at 22° C, 100% light; and 8 hours at 17° C, 0% light. Temperature settings followed standard nursery practices for temperate heathers as well as optimum guidelines (Thomas and Davies, 2002). As individual plant root systems reached the base of the tubes, they were potted on to three-inch square pots (then 5, 8, etc.) and moved to a glasshouse with a temperature range of 12° C – 24 °C, and 60% average humidity. The same compost mix and irradiation procedures were followed when repotting (see section 4.2.1.1). Pots were placed in shallow trays and watered regularly to keep the compost moist for most of the time, but not waterlogged. Efforts were made to imitate a wet heath environment, which can be seasonably damp and anaerobic, alternating with periods of dryness. Thus, the pots were periodically drenched followed by days of no watering. Periodically, ‘hair’ roots were removed for examination under a microscope (40x and 60x) to check they were not colonized prior to treatment.

The preparation for Cutting Experiments 1A, 1B and 1C followed the same protocols as the pre-trial but were amended to systematically measure the same height above and below ground for each cutting, thereby removing the necessity for baseline measurements. Experiment 1A and 1B’s height was 6.4 cm aboveground and 2.5 cm belowground (Fig. 18). This was refined in Experiment 1C to 3 cm aboveground and 2 cm below, plus heel cuttings for the *Calluna* (following recommendation of the Heather Society).



Fig. 18 - Preparation of *Calluna* cuttings to precise above- and belowground lengths (L) before placing in pots (R).

The container sizes differed for each of the experiments: 1A – 10 cm square; 1B – 5 cm square plug trays; and, 1C - 50 ml Ray Leach tubes. Glasshouse temperatures were also variable due to different control conditions in each of the glasshouse facilities available at Kew during the experiment period.

4.2.1.3 Preparing the seeds for sowing and growing

Prior to setting out a large-scale experiment, trials were run to determine appropriate germination conditions. Despite high germination rates in trays, transfer was difficult, resulting in low survival rates. *Calluna* germination was compared to *Erica*. Time to germination was noted (88 days to first cotyledons) and *Calluna* produced 5-6x more cotyledons than *Erica* for the largest trial.

The seeds had been stored in a freezer (at -18° C) between collection and sowing. To remove potential fungal spores, seeds were surface-sterilized for 30 seconds in 1.5% calcium hypochlorite and then rinsed with distilled water through a fine mesh, followed by filtered paper. The seeds were then sown directly in the 50 ml Ray Leach tubes, five at a time (using a dissecting microscope to ensure it was indeed a seed and not another particle as the seeds are minute). The surface of the tube was sprinkled with sand to keep the lightweight seeds in place, and gently watered using the narrow nozzle of the laboratory water bottles. Each tube was then placed in a bag (250ml long), and sealed on top. The tubes were placed in a glasshouse maintained between 22° C and 30° C, 75% humidity, and monitored and watered regularly to keep the compost moist (allowing occasional dry spells for a few days) replicating patterns found in nature. Once the seeds germinated, they were moved to a glasshouse at 12° C - 24° C with 60% humidity, and cuts were made in the bag bottom for drainage.

Seedling Experiment 2's tubes were sown late-October 2012 and potted on to three-inch square pots approximately four months later, once the seedlings outgrew their tubes and bags. Cultivation began in a growth chamber (temperature and light settings 14 h at 22 C°, 100 % light; and 8 h at 17 C°, 0 % light) but the seedlings were later moved and maintained in glasshouses at the same temperature and humidity settings as in the above-described trials. Seedling Experiments 2A and 3 were both sown in March 2013 (with seeds collected from the Norfolk site in November 2012) in glasshouse conditions.

4.2.2 Nursery inoculation trials: Vascular plants co-planted with liverworts (resynthesized with *Pezoloma ericae*) or *P. ericae* isolate

4.2.2.1 Determining ErM colonization in treatment liverworts

Prior to commencing the nursery interaction experiments, the presence of *P. ericae* in the liverwort rhizoids was verified, both visually, by examining their rhizoids under the microscope and observing ErM fungal hyphae, and through molecular testing.

4.2.2.2 Experimental design

Prior to commencing the nursery experiments I conducted a series of pre-trials (Appendix 7) to test design parameters; the experimental design was improved accordingly.

A summary of the cuttings and seedlings experiments can be seen below in Table 3.

Table 3 – Initial number of plants and treatments tested for each experiment

CUTTINGS	1A		1B		1C	
Treatment:	<i>Erica</i>	<i>Calluna</i>	<i>Erica</i>	<i>Calluna</i>	<i>Erica</i>	<i>Calluna</i>
Control	48	49	72	72	77	76
Fungus isolate	47	52	-	-	-	-
Liverwort	52	47	62	76	76	76

SEEDLINGS	2		2A		3	
Treatment:	<i>Erica</i>	<i>Calluna</i>	<i>Erica</i>	<i>Calluna</i>	<i>Erica</i>	<i>Calluna</i>
Control	150	150	60	60	50	50
Fungus isolate 1	-	-	-	-	75	75
Fungus isolate 2	-	-	-	-	75	75
Liverwort	150	150	60	60	75	75

4.2.2.2.1 Cutting Experiment 1A

In September 2013, 148 *Calluna vulgaris* and 147 *Erica tetralix* cuttings were planted (section 4.2.1.2). Three treatments were applied to unrooted cuttings of both taxa: 1) intact liverworts (axenically-grown and resynthesized with *P. ericae* isolates), 15 - 20 individuals per stem, directed to the base of the cutting (T1); 2) suspension of fungal isolate (JK38.37 series) derived from leafy liverworts (1ml per cutting consisting of 120 mg macerated mycelium:1 ml distilled sterilized water, modified technique and proportions used by Powell and Bagyaraj (1984)) (T2); and, 3) negative control of no treatment (T3).

Liverwort symbionts were introduced to cuttings within two days of planting. Each individual plant pot was numbered and treatments were randomly dispersed using a complete block design with four replicates.

As the temperatures were seasonably low, the trays were placed on a propagation bench with under-heating to facilitate rooting. Bags were opened periodically to allow



condensation to disperse and to facilitate airflow (Fig. 19).

Fig. 19 - View of plants on nursery bench with bags open.

The cuttings were lifted to assess rooting success seven weeks after planting. Once rooting occurred, the pots were covered with cling film and maintained as in the pre-trial but the plants were drenched with water for three weeks. An analysis, by species, of growth responses and survival followed.

4.2.2.2.2 Cutting Experiment 1B

A total of 282 cuttings were prepared in May 2014. Approximately half of the plugs contained *Erica* and *Calluna* controls and the other half were treated with intact liverworts (protocol the same as Experiment 1A but no isolate treatment).

To reduce cross-contamination potential by spores in the liverwort treatment group, a corrugated plastic shield was tied to the posts dividing the mist unit. Temperature and relative humidity were set at a ceiling of 23°C and 90% humidity respectively and monitored daily on both the control and treatment side of the shield. Relative humidity and temperature readings were recorder over a random five-day period (Appendix 8). There were several unseasonably hot days causing the temperature to exceed this maximum. This was normalized within four days. Thereafter, the mist unit was maintained between 17°C and 26°C. Once rooting commenced, the pots were removed from the mist unit to begin hardening off with less relative humidity and temperatures ranging from 12° C to 19° C.

Growth response measures recorded root establishment, new growth, and number of branches. Initial overall plant health was assessed after five weeks using the following three criteria: 'Good' - greater than one-half of cutting is green/alive; 'Poor' - less than one-half; or 'Dead' - determined by scraping the stem plus no appearance of new growth. At nine weeks, survival data were collected.

4.2.2.2.3 Cutting Experiment 1C

A total of 305 cuttings were prepared with four replicate blocks of both taxa ($n=19$) and the treatment inoculation regime followed Experiment 1B. Statistical power tests were run to determine an appropriate replicate block size (11 was deemed reasonable). These cuttings were maintained at 17° C at night (8 h) and 21° C at day (14 h). The light source was natural with a tinted roof providing daytime shade. The relative humidity was set to 85%.

Several weeks after planting, monitoring began for phenology records, e.g. first flowers. Growth response variables (including root establishment, new growth, height and branching) were measured periodically for statistical analyses. At 11 weeks, the roots were harvested to examine colonization success and check the controls were not colonized.

4.2.2.2.4 Seedling Experiment 2

A total of 600 seeds were sown, five per tube for both treatments (Fig. 20). Serially-washed naturally sourced liverworts were introduced three days after sowing. The seedlings remained in their tubes through initial measurements.



Fig. 20 - *Erica* seedlings growing in the nursery, bags open to remove excess condensation.

Initially, germination rate and days to germinate were recorded. At 11 weeks, and 18 weeks, height, leaf count and branching were measured and tested for statistical significance. A randomized selection of seedling hair roots was harvested from both treatment and control groups to assess fungal colonization. Randomization

(using the random function in Excel) was achieved by selecting two numbers between 1 and 30 for each taxa and treatment.

4.2.2.2.5 Seedling Experiment 2A

In January 2014, a repeat of Experiment 2 commenced. This time, however, the treatment was applied after the first signs of germination to test whether rooted seedlings are more responsive to inoculation, and three seeds (rather than five) were sown per tube. These were maintained as described in Experiment 2. After measuring, these seedlings were repotted following compost and cling film protocols established in this study. Germination rate and days to germinate were recorded. The cut-off date for germination was 11 weeks. At 20 weeks, growth response data were collected and analysed.

4.2.2.2.6 Seedling Experiment 3

In this seedling trial, treatments of wild liverworts (serially washed) were compared with treatments of isolated fungi cultures applied directly to young seedlings (approximately 16 weeks old). There were 75 seeds sown (one per tube) for each treatment, and 50 for the controls. The treatments were: 1) wild liverworts intact (T1); 2) a suspension of pure fungal isolate using an ericaceous hair root culture isolate (T2); 3) a suspension of pure fungal isolate using a typical leafy liverwort culture isolate (T3); and, 4) a control with no fungus added (T4). Proportions of fungal extract concentration to water was modified from Kosola *et al.* (2007).

4.2.2.3 Determining colonization of heathers post treatment

An assortment of Ericaceae plant hair roots were harvested, being careful to select roots from the top few centimetres of compost where the most actively growing roots are found (Leake and Read, 1991). These roots were rinsed under tap water (in a sieve) and placed in Petri dishes to carefully remove any further soil particles. Once clean, they were examined with a compound microscope (40x, 60x) to ascertain whether root cells were colonized by typical ErM fungi which form coils in the epidermal cells (Read, 1996). Colonization was deemed present if at least three cells were counted with typical ErM coils, which provided assurance this was not an aberration. In actuality there were always many more than three per hair root when present. Other studies have relied on just one cell (Starrett *et al.*, 2001) or percentage of root colonization on already colonized plants (Olsrud *et al.*, 2004).

4.2.2.4 Aboveground growth measurements of heathers

Initial height measurements were recorded before and after colonization. Height was used as a proxy for biomass after establishing a correlation between stem height and dry weight, at least in plants about one-year old (Appendix 9).

Using a tape measure, maximum height of the plants was measured from the base of the stem to the highest point on the plant, when extending the leading branch. These were repeated periodically. Diameter measurements were initially taken, but due to the difficulty measuring such small variations in diameter, this was discontinued. Data on leaf count, number of branches and new growth measures were also collected where practical; at around five months, e.g. it became impractical to count all the branches for *Calluna* but given the form of *Erica*, this was repeatable. As the plants matured, total height was added to include the lead stem plus the length of other branches (greater than 5 mm).

4.2.2.5 Germination, rooting and survival rates

The proportion of plants to germinate, the appearance of first leaves and number of days to germinate were all measured for seedling experiments 2, 2A and 3. Rooting success was examined for experiment 1B. All plants were lifted and examined for root development. If there were three or more roots measurable at least 5 mm long (visible to the eye), the plant was deemed to have positive root development. Three roots is grounding, tripod-like, and usually promotes success. In fact, there were either always more than three roots at the time of examination, or none. Thus, the threshold shifted to present or absent.

Survival rates for both cuttings and seedlings were also documented and analysed. A plant was considered 'dead' if no living tissue was detected following a gentle surface scratch on the stem (using a fingernail), there was no visible fresh growth and, more than one-half of the plant appeared brown.

4.2.2.6 Statistical tests

Populations were statistically tested for normal distribution and variance before choosing the correct statistical tests. Student t-tests were used to compare two sample means between the treatment and control group data, e.g. growth response variables such as height. ANOVA was also considered when more than two treatments were compared. Here the null hypothesis is that variation within the groups is the same as variation between the groups. When using t-tests and ANOVA, the assumption was the data are continuous, approximately normally distributed and the variance of the data sets is the same. Paired t-tests were carried out if an individual was measured more than once. Mann-Whitney or Wilcoxon-Mann-Whitney is preferred when variance is not deemed homogeneous or the distribution not normal. It is less powerful than the t-test or ANOVA; however, it is less likely to find a statistically significant result when there is no real difference (Dytham, 2003). When using categorical data (e.g. dead or alive,

rooting or not), chi-squared and proportion tests were employed. Taken together, these measures provide a stronger test for growth or establishment success, as a result of treatment. Rstudio (2009-2012) was usually used to carry out the statistical analyses; occasionally Excel or Prism Version 6.0f (GraphPad Software) was used for simple comparisons. Prism graphics replaced Rstudio for the most recent presentation due to its easier user interface.

Benchmarks for statistical significance use norms described by Motulsky (2014) whereby a *P-value* less than 0.05 is deemed significant and denoted by a single asterisk (*); less than 0.01 is highly significant (**); and, less than 0.001 is extremely significant (***). There is a difference between statistically significant and ecologically significant; low *P-values* do not necessarily suggest large effects (Burk and Ascough, 2005). The *P-value* is used due to its prevalence in the literature, although it must be interpreted carefully (Martinez-Abram, 2008). Here, they are used as a tool to help understand a data set's consistency with the null hypothesis.

4.3 Results

4.3.1 Summary of plant survival, colonization and growth response measurements

Establishment success, particularly when stress was introduced, was enhanced by liverwort treatments, particularly with *Erica* plants in cuttings experiments; *Erica* plants were generally more responsive to treatment than *Calluna*. Colonization of the roots, both for cuttings and seedlings is unquestionably statistically significant, regardless of whether treatment was delivered at the earliest stages of germination or after rooting. Growth response measurements indicate the treatment of liverworts resynthesized with ErM contribute to more vigorous plants (in the glasshouse) with increased biomass (using stem height as a proxy). Below is the full account of results, with significant findings summarized in Table 4. Rstudio transcripts are in the Appendices.

4.3.1.1 Establishment success rate in cuttings and seedlings

The three cuttings experiments (i.e. 1A, 1B and 1C) yielded statistically significant results when examining survival of cuttings following treatment. In Experiments 1A and 1B, *Erica* was more sensitive to treatment, however only *Calluna* responded significantly to treatment in Experiment 1C. Seedling survival was not affected by treatment in the one remaining seedlings experiment, Experiment 2A.