From rhizoids to roots? Experimental evidence of mutualism between liverworts and ascomycete fungi

Jill Kowal1,2,3,*, Silvia Pressel3, Jeffrey G. Duckett3, Martin I. Bidartondo1,2 and Katie J. Field4

1Imperial College London, London SW7 2AZ, UK, 2Royal Botanic Gardens, Kew, Richmond TW9 3DS, UK, 3Natural History Museum, Cromwell Road, London SW7 5BD, UK and 4Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

*For correspondence. E-mail j.kowal@nhm.ac.uk

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RESEARCH IN CONTEXT

From rhizoids to roots? Experimental evidence of mutualism between liverworts and ascomycete fungi

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INTRODUCTION

Mycorrhizas are intimate symbioses formed between plant roots and soil fungi that are prevalent across the globe in >80% of extant land plants (Smith and Read, 2008). Through mycorrhizal associations, many plants engage in bi-directional exchange of photosynthesis-derived plant carbon (C) and fungal-acquired nutrients, scavenged from sources beyond the root depletion zone or from soil pores too small for roots to access (Smith and Read, 2008; Leake and Read, 2016). The role of mycorrhizas in supplying extant land plants with nutrients, together with evidence of mycorrhiza-like associations in Rhynie Chert plant fossils (Remy et al., 1994), has led to the hypothesis that mycorrhizal fungi probably facilitated the evolution of land plants >470 Mya (Pirozynski and Malloch, 1975; Bidartondo et al., 2011; Leake, 2015). Additionally, recent studies have shown that the genes required for mycorrhization are conserved across all land plant lineages (Wang et al., 2010), including the earliest diverging clade of liverworts—Haplomitriopsida (Fig. 1A, after Crandall-Stotler et al., 2009). The later-derived leafy liverworts (Jungermanniidae) have not been incorporated in such analyses, and a critical caveat of molecular studies is that the presence of genes does not necessarily imply functional significance. Indeed, for many groups of extant plants that form mycorrhizas and mycorrhiza-like associations where roots are absent, knowledge regarding the physiological function of the symbiosis has been severely limited (Read et al., 2000; Field et al., 2015a). To date, amongst fungus-associated early-branching land plants (i.e. liverworts, hornworts and lycophytes), nutritional mutualisms have only been demonstrated in a handful of early-diverging thalloid and Haplomitriopsida liverworts, which originated in the Triassic and are sister to all other jungermannialean liverworts associated with fungi, our findings point toward an early origin of ascomycete–liverwort symbioses, possibly pre-dating their evolution in the Ericesales by some 150 million years.

Key words: Cephalozia bicuspidata, ericoid mycorrhizal fungi, liverwort, Pezoloma ericae, plant-fungus interactions, carbon-for-nutrient exchange, mycorrhizas, mutualism.

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Kowal et al. — Mycorrhizal fungi of Ericaceae are mutualistic with non-vascular plants

species worldwide compared with <100 with basidiomycetes, glomeromycetes and mucoromycetes (see Supplementary Data Table S2 for a detailed breakdown). Reinforcing their symbiotic rather than opportunistic status, the ascomycetes (1) induce swelling, branching and septation of the rhizoids and (2) are ubiquitous rather than sporadic, in species where they occur. In addition, transmission electron micrographs show healthy hyphae in healthy host cells (Duckett et al., 1991; Pressel et al., 2010).

There are several families in the leafy liverworts (Jungermanniidae), including Schistochilaceae, Lepidoziaceae, Calypogeiaceae, Cephaloziaceae and Cephaloziellaceae, which consistently associate with Ascomycota fungi (Fig. 1A) (Pressel et al., 2010). These fungal symbionts include Pezoloma ericae (D.J. Read) Baral (syn. Rhizoscyphus ericae (D.J. Read) W.Y. Zhuang and Korf, 2004; Hymenoscyphus ericae (D.J. Read) Korf and Kernan, 1983; and Pezizella ericae (D.J. Read, 1974) (Duckett and Read, 1995; Read et al., 2000; Pressel et al., 2010). Notably, Pezoloma ericae is known to form ericoid mycorrhizas (ErMs) with the roots of Ericaceae plants and has previously been shown to provide nutrients to their vascular plant hosts in exchange for fixed C (Read et al., 2004; Smith and Read, 2008).

Ericaceous habitats are typically low in plant-available soil nutrients, including nitrogen (N) and/or phosphorus (P) (Stribley and Read, 1974; Mitchell and Read, 1981; Leake et al., 1990; Bolan, 1991; Myers and Leake, 1996). The vascular plants inhabiting these habitats, such as Calluna, Erica, Rhododendron and Vaccinium, grow together with non-vascular plants, including the widespread leafy liverworts in the Cephaloziaceae (Chambers et al., 1999; Upson et al., 2007) with which they share fungal symbionts (Duckett and Read, 1995; Read et al., 2000). It was recently shown that that this shared mycobiont can bring benefits in terms of establishment and survival to ericaceous plants (Kowal et al., 2015), and that P. ericae-colonized liverworts may serve as a source of fungal inoculum for vascular plants. It is possible that this effect is driven by fungal-enhanced nutrition in liverworts and then in the vascular plant species sharing the fungal symbiont, analogous to the nutritional role of arbuscular mycorrhizal fungi (Glomeromycotina fungi) that associate with some thalloid liverworts (Field et al., 2012). It may be that P. ericae associates in leafy liverworts play a similar role to the Glomeromycotina or Mucoromycotina fungal partners of thalloid liverworts by supplementing plant P assimilation (Field et al., 2016).
Here, we aim to address the fundamental question of whether the ascomycete fungus *P. ericae* forms mycorrhiza-like associations with leafy liverworts equivalent to those formed by Glomeromycotina or Mucoromycotina fungi and thalloid liverworts. We traced the movement of P from *P. ericae* fungal hyphae to *Cephalozia bicuspidata* liverworts and the movement of C from liverworts to the fungi using isotope tracers. We determined liverwort growth responses to colonization by *P. ericae* fungi by measuring the size and mass of liverworts, grown both with and without *P. ericae* fungal symbionts.

**MATERIALS AND METHODS**

We collected *Cephalozia bicuspidata* from Thursley Common, Surrey, in autumn 2012 (OS grid reference SU900416). Mature sporophytes were harvested, surface-sterilized and spores were cultured axenically (Duckett and Read, 1995) on 1.5 % Phytagel™ (Sigma-Aldrich; ICP elemental analysis of Phytagel provided by Sigma-Aldrich: 0.85 % Ca, 0.35 % Mg, 1.70 % K, 0.15 % P and 0.45 % Na). No additional nutrients were added to the culture medium. *Pezoloma ericae* was isolated from the same liverwort collection and resynthesized with axenically-grown *C. bicuspidata* using published methods (Kowal et al., 2015), thus satisfying Koch’s postulates. Molecular identification of the fungal isolate as *P. ericae* was carried out previously (Kowal et al., 2015). Nomenclature for plants and fungi follows Hill et al. (2008) and www.speciesfungorum.org, respectively.

**Fungus to plant phosphorus transfer**

We grew *C. bicuspidata* and *P. ericae* together in one compartment (‘liverwort and fungus’) of 9 cm split-plate microcosms (Supplementary Data Fig. S1) filled on both sides of the divide (‘B’ in Fig. S1a) with 1.5 % sterile Phytagel. Fragments of *P. ericae* isolate (approx. 7 mm²) were inserted beneath the surface of the Phytagel, and an axenically grown leafy liverwort stem (two per microcosm, approx. 2 cm apart) was gently pressed onto the surface, directly above the fungus fragment. Following establishment of liverwort–fungal symbiosis, which is confined to the rhizoids (Fig. 1B), and growth of extraradical fungal hyphae (11 weeks after planting), we introduced 0.1 MBq H³²PO₄ (i.e. 0.03 μg of ³²P, specific activity 111 GBq mmol⁻¹; Hartmann Analytics, Braunschweig, Germany) into a well within the medium in the contiguous compartment of the plate. Each ³²P-labelled well was then filled with 1.5 % sterile Phytagel. The barrier dividing the microcosm prevented the liverwort from encroaching into the compartment containing ³²P while fungal hyphae were able to grow over the barrier and colonize the medium in both compartments (see Supplementary Data Fig. S1). We prepared a total of 16 microcosms with an additional fungus-free (control) microcosm to measure non-fungal-mediated diffusion of ³²P into liverwort tissue. We also tested the effectiveness of the barriers in eight undivided microcosms containing *C. bicuspidata* without fungus, with ³²P-labelled wells placed at the same distance from plants as in divided microcosms. All microcosms were sealed with Parafilm ‘M’ (Sigma), and placed in a controlled environment chamber (BDR16, Conviron, Winnipeg, MB, Canada). The temperature regime was typical of late-spring/summer for south-eastern England with light intensity reflecting that at ground level beneath canopy vegetation, similar to that experienced by the liverworts in their natural environment (irradiance of 50 μmol m⁻² s⁻¹, 12 h:12 h, light:dark, 16 °C:14 °C day:night, 80 % relative humidity and 440 ppm [CO₂]). After 8 weeks, we removed the liverworts from the microcosms, and freeze-dried all plant tissues and growth medium containing fungal hyphae.

Between 10 and 30 mg of plant tissue and growth medium for each microcosm were digested in 1 mL of concentrated H₂SO₄ for 2 h and then heated to 365 °C for 15 min. After cooling, 100 μL of hydrogen peroxide was added to each sample before re-heating to 365 °C for 2 min, resulting in a clear solution. Samples were diluted up to 10 mL with distilled water before 2 mL of the diluted digest solution was mixed with 10 mL of the liquid scintillant Emulsify Safe (Perkin Elmer). The activity of the samples was determined using liquid scintillation counting (Packard Tri Carb 3100, Isotech, Chesterfield, UK). The amount of ³³P transferred to the plants by the fungus in each microcosm was calculated using equations from Cameron et al. (2007). This figure was then adjusted for passive movement of ³³P from the substrate via diffusion by subtracting the mean amount of ³³P measured in liverworts harvested from fungus-free control microcosms.

**Plant to fungus carbon transfer**

We filled nine cube-shaped vessels (Sigma Magenta GA-7-3, 77 × 77 × 97 mm) with Phytagel (1.5 %) to a depth of 30 mm to prepare three replicate microcosms with fungus only and six ‘complete’ microcosms with liverwort and fungus. A fragment of *P. ericae* isolate (approx. 7 mm²) was inserted beneath the surface of the Phytagel for the fungus-only and complete microcosms. For the complete microcosms, an axenically grown leafy liverwort stem was gently pressed onto the surface of the medium, directly above the fungus fragment. We maintained the plants in the same controlled environment chamber and conditions as above. After 6 months of growth, we labelled the microcosms with ¹⁴CO₂, separating the growth medium from the chamber headspace with polythene (pre-cut with 25 mm diameter holes for the liverworts) in order to minimize direct diffusion of ¹⁴CO₂ into the substrate. The polythene was sealed with anhydrous lanolin where it met the substrate and along the edges of the Magenta vessels (Supplementary Data Fig. S2b). We generated 0.5 MBq of ¹⁴CO₂ gas by adding 6.8 μL of [¹⁴C]sodium bicarbonate to tubes in each microcosm before introducing 500 μL of 25 % lactic acid. We allowed plants to fix ¹⁴CO₂ for 5 h in the middle of the day before introducing two Eppendorf tubes containing 1 mL of 2M KOH into each microcosm for 30 min to trap any remaining ¹⁴CO₂ from the headspace of each microcosm. We then harvested, separated and freeze-dried the liverworts and Phytagel-containing fungal hyphae (see Supplementary Data Methods S1) before weighing, homogenizing and determining ¹⁴C content by sample oxidation (Packard Sample Oxidiser) and liquid scintillation counting (Packard Tri-Carb 3100).

We calculated total C (¹³C plus ¹⁴C) fixed by the plant and transferred to the fungus as a function of the total volume and CO₂ content of the vessel headspace and the proportion of
supplied $^{14}$CO$_2$, label fixed by the plants, using equations from Cameron et al. (2008). The specific activity of the source was 2.04 TBq mol$^{-1}$.

To account for C movement through passive diffusion, and to assess the effectiveness of the polythene–lanolin barrier, total plant-fixed fungal C in the complete microcosms was determined by subtracting the mean C measured in the fungus from the fungus-only control microcosms from each of the complete microcosms.

Liverwort growth

We measured C. bicuspidata growth both with and without fungal inoculation, looking first at changes in leafy liverwort surface area and secondly at liverwort biomass. In the first experiment, we introduced small fragments (approx. 7 mm$^2$) of P. ericae to 9 cm plates containing 1.5% Phytagel with axenically grown leafy liverworts (approx. 1 year old). After fungal colonization of the liverwort rhizoids was confirmed microscopically (Fig. 1B), we re-plated colonized liverworts and fungus-free control liverworts individually using the same medium. Surface area measurements of each liverwort stem (including leaves) were made before and after 6 weeks using images taken with a Nikon Coolpix S10 digital camera and analysed using ImageJ software (Rasband, 1997–2012) (Supplementary Data Fig. S3). For the second measurement of the effect of P. ericae on C. bicuspidata growth, we freeze-dried and weighed the liverworts from the complete microcosms used for the $^{14}$C transfer experiment (above) after they were harvested at 6 months and compared the mean with that of plants from liverwort-only microcosms ($n = 6$). This allowed us to test for differences in mean plant biomass in liverwort growth with or without fungus over a longer time period than the surface area growth experiment described above. Liverwort stems were weighed before initial planting to ensure similarity in mass between sample groups.

Statistics

After analysing the data for normal distribution and homogeneity of variance, we applied the appropriate statistical tests, i.e. parametric or non-parametric, using GraphPad Prism (version 6.0h). The Mann–Whitney U-test was used both for the dry mass experiment and for testing the barrier effect in the $^{33}$P experiment, owing to the low number of data points; Student’s $t$-test was used for other data sets.

RESULTS AND DISCUSSION

The leafy liverwort–Pezoloma ericae symbiosis is mutualistic and mycorrhiza-like

In our radiolabelled liverwort microcosms, 1.786 ng g$^{-1}$ of $^{33}$P was assimilated from P. ericae into C. bicuspidata tissues (Fig. 2A; Supplemenary Data Table S1). There was a significant difference in the liverwort $^{33}$P content and concentration between plant-only microcosms with barriers and plant-only microcosms without barriers [Mann–Whitney U = 0; $n_1 = 2$, $n_2 = 13$; $P = 0.019$ two-tailed (data not shown)], further demonstrating the efficacy of the barriers in our microcosms for preventing direct plant access to $^{33}$P-labelled wells. While we cannot exclude the possibility that the P content of the Phytagel substrate used in our microcosms may have been directly assimilated by the liverworts within the systems and therefore reduced plant demand for fungal-acquired $^{33}$P, our data unequivocally show movement of $^{33}$P from the fungus to the leafy liverwort.

The leafy liverworts transferred 0.019 ng (0.22 ng g$^{-1}$) of plant-fixed C to their fungal partners (Fig. 2B), equivalent to 0.27% of the total amount of C fixed during the labelling period (Supplementary Data Table S1). By demonstrating unequivocal exchange of fungal-acquired P and plant-fixed C between symbionts, our results provide the first experimental evidence that associations between non-vascular plants and ascomycete fungal symbionts are nutritionally mutualistic. This confirms previous hypotheses based on cytological evidence (van der Heijden et al., 2015) and culturing experiments (Duckett and Read, 1991; Upson et al., 2007; Kowal et al., 2015). Additionally, liverworts resynthesized with P. ericae grew significantly larger than liverworts grown under identical conditions without the fungus for both growth experiments (Fig. 3A, B). Surface area after 6 weeks almost doubled in the P. ericae-treated microcosms compared with the liverwort-only microcosms ($t$-test; $n_1 = 22$, $n_2 = 16$; $P < 0.01$), and total biomass of the liverworts (dry weight; g) after 6 months of growth...
was significantly greater with *P. ericae* (Mann–Whitney *U* = 2; *P* ≤ 0.01; *n* = 6).

Together with our evidence of C for nutrient exchange between symbionts, it is clear that the symbiosis between non-vascular leafy liverworts and their fungal partners is mutualistic and mycorrhiza-like. This lays the foundation to investigate further functional differences between liverwort–ascomycete partnerships and previously documented exchanges between *P. ericae* and vascular plants (Pearson and Read, 1973; Read and Stribley, 1973; Upson et al., 2008, Kowal et al., 2015).

Our experiments aimed to uncover whether there is any movement of plant-fixed C and fungal-acquired P between *C. bicuspidata* liverworts and *P. ericae*. The evidence of 33P transfer from fungus to plant in our experimental microcosms (Fig. 2A) strongly supports a nutritional role for *P. ericae* that is particularly significant given that P can be one of the most limiting nutrients in the ericaceous habitats where *C. bicuspidata* grows. It is possible that the enhanced growth we observed in liverworts with fungal symbionts compared with those without (Fig. 3) was a result of fungal remineralization of organic compounds leached from the liverwort, rather than a direct result of increased uptake of fungal-acquired P. However, the provision of 33P by the fungus, as shown here, supports the hypothesis that the fungus plays a role in leafy liverwort P nutrition in natural environments.

**Observations on previously reported liverwort–fungal symbioses**

Although not directly comparable, given considerable differences in the physiology of the liverwort hosts and in experimental design (i.e. axenic and edaphic conditions), our finding of mycorrhiza-like associations between ascomycete fungi and leafy liverworts now invites further comparisons with more ancient lineages of liverworts, i.e. Haploomitriopsida (*Treubia* and *Haplanitrium*) and complex thalloid liverworts (*Neohodgsonia*, *Allisonia* and *Marchantia*) and fungi, i.e. Mucoromycotina and/or Glomeromycotina (Field et al., 2015a, 2016) (see Fig. 1A; Supplementary Data Table S1). When fungal-acquired 33P uptake is normalized to biomass (Fig. 2A; Table S1), it appears that *C. bicuspidata* gains significantly less 33P from its fungal partner than *Haplanitrium*, *Treubia* (with Mucoromycotina only), *Allisonia* and *Neohodgsonia* (with Mucoromycotina and Glomeromycotina), but a roughly similar amount to *Marchantia* (with Glomeromycotina only). *Cephalozia bicuspidata* C allocation (in terms of absolute amount, Fig. 2B) to its symbiotic fungus also points to a relatively low C demand by *P. ericae* on its liverwort host, especially when compared with other liverwort lineages (0.27 % of plant-fixed C transferred to the symbiotic fungus vs. 2.2–14.2 %, see Table S1). This may be partially influenced by the facultative biotrophic nature of *P. ericae* allowing it to gain at least some organic C from dead organic matter, and the extent of colonization (i.e. restricted to rhizoids vs. extensive through the thallus). It is possible that the fungus in our experiments was able to derive some C directly from the Phytagel substrate (which contains glucose and trace levels of nutrients; see the Materials and Methods). Regardless of the P for C measurements presented in Table S1, it is evident that the relative ‘cost’ of maintaining mycorrhiza-like symbioses varies between liverwort–fungal
symbioses, with ErM-like associations potentially requiring less liverwort investment in terms of photosynthetic allocation, and the liverwort–Mucoromycotina partnership requiring the most (Field et al., 2016).

Having established that there is a mycorrhizal-like nutritional exchange between the leafy liverwort C. bicuspidata and its fungal symbiont P. ericae, investigations are now needed which include fungus to plant N transfer and more natural experimental conditions to provide a robust platform to investigate functional variation in plant–fungal symbioses across evolutionary lineages and ecological gradients.

**Future directions**

The diversity of fungal symbionts across the land plant phylogeny is becoming increasingly apparent; however, there is still a relative dearth of information regarding the function of plant–fungal symbioses in many clades, particularly the bryophytes. The largest remaining functional knowledge gaps now are the basidiomycete symbioses in thalloid Aneuraceae and Arnelliacaeae liverworts (Bidartondo and Duckett, 2010). In pteridophytes, only two pioneering studies to date have demonstrated the bi-directional exchange of plant-fixed C for fungal-acquired N and P between green sporophytes of Osmunda regalis and Ophioglossum vulgatum and their Glomeromycotina fungal symbionts (Field et al., 2012; Pressel et al., 2016).

Although we show transfer of C from C. bicuspidata to fungi *in vitro*, in habitats where C. bicuspidata grows, often in deep shade under a canopy of ericaceous plants, the fungal symbiont of C. bicuspidata may be getting a large proportion of its organic C from surrounding vascular plants via a shared fungal hyphal network, as demonstrated previously between Betula and the mycoheterotrophic liverwort Cryptothallus (Aneurca) mirabilis via a shared basidiomycete fungus (Read et al., 2000; Bidartondo et al., 2003). How this affects ecosystem C and nutrient budgets in terms of storage and cycling remains to be uncovered.

In conclusion, our findings provide a novel and important example of myccorrhiza-like functioning in an additional fungal lineage with non-vascular plants. Thus, our demonstration of symbiotic ErM-like functioning of *P. ericae* with a leafy liverwort now adds the Ascomycota to the list of fungal groups engaging in mutualistic C for nutrient exchange across the land plant phylogeny. It has been proposed that *C. bicuspidata* liverworts with ErM fungal symbionts can be used to restore threatened heathlands by promoting establishment of native ericaceous plants (Kowal et al., 2015) that are limited by ErM inoculum availability (Diaz et al., 2006). Our results suggest that at least one of the mechanisms underpinning the potential role of *C. bicuspidata* in habitat restoration strategies is likely to be nutritional. The potential ecological applications of pioneer non-vascular plants as myccorrhizal reservoirs and vectors, particularly in habitat restoration, are only just becoming apparent. Evidence of a mutualism between a widespread non-vascular plant and ascomycete fungi that form myccorrhizas with dominant vascular plants should now encourage further ecological and physiological experiments.

**SUPPLEMENTARY DATA**

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: diagrams and photographs of the 32P transfer experiment showing the split plate experimental design set up before and after the fungus crossed the barrier. Figure S2: diagram showing microcosm design for carbon transfer experiments. Figure S3: example of digitized pictures used to measure perimeter area of liverworts. Table S1: summary of carbon for nutrient exchange between *Cryptothallus bicuspidata* and *Pezoloma ericae*, alongside data from previous studies of early-diverging liverwort lineages and their fungal partners. Table S2: estimates of likely species numbers of liverworts worldwide with fungal symbionts. Methods S1: methods for harvesting plants and fungus for 32P acid digestion, liquid scintillation and nutrient budgeting.

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**LITERATURE CITED**


