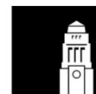

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1 **RESEARCH IN CONTEXT**

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

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1 **Abstract**

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

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

16 Thus, this ascomycete-liverwort symbiosis can now be described as
17 mycorrhiza-like, providing further insights into ericoid mycorrhizal evolution and
18 adding Ascomycota fungi to mycorrhizal fungal groups engaging in mutualisms with
19 plants across the land plant phylogeny. As P. ericae also colonizes the rhizoids of
20 Schistochilaceae liverworts, which originated in the Triassic and are sister to all other
21 jungermannialean liverworts associated with fungi, our findings point toward an early
22 origin of ascomycete-liverwort symbioses, possibly predating their evolution in the
23 Ericales by some 150 MY.

24

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

3

4 **Introduction**

5 Mycorrhizas are intimate symbioses formed between plant roots and soil fungi
6 that are prevalent across the globe in more than 80% of extant land plants (Smith and
7 Read, 2008). Through mycorrhizal associations, many plants engage in bi-directional
8 exchange of photosynthesis-derived plant carbon and fungal-acquired nutrients,
9 scavenged from sources beyond the root depletion zone or from soil pores too small
10 for roots to access (Smith and Read, 2008; Leake and Read, 2016). The role of
11 mycorrhizas in supplying extant land plants with nutrients, together with evidence of
12 mycorrhiza-like associations in Rhynie Chert plant fossils (Remy et al., 1994) has led
13 to the hypothesis that mycorrhizal fungi likely facilitated the evolution of land plants
14 >470 Mya (Pirozynski et al., 1975; Bidartondo et al., 2011; Leake, 2015).
15 Additionally, recent studies have shown that the genes required for mycorrhization
16 are conserved across all land plant lineages (Wang et al., 2010), including the earliest
17 diverging clade of liverworts – Haplomitriopsida (Fig. 1a, after Crandall-Stotler et
18 al., 2009). The later-derived leafy liverworts (Jungermanniidae) have not been
19 incorporated in such analyses and a critical caveat of molecular studies is that the
20 presence of genes does not necessarily imply functional significance. Indeed, for
21 many groups of extant plants that form mycorrhizas and mycorrhiza-like associations
22 where roots are absent, knowledge regarding the physiological function of the
23 symbiosis has been severely limited (Read et al., 2000, Field et al., 2015a). To date,
24 amongst fungus-associated early-branching land plants (i.e. liverworts, hornworts and
25 lycophytes) nutritional mutualisms have only been demonstrated in a handful of early

1 diverging thalloid and Haplomitriopsida liverworts (Field et al., 2012; 2015a; 2016),

2 (Fig. 1a), all which form symbioses with Mucoromycota fungi (Glomeromycotina

3 and/or Mucoromycotina) (Field et al. 2015b).

4 As far as can be established from published surveys and incidental

5 illustrations in floras (Pocock et al.,1984; Pocock & Duckett, 1985; Duckett et

6 al.,1991, Ligrone et al. 2007) rhizoidal ascomycete associations occur in far more

7 liverwort species than those containing basidiomycetes, glomeromycetes and

8 mucoromycetes (Pressel et al., 2010). Following the accepted species names in the

9 Plant List (2013), we estimate that up to 1,000 late-diverging leafy liverworts are

10 likely to have ascomycete fungal symbionts, i.e. around 20% of liverwort species

11 worldwide compared to less than 100 with basidiomycetes, glomeromycetes and

12 mucoromycetes (see Table S2 for detailed breakdown). Reinforcing their symbiotic

13 rather than opportunistic status, the ascomycetes 1) induce swelling, branching and

14 septation of the rhizoids; and, 2) are ubiquitous rather than sporadic, in species where

15 they occur. In addition, transmission electron micrographs show healthy hyphae in

16 healthy host cells (Duckett et al.,1991; Pressel et al., 2010).

17 There are several families in the leafy liverworts (Jungermanniideae),

18 including Schistochilaceae, Lepidoziaceae, Calypogeiaceae, Cephaloziaceae and

19 Cephaloziellaceae, which consistently associate with Ascomycota fungi (Fig. 1a)

20 (Pressel et al., 2010). These fungal symbionts include Pezoloma ericae (D.J. Read)

21 Baral (syn. Rhizoscyphus ericae (D.J. Read) W.Y. Zhuang and Korf, 2004;

22 Hymenoscyphus ericae (D.J. Read) Korf and Kernan, 1983; and Pezizella ericae (D.J.

23 Read, 1974) (Duckett and Read, 1995; Read et al., 2000; Pressel et al., 2010)).

24 Notably, Pezoloma ericae is known to form ericoid mycorrhizas (ErM) with the roots

25 of Ericaceae plants and has previously been shown to provide nutrients to their

1 vascular plant hosts in exchange for fixed carbon (Read et al., 2003; Smith and Read,
2 2008).
3 Ericaceous habitats are typically low in plant-available soil nutrients,
4 including N and/or P (Stribley and Read, 1974; Mitchell and Read, 1981; Leake et al.
5 1990; Bolan, 1991; Myers and Leake, 1996). The vascular plants inhabiting these
6 habitats, such as Calluna, Erica, Rhododendron and Vaccinium, grow together with
7 non-vascular plants, including the widespread leafy liverworts in the Cephaloziaceae
8 (Chambers et al., 1999; Upson et al., 2007) with which they 'share' fungal symbionts
9 (Duckett and Read, 1995; Read et al., 2000). It was recently shown that that this
10 shared mycobiont can bring benefits in terms of establishment and survival to
11 ericaceous plants (Kowal et al., 2015), and that the P. ericae-colonized liverwort may
12 serve as a source of fungal inoculum for vascular plants. It is possible that this effect
13 is driven by fungal-enhanced nutrition in liverworts and then in the vascular plant
14 species sharing the fungal symbiont, analogous to the nutritional role of arbuscular
15 mycorrhizal fungi (Glomeromycotina fungi) that associate with some thalloid
16 liverworts (Field et al., 2012). It may be that P. ericae associates in leafy liverworts
17 play a similar role to the Glomeromycotina or Mucoromycotina fungal partners of
18 thalloid liverworts by supplementing plant phosphorus (P) assimilation (Field et al.,
19 2016).
20 Here, we aim to address the fundamental question of whether the ascomycete
21 fungus P. ericae forms mycorrhiza-like associations with leafy liverworts equivalent
22 to those formed by Glomeromycotina or Mucoromycotina fungi and thalloid
23 liverworts. We traced the movement of P from P. ericae fungal hyphae to Cephalozia
24 bicuspidata liverworts and the movement of carbon (C) from liverworts to the fungi
25 using isotope tracers. We determined liverwort growth responses to colonization by

1 P. ericae fungi by measuring the size and mass of liverworts, grown both with and
2 without P. ericae fungal symbionts.
3

4 **Materials and Methods**

5 We collected Cephalozia bicuspidata from Thursley Common, Surrey, in
6 autumn 2012 (OS grid reference SU900416). Mature sporophytes were harvested,
7 surface-sterilized and spores were cultured axenically (Duckett and Read, 1995) on
8 1.5% Phytagel^{TM} (Sigma-Aldrich; ICP elemental analysis of Phytagel provided by
9 Sigma-Aldrich: 0.85% Ca, 0.35% Mg; 1.70% K, 0.15% P and 0.45% Na). No
10 additional nutrients were added to the culture medium. Pezoloma ericae was isolated
11 from the same liverwort collection and resynthesized with axenically-grown C.
12 bicuspidata using published methods (Kowal et al., 2015), thus satisfying Koch's
13 postulates. Molecular identification of the fungal isolate as P. ericae was carried out
14 previously (Kowal et al., 2015). Nomenclature for plants and fungi follows Hill et al.
15 (2008) and www.speciesfungorum.org, respectively.
16

17 Fungus-to-plant phosphorus transfer

18 We grew C. bicuspidata and P. ericae together in one compartment
19 ('liverwort and fungus') of 9 cm split-plate microcosms (Fig. S1) filled on both sides
20 of the divide ('B' in Fig. S1a) with 1.5% sterile Phytagel. Fragments of P. ericae
21 isolate (approximately 7 mm^2) were inserted beneath the surface of the Phytagel and
22 an axenically-grown leafy liverwort stem (two per microcosm, c. 2 cm apart) was
23 gently pressed onto the surface, directly above the fungus fragment. Following
24 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

1 containing 1 mL 2 M KOH into each microcosm for 30 min to trap any remaining

2 $^{14}CO_2$ from the headspace of each microcosm. We then harvested, separated and

3 freeze-dried the liverworts and Phytagel containing fungal hyphae (see Methods S1)

4 before weighing, homogenizing and determining ^{14}C content by sample oxidation

5 (Packard Sample Oxidiser) and liquid scintillation counting (Packard Tri-Carb 3100).

6 We calculated total carbon (^{12}C plus ^{14}C) fixed by the plant and transferred to

7 the fungus as a function of the total volume and CO_2 content of the vessel headspace

8 and the proportion of supplied $^{14}CO_2$ label fixed by the plants, using equations from

9 Cameron *et al.* (2008). The specific activity of the source was 2.04 TBq Mol $^{-1}$.

10 To account for carbon movement through passive diffusion, and to assess the

11 effectiveness of the polythene/lanolin barrier, total plant-fixed fungal carbon in the

12 complete microcosms was determined by subtracting the mean carbon measured in

13 the fungus from the fungus-only control microcosms from each of the complete

14 microcosms.

15

16 Liverwort growth

17 We measured C. bicuspidata growth both with and without fungal inoculation

18 looking first at changes in leafy liverwort surface area, and second at liverwort

19 biomass. In the first experiment, we introduced small fragments (c. 7 mm^2) of P.

20 ericae to 9 cm plates containing 1.5% Phytagel with axenically-grown leafy

21 liverworts (c. one year old). After fungal colonization of the liverwort rhizoids was

22 confirmed microscopically (Fig. 1b), we re-plated colonized liverworts and fungus-

23 free control liverworts individually using the same medium. Surface area

24 measurements of each liverwort stem (including leaves) were made before and after

1 six weeks using images taken with a Nikon Coolpix S10 digital camera and analyzed
2 using ImageJ software (Rasband, 1997-2012) (Fig. S3). For the second measurement
3 of the effect of P. ericae on C. bicuspidata growth, we freeze-dried and weighed the
4 liverworts from the complete microcosms used for the ^{14}C transfer experiment
5 (above) after they were harvested at six months and compared the mean to that of
6 plants from liverwort-only microcosms (n = 6). This allowed us to test for
7 differences in mean plant biomass in liverwort growth with or without fungus over a
8 longer time period than the surface area growth experiment described above.
9 Liverwort stems were weighed before initial planting to ensure similarity in mass
10 between sample groups.

11

12 Statistics

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

18

19 **Results and discussion**

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

21 In our radio-labeled liverwort microcosms, 1.786 ng g^{-1} of ^{33}P was
22 assimilated from P. ericae into C. bicuspidata tissues (Fig. 2a, Table S1). There was
23 a significant difference in the liverwort ^{33}P content and concentration between plant-
24 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).

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

12

13 Evolutionary context

14 Our demonstration that the association between a leafy liverwort and the
15 ericoid mycorrhizal fungus P. ericae can be reciprocally beneficial provides
16 additional clues about the evolution of ErM, and lends further weight to the idea that
17 mutualisms between leafy liverworts and ascomycete fungi pre-dated their formation
18 in the Ericales, estimated at less than 100 MYA (Brundrett, 2004). Following their
19 discovery that ascomycetes belonging to the P. ericae group colonize the rhizoids of
20 Schistochilaceae liverworts, thought to have originated in the Triassic (Heinrichs et
21 al., 2007) as the sister group to all other fungus-containing lineages in the
22 Jungermanniales, Pressel et al. (2008) suggested that this association arose more than
23 250 MYA. Nonetheless, divergence time estimates for ascomycete lineages remain a
24 matter of debate particularly as the presumed recent origin of Leotiomycetes, the
25 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

1 Ophioglossum vulgatum and their Glomeromycotina symbionts (Field et al., 2012,
2 2015c; Pressel et al., 2016).

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

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

1

2

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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

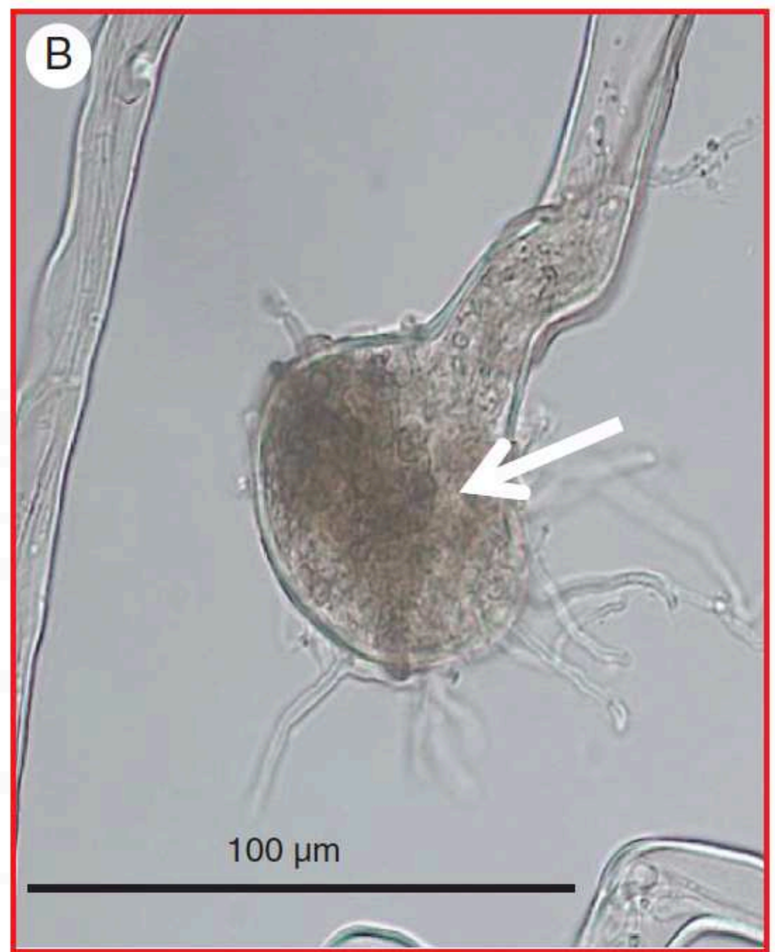
fungal hyphae (blue). Dotted black line shows placement of polythene with lanolin seal in yellow. Polythene barrier minimizes $^{14}CO_2$ diffusion into Phytagel.

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.

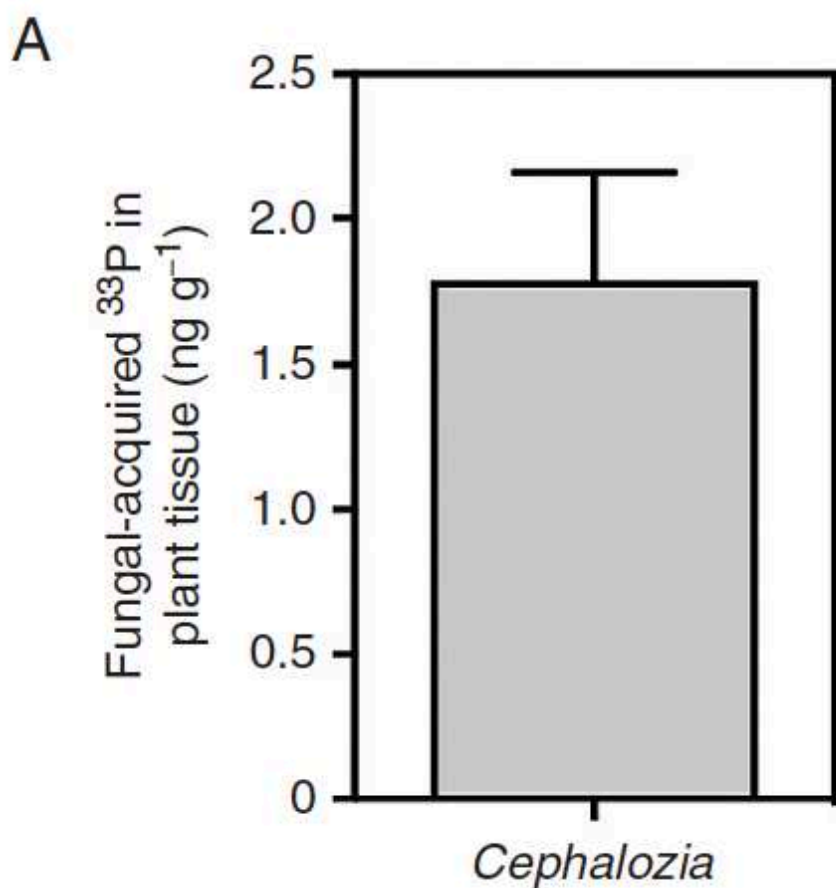
Table S1 Summary of carbon-for-nutrient exchange between *Cephalozia bicuspidata* and *Pezoloma ericae*, alongside data from previous studies of early-diverging liverwort lineages and their fungal partners (below dotted line) (Field *et al.*, 2015a).

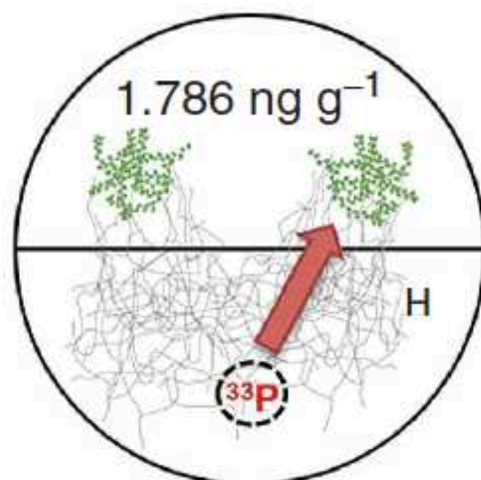
Table S2 Estimates of likely species numbers of liverworts worldwide with fungal symbionts.



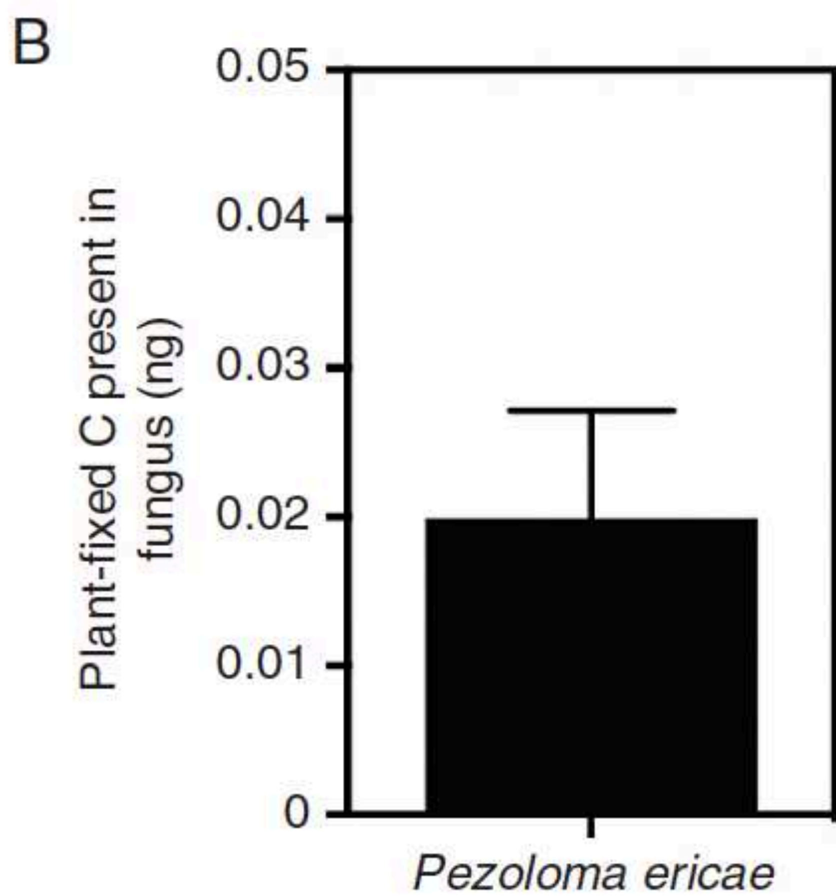
A
Fungal associates
None
Mucoromycotina
Glomeromycotina
Basidiomycota
Ascomycota
Jungermaniidae
including:
Myliaceae
Arnelliaceae
Calypogeiaceae
Cephaloziineae
including:
Cephaloziaceae
Cephaloziellaceae
Scapaniaceae
Geocalycaceae
Lophocoleineae
including:
Lepidoziaceae
Personiellineae
including:
Schistochilaceae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

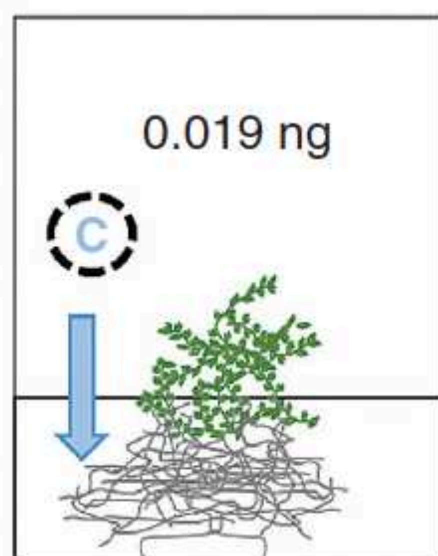
A

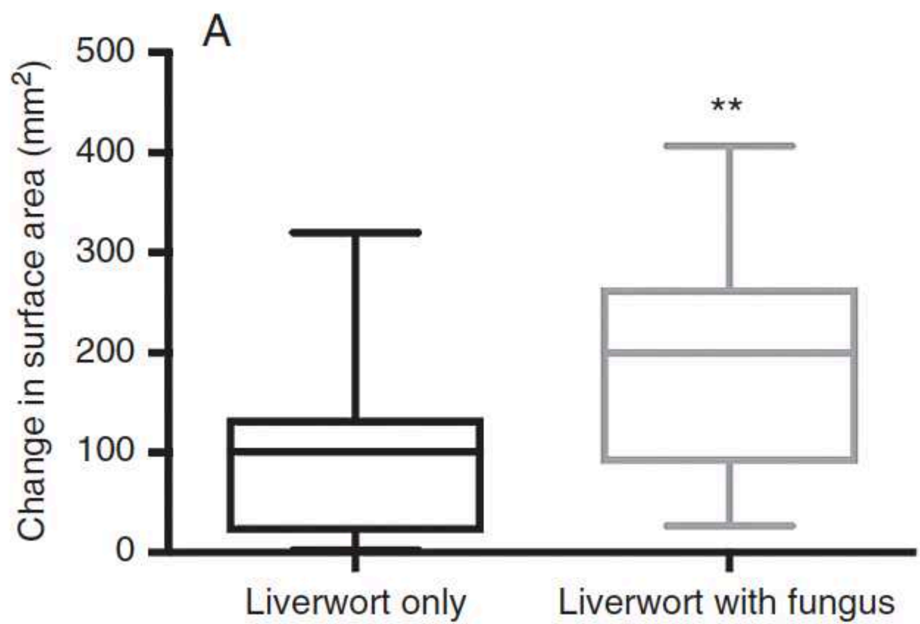


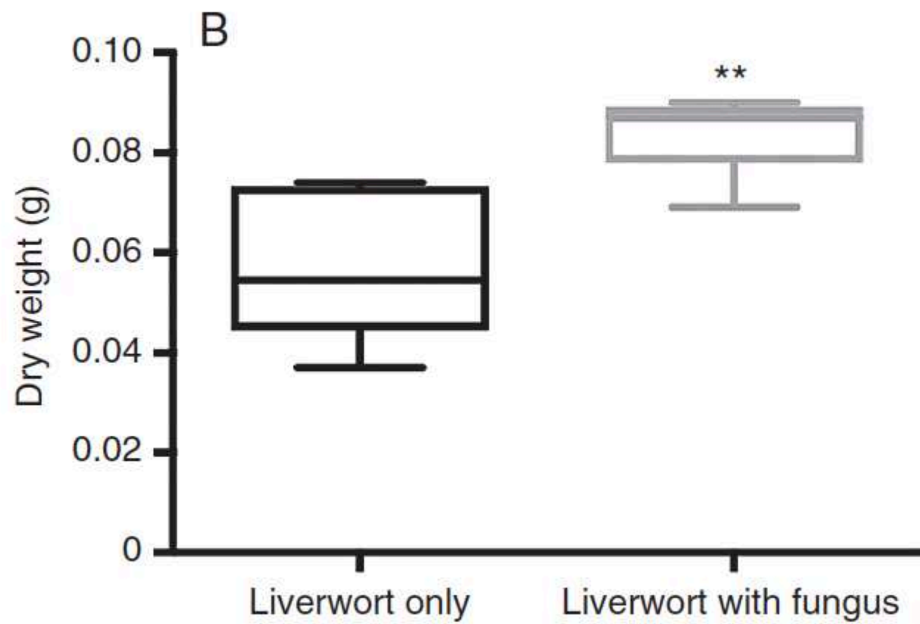


B






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