Article:

Kowal, J., Pressel, S., Duckett, J.G. et al. (2 more authors) (2018) From rhizoids to roots? Experimental evidence of mutualism between liverworts and ascomycete fungi. Annals of Botany, 121 (2). pp. 221-227. ISSN 0305-7364

https://doi.org/10.1093/aob/mcx126

RESEARCH IN CONTEXT

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

Jill Kowal[1,2,3], Silvia Pressel[3], Jeffrey G. Duckett[3], Martin I. Bidartondo[1,2], Katie J. Field[4]

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

Author for correspondence: Jill Kowal - j.kowal@nhm.ac.uk

Tel: +44 (0)20 7942 5276

Emails of co-authors: s.pressel@nhm.ac.uk; j.g.duckett@qmul.ac.uk; m.bidartondo@imperial.ac.uk; k.field@leeds.ac.uk

Date of submission: May 6, 2017

Total word count (excluding summary, references and legends): 5873

Abstract: 208

Introduction: 881

Materials and Methods: 1449

Results and discussion: 1553

Acknowledgements: 36

No. of Figures: 3 (Fig. 1 and 2 in colour)

Supplemental data files: 5 (Table S1; Fig. S1,S2,S3; Methods S1)

Abstract

The rhizoids of leafy liverworts (Jungermanniales, Marchantiophyta) are commonly colonized by the ascomycete fungus Pezoloma ericae. These associations are hypothesized to be functionally analogous to the ericoid mycorrhizas (ErM) formed by P. ericae with the roots of Ericaceae plants in terms of bi-directional phosphorus-for-carbon exchange; however, this remains unproven. Here, we test whether associations between the leafy liverwort Cephalozia bicuspidata and P. ericae are mutualistic.

We measured movement of phosphorus and carbon between C. bicuspidata and P. ericae using ^{33}P-orthophosphate and $^{14}CO_2$ isotope tracers in monoxenic cultures. We also measured leafy liverwort growth, with and without P. ericae. We present the first demonstration of nutritionally mutualistic symbiosis between a non-vascular plant and an ErM-forming fungus, showing transfer of fungal-acquired P to the liverwort and of liverwort-fixed C to the fungus alongside increased growth in fungus-colonized liverworts.

Thus, this ascomycete-liverwort symbiosis can now be described as mycorrhiza-like, providing further insights into ericoid mycorrhizal evolution and adding Ascomycota fungi to mycorrhizal fungal groups engaging in mutualisms with plants across the land plant phylogeny. As P. ericae also colonizes the rhizoids of Schistochilaceae 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 predating their evolution in the Ericales by some 150 MY.

Key words: Cephalozia bicuspidata; ericoid mycorrhizal fungi; liverwort; Pezoloma ericae; symbiosis; carbon-for-nutrient exchange; mycorrhizas; mutualism

Introduction

Mycorrhizas are intimate symbioses formed between plant roots and soil fungi that are prevalent across the globe in more than 80% of extant land plants (Smith and Read, 2008). Through mycorrhizal associations, many plants engage in bi-directional exchange of photosynthesis-derived plant carbon 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 likely facilitated the evolution of land plants >470 Mya (Pirozynski et al., 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 (Field et al., 2012; 2015a; 2016), (Fig. 1a), all which form symbioses with Mucoromycota fungi (Glomeromycotina and/or Mucoromycotina) (Field et al. 2015b).

As far as can be established from published surveys and incidental illustrations in floras (Pocock et al.,1984; Pocock & Duckett, 1985; Duckett et al.,1991, Ligrone et al. 2007) rhizoidal ascomycete associations occur in far more liverwort species than those containing basidiomycetes, glomeromycetes and mucoromycetes (Pressel et al., 2010). Following the accepted species names in the Plant List (2013), we estimate that up to 1,000 late-diverging leafy liverworts are likely to have ascomycete fungal symbionts, i.e. around 20% of liverwort species worldwide compared to less than 100 with basidiomycetes, glomeromycetes and mucoromycetes (see Table S2 for 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 (Jungermanniideae), 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 (ErM) with the roots of Ericaceae plants and has previously been shown to provide nutrients to their vascular plant hosts in exchange for fixed carbon (Read et al., 2003; Smith and Read, 2008).

Ericaceous habitats are typically low in plant-available soil nutrients, including N and/or 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 the P. ericae-colonized liverwort 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 phosphorus (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 carbon (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 (Fig. S1) filled on both sides of the divide ('B' in Fig. S1a) with 1.5% sterile Phytagel. Fragments of P. ericae isolate (approximately 7 mm^2) were inserted beneath the surface of the Phytagel and an axenically-grown leafy liverwort stem (two per microcosm, c. 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 (eleven weeks after planting), we introduced 0.1 MBq $H_3{}^{33}PO_4$ (i.e. 0.03 μg ^{33}P, specific activity 111 GBq mmol^{-1}; Hartmann Analytics, Braunschweig, DE) into a well within the medium in the contiguous compartment of the plate. Each ^{33}P-labelled well was then filled with 1.5% sterile Phytagel. The barrier dividing the microcosm prevented the liverwort from encroaching into the compartment containing ^{33}P while fungal hyphae were able to grow over the barrier and colonize the medium in both compartments (see Fig. S1). We prepared a total of 16 microcosms with an additional fungus-free (control) microcosm to measure non-fungal mediated diffusion of ^{33}P into liverwort tissue. We also tested the effectiveness of the barriers in eight undivided microcosms containing C. bicuspidata without fungus with ^{33}P-labelled wells placed 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 southeastern 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% RH and 440 ppm [CO_2]). After eight weeks, we removed the liverworts from the microcosms, and freeze-dried all plant tissues and growth medium containing fungal hyphae.

Between 10-30 mg of plant tissue and growth medium for each microcosm was digested in 1 mL concentrated H_2SO_4 for two hours and then heated to 365 °C for 15 minutes. After cooling, 100μL of hydrogen peroxide was added to each sample before reheating to 365 °C for two minutes, 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). Activity of the samples was determined using liquid scintillation counting (Packard TriCarb 3100, Isotech, Chesterfield, UK). The amount of ^{33}P 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 ^{33}P from the substrate via diffusion by subtracting the mean amount of ^{33}P 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 mm × 77 mm × 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 (ca. 7 mm^2) 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 six months of growth, we labeled the microcosms with $^{14}CO_2$, 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 $^{14}CO_2$ into the substrate. The polythene was sealed with anhydrous lanolin where it met the substrate and along the edges of the Magenta vessels (Fig. S2b). We generated 0.5 MBq of $^{14}CO_2$ gas by adding 6.8 μL ^{14}C-sodium bicarbonate to tubes in each microcosm before introducing 500 μL 25% lactic acid. We allowed plants to fix $^{14}CO_2$ for five hours in the middle of the day before introducing two Eppendorf tubes containing 1 mL 2 M KOH into each microcosm for 30 min to trap any remaining $^{14}CO_2$ from the headspace of each microcosm. We then harvested, separated and freeze-dried the liverworts and Phytagel containing fungal hyphae (see Methods S1) before weighing, homogenizing and determining ^{14}C content by sample oxidation (Packard Sample Oxidiser) and liquid scintillation counting (Packard Tri-Carb 3100).

We calculated total carbon (^{12}C plus ^{14}C) fixed by the plant and transferred to the fungus as a function of the total volume and CO_2 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 carbon movement through passive diffusion, and to assess the effectiveness of the polythene/lanolin barrier, total plant-fixed fungal carbon in the complete microcosms was determined by subtracting the mean carbon 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 second at liverwort biomass. In the first experiment, we introduced small fragments (c. 7 mm^2) of P. ericae to 9 cm plates containing 1.5% Phytagel with axenically-grown leafy liverworts (c. one 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 six weeks using images taken with a Nikon Coolpix S10 digital camera and analyzed using ImageJ software (Rasband, 1997-2012) (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 six months and compared the mean to 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 analyzing 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 barrier effect in the ^{33}P experiment, owing to the low number of datapoints; Student's t-test was used for other datasets.

Results and discussion

Leafy liverwort-Pezoloma ericae symbiosis is mutualistic and mycorrhiza-like

In our radio-labeled liverwort microcosms, 1.786 ng g^{-1} of ^{33}P was assimilated from P. ericae into C. bicuspidata tissues (Fig. 2a, Table S1). There was a significant difference in the liverwort ^{33}P content and concentration between plant-only microcosms with barriers and in 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 barriers in microcosms for preventing direct plant access to ^{33}P-labeled 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 carbon to their fungal partners (Fig. 2b); equivalent to 0.27% of the total amount of carbon fixed during the labelling period (Table S1). By demonstrating unequivocal exchange of fungal-acquired phosphorus and plant-fixed carbon 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 ($P < 0.01$ for both, Fig. 3a,b). Together with our evidence of carbon-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 carbon and fungal-acquired P between C. bicuspidata liverworts and P. ericae. The evidence of ^{33}P transfer from fungus to plant in our experimental microcosm (Fig. 2a) strongly supports a nutritional role for P. ericae that is particularly significant given that phosphorus 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 to 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 ^{33}P by the fungus, as shown here, supports the hypothesis that the fungus plays a role in leafy liverwort P nutrition in natural environments.

Evolutionary context

Our demonstration that the association between a leafy liverwort and the ericoid mycorrhizal fungus P. ericae can be reciprocally beneficial provides additional clues about the evolution of ErM, and lends further weight to the idea that mutualisms between leafy liverworts and ascomycete fungi pre-dated their formation in the Ericales, estimated at less than 100 MYA (Brundrett, 2004). Following their discovery that ascomycetes belonging to the P. ericae group colonize the rhizoids of Schistochilaceae liverworts, thought to have originated in the Triassic (Heinrichs et al., 2007) as the sister group to all other fungus-containing lineages in the Jungermanniales, Pressel et al. (2008) suggested that this association arose more than 250 MYA. Nonetheless, divergence time estimates for ascomycete lineages remain a matter of debate particularly as the presumed recent origin of Leotiomycetes, the clade containing P. ericae, is in the Cretaceous (James et al., 2006; Prieto and Wedin, 2013). Until ever-improving phylogenetic methods integrating fossil evidence (Beimforde et al., 2014) provide more accurate date estimates, recent origins for the association with multiple instances of host shifting between leafy liverworts and Ericaceae plants, remain a plausible alternative scenario (Selosse, 2005; Pressel et al., 2008).

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 mycorrhizal-like associations between ascomycete fungi and leafy liverworts now invites further comparisons with more ancient lineages of liverworts, i.e. Haplomitriopsida (Treubia and Haplomitrium) and complex thalloid liverworts (Neohodgsonia, Allisonia and Marchantia) and fungi - Mucoromycotina and/or Glomeromycotina (Field et al. 2015a and 2016) (see Table S1; Fig. 1a). When fungal-acquired ^{33}P uptake is normalized to biomass (Fig. 2a; Table S1), it appears C. bicuspidata gains significantly less ^{33}P from its fungal partner than Haplomitrium, Treubia (Mucoromycotina only), Allisonia and Neohodgsonia (Mucoromycotina and Glomeromycotina), but a roughly similar amount to Marchantia (Glomeromycotina only). Cephalozia bicuspidata carbon allocation (in terms of absolute amount, Fig. 2b) to its symbiotic fungus also points to a relatively low carbon demand by P. ericae on its liverwort host, especially when compared to other liverwort lineages (0.27% of plant-fixed carbon 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 carbon from dead organic matter, and the extent of colonisation (i.e. restricted to rhizoids vs. extensive through 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 materials and methods). Regardless of the phosphorus-for-carbon 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 photosynthate 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 leafy Scapaniaceae and Arnelliaceae liverworts (Bidartondo and Duckett, 2010). In pteridophytes only two pioneering studies to date have demonstrated the reciprocal exchange of plant-fixed C for fungal-acquired N and P between green sporophytes of Osmunda regalis and

Ophioglossum vulgatum and their Glomeromycotina symbionts (Field et al., 2012, 2015c; 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 network, as demonstrated previously between Betula and the mycoheterotrophic liverwort Cryptothallus (Aneura) mirabilis via a shared basidiomycete fungus (Read et al., 2000; Bidartondo et al., 2003). How this affects ecosystem carbon and nutrient budgets in terms of storage and cycling remains to be uncovered.

In conclusion, our findings provide a novel and important example of mycorrhizal functioning of 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 carbon-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 mycorrhizal 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 mycorrhizas with dominant vascular plants should now encourage further ecological and physiological experiments.

Acknowledgements:

J.K. thanks BSBI for funding for laboratory work and the University of Sheffield for use of growth and analytical facilities. K.F. is supported by a BBSRC Translational Fellowship (BB/M026825/1). All authors are supported by NERC (NE/N009665/1). We thank the editor and reviewers for their constructive comments on an earlier version of this manuscript.

Literature Cited

Bajwa R, Read DJ. 1985. The biology of mycorrhiza in the Ericaceae IX. Peptides as nitrogen sources for the ericoid endophyte and for mycorrhizal and non-mycorrhizal plants. New Phytologist **101**: 459-467.

Bajwa R, Read DJ. 1986. Utilization of mineral and amino N sources by the ericoid mycorrhizal endophyte Hymenoscyphus ericae and by mycorrhizal and non-mycorrhizal seedlings of Vaccinium. Transactions of the British Mycological Society **87**: 269-277.

Beimforde C, Feldberg K, Nylinder S, Rikkinen J, Tuovila H, Dörfelt H, Gube M, Jackson DJ, Reitner J, Seyfullah LJ, Schmidt AR. 2014. Estimating the Phanerozoic history of the Ascomycota lineages: combining fossil and molecular data. Molecular Phylogenetics and Evolution **78:** 386-398.

Bidartondo MI, Bruns TD, Weiss M, Sergio S, Read DJ. 2003. Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. Proceedings of the Royal Society B-Biological Sciences **270:** 835-842.

Bidartondo MI, Duckett JG. 2010. Conservative ecological and evolutionary patterns in liverwort-fungal symbioses. Proceedings of the Royal Society B-Biological Sciences **277**: 485-492.

Bidartondo MI, Read DJ, Trappe JM, Merckx V, Ligrone R, Duckett JG. 2011. The dawn of symbiosis between plants and fungi. Biology Letters **7**: 574-577.

Bolan NS. 1991. A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant and Soil **134**: 189-207.

Brundrett MC. 2004. Diversity and classification of mycorrhizal associations. Biological Reviews **79:** 473-495.

Cameron DD, Leake JR, Read DJ. 2006. Mutualistic mycorrhiza in orchids: evidence from plant– fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid Goodyera repens. New Phytologist **171**: 405-416.

Cameron DD, Johnson I, Leake JR, Read DJ. 2007. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid Goodyera repens. Annals of Botany **99**: 831-834.

Cameron DD, Johnson I, Read DJ, Leake JR. 2008. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, Goodyera repens. New Phytologist **180**: 176-184.

Chambers SM, Williams PG, Seppelt RD, Cairney JWG. 1999. Molecular identification of Hymenoscyphus sp. from rhizoids of the leafy liverwort Cephaloziella exiliflora in Australia and Antarctica. Mycological Research **103**: 286-288.

Crandall-Stotler B, Stotler RE, Long, DG. 2009. Phylogeny and classification of the Marchantiophyta. Edinburgh Journal of Botany **66**: 155–198.

Diaz A, Green I, Benvenuto M, Tibbett M. 2006. Are ericoid mycorrhizas a factor in the success of Calluna vulgaris heathland restoration? Restoration Ecology **14**: 187-195.

Duckett JG, Read DJ. 1991. The use of the fluorescent dye, 3,3'-dihexyloxacarbocyanine iodide, for selective staining of ascomycete fungi associated with liverwort rhizoids and ericoid mycorrhizal roots. New Phytologist **118**: 259-272.

Duckett JG, Renzaglia KS & Pell K. **1991**. A light and electron microscope study of rhizoid-ascomycete associations and flagelliform axes in British hepatics: with observations on the effects of the fungus on host morphology. New Phytologist, **118**: 233-257.

Duckett JG, Read DJ. 1995. Ericoid mycorrhizas and rhizoid-ascomycete associations in liverworts share the same mycobiont - isolation of the partners and resynthesis of the associations in-vitro. New Phytologist **129**: 439-477.

Field KJ, Cameron DD, Leake JR, Tille S, Bidartondo MI, Beerling DJ. 2012. Contrasting arbuscular mycorrhizal responses of vascular and non-vascular plants to a simulated Palaeozoic CO_2 decline. Nature Communication **3**: 1-8.

Field KJ, Pressel S, Rimington WR, Duckett JG, Bidartondo MI. 2015a. Symbiotic options for the conquest of land. Trends in Ecology and Evolution **30**: 477-486.

Field KJ, Rimington WR, Bidartondo MI, Allinson KE, Beerling DJ, Cameron DD, Duckett JG, Pressel S. 2015b. First evidence of mutualism between ancient plant lineages (Haplomitriopsida liverworts) and Mucoromycotina fungi and its response to simulated Palaeozoic changes in atmospheric CO_2. New Phytologist **205**: 743-756.

Field, KJ, Leake JR, Tille S, Alllinson KE, Rimington WR, Bidartondo MI, Beerling DJ, Cameron DD. 2015c. From mycoheterotrophy to mutualism: mycorrhizal specificity and functioning in Ophioglossum vulgatum sporophytes. New Phytologist **205**: 1492-1502.

Field KJ, Rimington WR, Bidartondo MI, Allinson KE, Beerling DJ, Cameron DD, Duckett JG, Leake JR, Pressel S. 2016. Functional analysis of liverworts in dual symbiosis with Glomeromycota and Mucoromycotina fungi under a simulated Palaeozoic CO_2 decline. The ISME Journal **10**: 1514-1526.

Heinrichs J, Hentschel J, Wilson R, feldberg K, Schneider H. 2007. Evolution of leafy liverworts (Jungermanniidae, Marchantiophyta): estimates divergence times from chloroplast DNA sequences using penalized likelihood with integrated fossil evidence. Taxon **56:** 31-44.

Hendry GAF, Grime JP. 1993. Methods in Comparative Plant Ecology: A Laboratory Manual. London: Chapman and Hall.

Hill MO, Blackstock TH, Long DG, Rothero GP. 2008. A checklist and census catalogue of British and Irish bryophytes: Cheshire: British Bryological Society.

Humphreys CP, Franks PJ, Rees M, Bidartondo MI, Leake JR, Beerling DJ. 2010. Mutualistic mycorrhiza-like symbiosis in the most ancient group of land plants. Nature Communication **1:** 1-7.

James TY, Kauff F, Schoch CL et al. 2006. Reconstructuing the early evolution of fungi using a six-gene phylogeny. Nature **443:** 818-822.

Kerley SJ, Read DJ. 1997. The biology of mycorrhiza in the Ericaceae .19. Fungal mycelium as a nitrogen source for the ericoid mycorrhizal fungus Hymenoscyphus ericae and its host plants. New Phytologist **136**: 691-701.

Kowal J, Pressel S, Duckett JG, Bidartondo MI. 2015. Liverworts to the rescue: an investigation of their efficacy as mycorrhizal inoculum for vascular plants. Functional Ecology **30**: 1014–1023.

Leake JR, Shaw G, Read DJ. 1990. The Role of Ericoid Mycorrhizas in the Ecology of Ericaceous Plants. Agriculture Ecosystems and Environment **29**: 237-250.

Leake JR. 2015. Box 12.1 Biogeochemical effects of coevolution of plants and fungi. In: Bronstein JL ed. Mutualism. Oxford, UK: Oxford University Press, 2.

Ligrone R, Carafa A, Lumini E, Bianciotto V, Bonfante P, Duckett JG. **2007**. Glomeromycotean associations in liverworts: A molecular cellular and taxonomic analysis. American Journal of Botany **94**: 1756-1777.

Mitchell DT, Read DJ. 1981. Utilization of organic and inorganic phosphates by the mycorrhizal endophytes of Vaccinium macrocarpon and Rhododendron ponticum. Transactions of the British Mycological Society **76**: 255-260.

Myers MD, Leake JR. 1996. Phosphodiesters as mycorrhizal P sources II. Ericoid mycorrhiza and the utihzation of nuclei as a phosphorus and nitrogen source by Vaccinium macrocarpon. New Phytologist **132**: 445-451.

Perotto S, Martino E, Abba S, Vallino M. 2012. Genetic diversity and functional aspects of ericoid mycorrhizal fungi. In: Hock B ed. Fungal associations, 2nd Edition. Berlin: Springer-Verlag.

Pearson V, Read DJ. 1973. The biology of mycorrhiza in the Ericaceae. New Phytologist **72**: 1325-1331.

Pietro M, Wedin M. 2013. Dating the diversification of the major lineages of Ascomycota (Fungi). PLOSone **8:** e65576.

Pirozynski KA, Malloch DW. 1973. The origin of land plants: a matter of mycotropism. Biosystems **6,** 153–164.

The Plant List. 2013. Version 1.1. Published on the Internet; http://www.theplantlist.org/ (accessed 1st January).

Pocock K, Duckett JG, Grolle R, Mohamed MAH, Pang WC. 1984. Branched and swollen rhizoids in hepatics from montane rain forest in Peninsular Malaya. Journal of Bryology **13**: 241-246.

Pocock K, & Duckett JG. 1985. On their occurrence of branched and swollen rhizoids in British hepatics: their relationships with the substratum and associations with fungi. New Phytologist **99**: 281-304.

Pressel S, Ligrone R, Duckett JD, Davis, EC. 2008. A novel ascomycetous endophytic association in the rhizoids of the leafy liverwort family, Schistochilaceae (Jungermanniidae, Hepaticopsida). Americal Journal of Botany **95:** 531-541.

Pressel S, Bidartondo MI, Ligrone R, Duckett JG. 2010. Fungal symbioses in bryophytes: New insights in the Twenty First Century. Phytotaxa **9**: 238-253.

Pressel S, Bidartondo MI, Field KJ, Rimington WR, Duckett JG. 2016. Pteridophyte fungal associations: Current knowledge and future perspectives. Journal of Systematics and Evolution **54**: 666-678.

Rasband WS 1997-2012. ImageJ. Maryland, USA: U. S. National Institutes of Health.

Read DJ. 1993. Mycorrhiza in plant communities. Advances in Plant Pathology **9**: 1-31.

Read DJ, Stribley DP. 1973. Effect of mycorrhizal infection on nitrogen and phosphorus nutrition of ericaceous plants. Nature New Biology **244**: 81-82.

Read DJ, Duckett JG, Francis R, Ligrone R, Russell AJ. 2000. Symbiotic fungal associations in 'lower' land plants. Philosophical Transactions of the Royal Society B **355**: 815-830.

Read DJ, Leake JR, Perez-Moreno J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Canadian Journal of Botany **82**: 1243-1263.

Remy W, Taylor TN, Hass H, Kerp H. 1994. Four hundred million year old vesicular arbuscular mycorrhizae. Proceedings of the National Academy of Sciences, USA **91:** 11841–11843.

Selosse M-A. 2005. Are liverworts initiating mycorrhizas? New Phytologist **165:** 345-349.

Smith SE, Read DJ. 2008. Mycorrhizal symbiosis. Cambridge, UK: Academic Press.

Stribley, DP, Read, DJ. 1974. Biology of Mycorrhiza in Ericaceae IV. Effect of mycorrhizal infection on uptake of N-15 from labelled soil by Vaccinium macrocarpon Ait. New Phytologist, **73,** 1149-1155.

Stribley DP, Read DJ. 1980. The biology of mycorrhiza in the Ericaceae VII. The relationship between mycorrhizal infection and the capacity to utilize simple and complex organic nitrogen-sources. New Phytologist **86**: 365-371.

Stevens PF. 2001. Angiosperm Phylogeny Website. Version 12, July 2012 [and more or less continuously updated since]. [WWW document] URL http://www.mobot.org/MOBOT/research/APweb/. [accessed 1 November 2016].

Upson R, Read DJ, Newsham KK. 2007. Widespread association between the ericoid mycorrhizal fungus Rhizoscyphus ericae and a leafy liverwort in the maritime and sub-Antarctic. New Phytologist **176**: 460-471.

Walder F, van der Heijden MGA. 2015. Regulation of resource exchange in the arbuscular mycorrhizal symbiosis. Nature Plants **1:** 15159.

Wang B, Yeun LH, Xue JY, Liu Y, Ane JM, Qiu YL. 2010. Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. New Phytologist **186**: 514-525.

Figures

Fig. 1 (a) Liverwort phylogram showing diversity in liverwort-fungal associations (after Crandall-Stotler et al., 2009) based on extensive sampling of nearly all the fungus-containing liverwort genera worldwide (Ligrone et al. 2007). (b) Cephalozia bicuspidata rhizoid resynthesized with Pezoloma ericae fungus (arrowed) in axenic culture.

Fig. 2 (a) Concentration of fungal-acquired ^{33}P in Cephalozia bicuspidata tissue (ng g^{-1}). (b) Total plant-derived carbon present in extraradical fungal mycelium after a five-hour labeling period (ng). Error bars denote 1 s.e. in both panels.

Fig. 3 (a) Change in surface area of leafy liverwort after six weeks growth with and without Pezoloma ericae fungal symbiont (n = 16, 22), $P < 0.01$** (using t-test). (b) Total biomass of liverworts (dry weight; g) after six months of growth with and without P. ericae (Mann-Whitney U = 2; P = 0.0087**; $n_{1,2} = 6$ two-tailed). Error bars denote 1 s.e. in both panels.

Supplementary Figures and Data

Fig. S1 Diagrams and photographs of ^{33}P transfer experiment showing split plate experimental design set up before (a,b) and after (c,d) the fungus crossed the barrier. L – liverwort; H – hyphae; W – well and B – barrier.

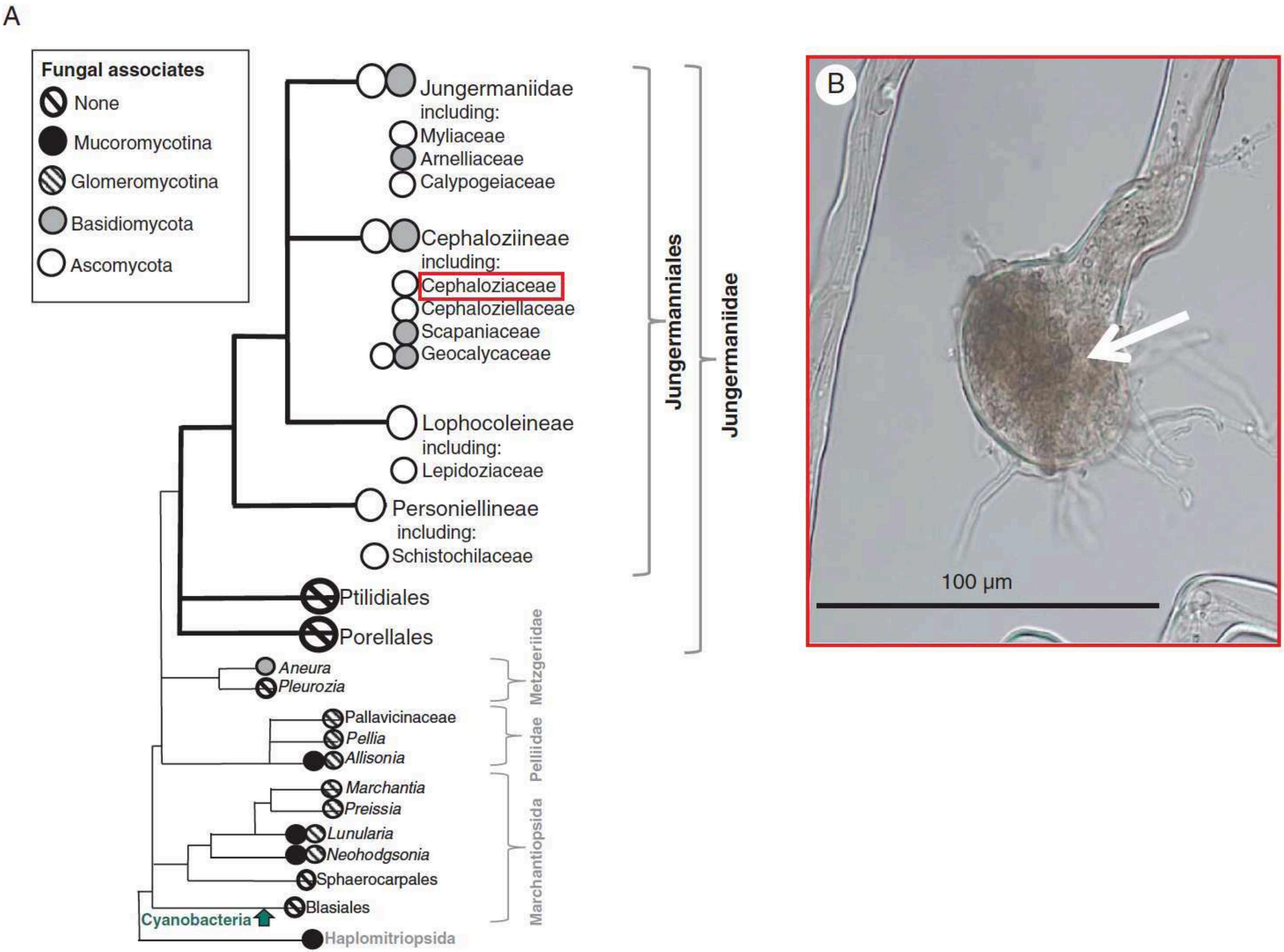
Fig. S2 Diagram showing microcosm design for carbon transfer experiments. (a) Cephalozia bicuspidata and Pezoloma ericae growing on 1.5% Phytagel (b) $^{14}CO_2$ released into chamber headspace, fixed by plant then transferred into fungal hyphae (blue). Dotted black line shows placement of polythene with lanolin seal in yellow. Polythene barrier minimizes $^{14}CO_2$ diffusion into Phytagel.

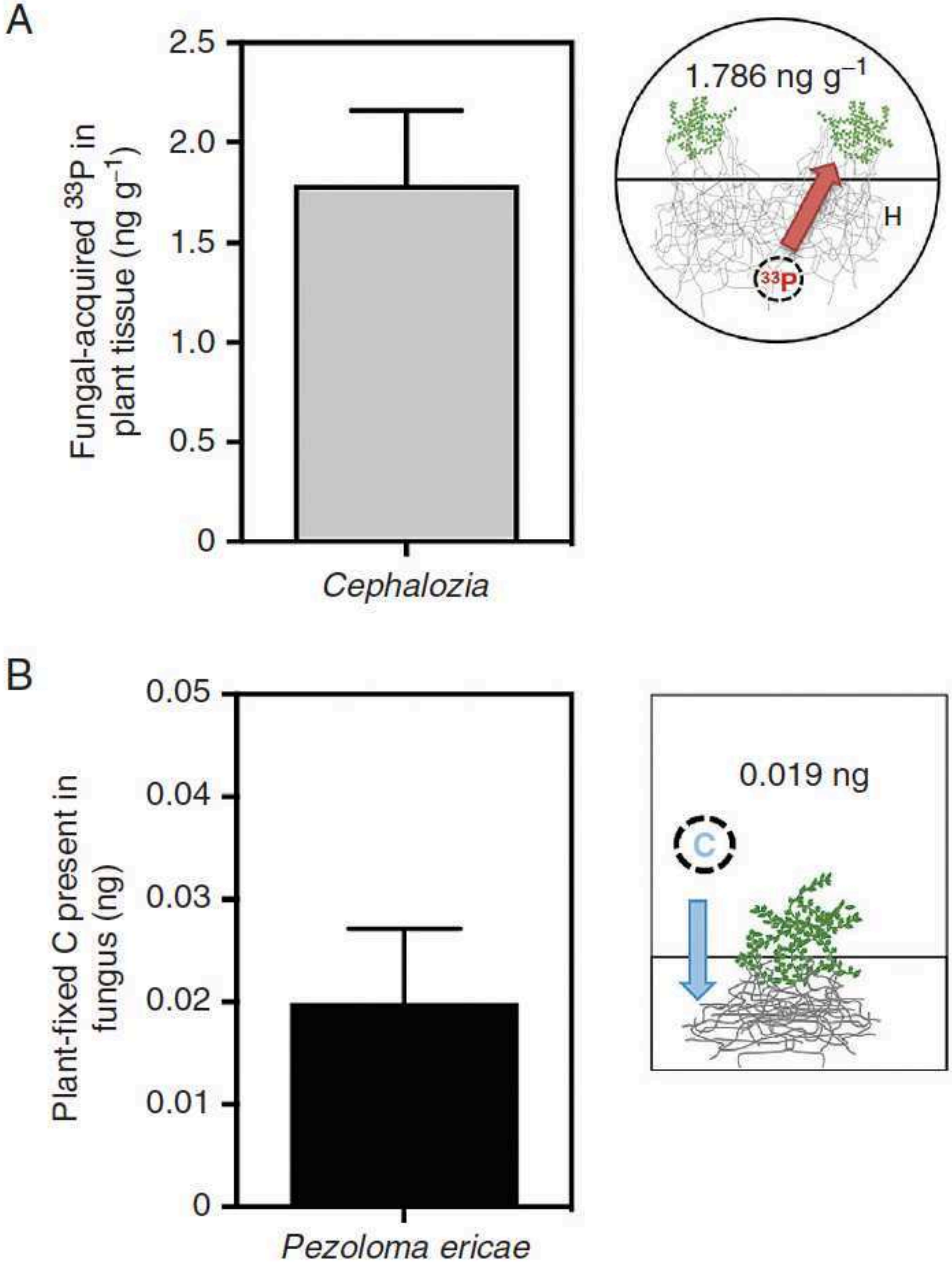
Fig. S3 Example of digitized pictures used to measure perimeter area of liverworts. (a) Control without fungus (b) Inoculated with fungus (arrow).

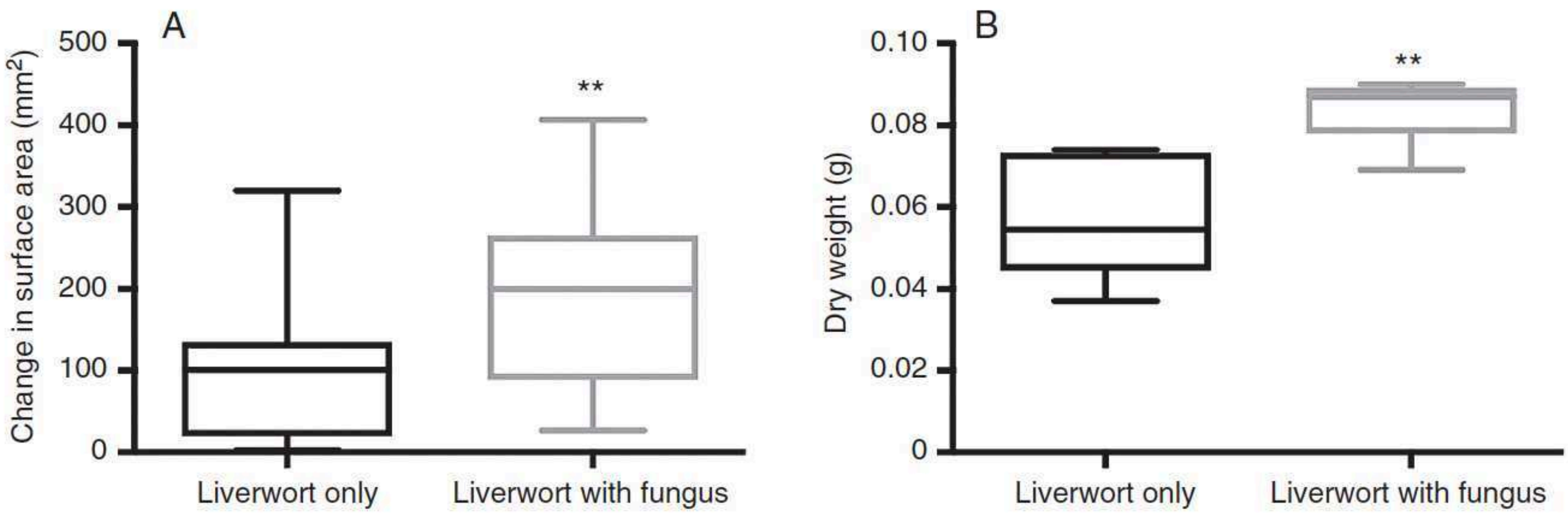
Methods S1 Methods for harvesting plants and fungus for ^{33}P acid digestion, liquid scintillation and nutrient budgeting.

Table S1 Summary of carbon-for-nutrient exchange between *Cephalozia bicuspidata* and *Pezoloma ericae*, alongside data from previous studies of early-diverging liverwort lineages and their fungal partners (below dotted line) (Field *et al.*, 2015a).

Table S2 Estimates of likely species numbers of liverworts worldwide with fungal symbionts.


A
Fungal associates
None
Mucoromycotina
Glomeromycotina
Basidiomycota
Ascomycota
Jungermaniidae
including:
Myliaceae
Arnelliaceae
Calypogeiaceae
Cephaloziineae
including:
Cephaloziaceae
Cephaloziellaceae
Scapaniaceae
Geocalycaceae
Lophocoleineae
including:
Lepidoziaceae
Personiellineae
including:
Schistochilaceae
Ptilidiales
Porellales
Aneura
Pleurozia
Pallavicinaceae
Pellia
Allisonia
Marchantia
Preissia
Lunularia
Neohodgsonia
Sphaerocarpales
Blasiales
Cyanobacteria
Haplomitriopsida
Jungermanniales
Jungermaniidae
Metzgeriidae
Pelliidae
Marchantiopsida
B
100 µm


A
2.5
2.0
1.5
1.0
0.5
0
Fungal-acquired 33P in plant tissue (ng g−1)
Cephalozia
1.786 ng g−1
H
33P
B
0.05
0.04
0.03
0.02
0.01
0
Plant-fixed C present in fungus (ng)
Pezoloma ericae
0.019 ng
C


A
Change in surface area (mm2)
500
400
300
200
100
0
**
Liverwort only
Liverwort with fungus
B
Dry weight (g)
0.10
0.08
0.06
0.04
0.02
0
**
Liverwort only
Liverwort with fungus