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Functional complementarity of ancient plant-fungal mutualisms: contrasting nitrogen, phosphorus and carbon exchanges between Mucoromycotina and Glomeromycotina fungal symbionts of liverworts

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Summary

- Liverworts, which are amongst the earliest-divergent plant lineages and important ecosystem pioneers, often form nutritional mutualisms with arbuscular mycorrhiza-forming Glomeromycotina and fine root endophyte Mucoromycotina fungi, both of which co-evolved with early land plants. Some liverworts, in common with many later-divergent plants, harbour both fungal groups, suggesting these fungi may complementarily improve plant access to different soil nutrients.

- We tested this hypothesis by growing liverworts in single and dual fungal partnerships under a modern atmosphere and under 1500 ppm [CO_2], as experienced by early land plants. Access to soil nutrients via fungal partners was investigated with ^{15}N-labelled algal necromass and ^{33}P orthophosphate. Photosynthate allocation to fungi was traced using $^{14}CO_2$.

- Only Mucoromycotina fungal partners provided liverworts with substantial access to algal ^{15}N, irrespective of atmospheric CO_2 concentration. Both symbionts increased ^{33}P uptake, but Glomeromycotina were often more effective. Dual partnerships showed complementarity of nutrient pool use and greatest photosynthate allocation to symbiotic fungi.

- We show there are important functional differences between the plant-fungal symbioses tested, providing new insights into the functional biology of Glomeromycotina and Mucoromycotina fungal groups that form symbioses with plants. This may explain the persistence of both fungal lineages in symbioses across the evolution of land plants.

Key words:

Symbiosis, arbuscular mycorrhiza, fine root endophyte, carbon-for-nutrient exchange, nitrogen, orthophosphate, CO_2, liverworts

Introduction

Mycorrhizal associations (and mycorrhizal-like associations in plants that lack roots, henceforth also referred to as mycorrhizas) are near-ubiquitous mutualisms between soil fungi and most land plant phyla (Wang & Qiu, 2006; Wang *et al.,* 2010) with key roles in plant nutrition and health (Smith & Read, 2008). The earliest-divergent extant branches of the land plant phylogeny supporting fungal symbionts, i.e. liverworts (Haplomitriopsida and thalloids), hornworts and lycophytes (Bidartondo *et al.,* 2011; Desirò *et al.,* 2013; Rimington *et al.,* 2015; Field *et al.,* 2015a), have recently been shown to form symbioses with members of two ancient fungal lineages, arbuscular mycorrhizal (AM) fungi falling within the Glomeromycotina, and the much less studied, and poorly understood, symbiotic fungi of the Mucoromycotina.

These findings, together with fossil evidence of Mucoromycotina-like and Glomeromycotina-like fungal associations in some of the first vascular plants of the early Devonian (Strullu-Derrien *et al.,* 2014), have led to the novel hypothesis that engagement with both fungal partners might have been a common strategy during

early land plant evolution (Field *et al.,* 2015a). Furthermore, new molecular evidence reveals the same Mucoromycotina fungal symbionts are shared by liverworts, early-divergent vascular plants and angiosperms (Hoysted *et al.,* 2019). This is consistent with the recent finding that the globally widespread fine root endophytes (FRE), originally classified as *Glomus tenue* (more recently *Planticonsortium tenue* (Walker *et al.,* 2018)*,* are not members of the Glomeromycotina as hitherto assumed, but instead fall within the Mucoromycotina (Orchard *et al.,* 2017a; 2017b). In angiosperms Mucoromycotina-FRE, when present, seem to always co-occur with Glomeromycotina in the same host (Orchard *et al.,* 2017a; 2017b; Hoysted *et al.,* 2019), while exclusive Mucoromycotina-FRE symbioses have been reported so far in only one lycophyte (Rimington *et al.,* 2015; 2016; Pressel *et al.,* 2016) and in the earliest-divergent Haplomitriopsida liverworts *Haplomitrium* and *Treubia*. The majority of early-divergent mycorrhizal plant clades, lycophytes, hornworts and (thalloid) liverworts (e.g. the complex thalloid *Neohodgsonia mirabilis*) engage in partnerships with both fungi, sometimes simultaneously in so-called 'dual' symbiosis (Desirò *et al.,* 2013; Field *et al.,* 2015a; 2016; Rimington *et al.,* 2015; 2016; Pressel *et al.,* 2016), paralleling the situation recently established for flowering plants. Only a few complex thalloid liverworts, such as species in the genus *Marchantia* appear to associate exclusively with Glomeromycotina fungi (Field *et al.,* 2015a; Rimington *et al.,* 2018). Taken together, these discoveries suggest that the ability of plants to form symbioses with both Mucoromycotina and Glomeromycotina has persisted from the ancient past (Strullu-Derrien *et al.,* 2014; Field *et al.,* 2015a; 2015b; Field & Pressel, 2018) to the present day, being now common in both natural and agricultural ecosystems (Orchard *et al.,* 2017a; 2017b). The apparent persistence of symbiosis with both fungal groups

through the 500 my-long evolutionary history of land plants (Morris *et al.,* 2018) and the widespread occurrence of them simultaneously in diverse plant lineages today suggests the ability to form associations with both Glomeromycotina and Mucoromycotina fungi likely provides functional advantages over associations with only one or other of these fungal groups. However, to date there is limited evidence to test this hypothesis.

Studies on liverwort-Glomeromycotina (Humphreys *et al.,* 2010), liverwort-Mucoromycotina (Field *et al.,* 2015a; 2015b) and dual symbioses with both fungal groups (Field *et al.,* 2016) have confirmed that all three partnerships are mycorrhiza-like mutualisms with the fungi receiving plant photosynthate in exchange for mineral nutrients they have acquired from soil. Significant functional differences between these three types of symbiotic partnership were revealed in response to experimental simulations of the large changes in atmospheric CO_2 concentrations experienced during the long co-evolutionary history of plants and mycorrhizal fungi. Liverworts associating only with Glomeromycotina fungi gained considerably more ^{33}P orthophosphate tracer via their fungal partners per unit of photosynthate C received at CO_2 concentrations simulating the atmosphere of the early-mid Paleozoic under which land plants first evolved (1,500 ppm a[CO_2]; Berner 2006; Lenton *et al.,* 2018) than under modern-day a[CO_2] of 440 ppm (Field *et al.,* 2016). In contrast, Mucoromycotina fungal symbionts, maintained or increased ^{33}P transfer to their hosts under the modern a[CO_2] compared to the simulated Paleozoic a[CO_2]. These liverwort P gains under modern a[CO_2] were further enhanced by the co-occurrence of Glomeromycotina fungi in dual symbiosis but at a greater carbon cost (Field *et al.,* 2016).

Whilst the importance of Glomeromycotina AM symbioses in plant P nutrition is well established (Smith *et al.,* 2015), their role in host plant N nutrition is much less clear (Hodge & Fitter, 2010; Bücking & Kafle, 2015; Hodge & Storer, 2015; Thirkell *et al.,* 2016). This ambiguity has increased with the recent evidence of widespread dual symbioses between FRE and Glomeromycotina fungi (Rimington *et al.,* 2015, Orchard *et al.,* 2017a). Nutritional effects previously ascribed to Glomeromycotina fungi may have been due to undetected Mucoromycotina as most primers used to characterize DNA from arbuscular mycorrhizal symbionts do not amplify Mucoromycotina DNA (Bidartondo *et al*., 2011). This major uncertainty increases the urgency of the need to resolve whether Mucoromycotina and Glomeromycotina fungi are functionally distinct with respect to their provisioning of host plants of both P and N, both of which are major macronutrients that are often plant growth limiting.

Mucoromycotina symbionts may be facultative saprotrophs, i.e. able to access and assimilate nutrients from organic matter for use in metabolism in a similar way to free-living saprotrophs (Lindhal & Tunlid, 2015), as it has sometimes been possible to achieve *in vitro* isolation and axenic culturing of these fungi (Field *et al.,* 2015b). In contrast, Glomeromycotina fungi are strict obligate biotrophs, entirely reliant on symbiosis with a living plant for organic carbon and thus considered unable to assimilate nutrients by saprotrophic extracellular digestion of organic matter (Smith & Read, 2008); it is possible therefore that the Mucoromycotina may be better able to access organic N than the Glomeromycotina. If this were the case, then we might expect there to be differences in the ability of liverworts associated with the two groups of fungi, either singly or in dual symbiosis, to utilise soil nutrients with dual

symbioses providing complementarity through increasing access both to inorganic P and to organic N compared to associations with only one type of fungus.

We set out to test this hypothesis by growing wild-collected liverworts, that naturally support single (*Haplomitrium gibbsiae* -Mucoromycotina; *Marchantia paleacea* -Glomeromycotina) or dual fungal (*Neohodgsonia mirabilis* – Mucoromycotina and Glomeromycotina) partnerships, in experimental microcosms on non-sterile soil containing native mycorrhizosphere microbial communities. Access to soil nutrients via the fungal partners was investigated with phosphorus provided as ^{33}P orthophosphate and ^{15}N in labelled necromass of the unicellular green alga *Chlorella* (Trebouxiophyceae). We selected *Chlorella* as a substrate that is likely to have co-existed with land plants from when they first transitioned from aquatic to terrestrial habitats (Edwards *et al.,* 2015). Both N and P sources were added to rhizoid-excluding soil compartments that could be accessed by fungal hyphae growing from their plant partners.

We conducted these experiments under both a modern 440 ppm a[CO_2], and the 1500 ppm a[CO_2] experienced by early land plants from the Ordovician/early Devonian periods as indicated by geochemical carbon cycle models (Berner, 2006; Lenton *et al.,* 2018). This allowed us to test the second hypothesis that mycorrhizal functioning in terms of fungal nutrient uptake from soil, and liverwort photosynthate allocation to external mycelium of fungal symbionts, is influenced by the changes in a[CO_2] that have occurred through Palaeozoic (Berner, 2006), as we have shown previously in relation to P assimilation (Field *et al.,* 2012; 2015b).

Materials and Methods

Plant material and growth conditions

Haplomitrium gibbsiae (Steph.) R.M. Schust. (known to exclusively associate with Mucoromycotina fungal symbionts (Bidartondo et al., 2011) and *Neohodgsonia mirabilis* Perss. (known to associate with both Mucoromycotina and Glomeromycotina symbionts (Field *et al.,* 2016) were collected from the South Island of New Zealand in December 2013 (for location details please see SI), vouchers for which are deposited in the Natural History Museum, London. *Marchantia paleacea* Bertol. plants (that exclusively associate with Glomeromycotina fungal symbionts, Humphreys *et al.,* 2010) were originally collected from cool temperate cloud forests in Veracruz, Mexico and symbiotic gemmae propagated from thalli maintained in controlled environment growth chambers (Conviron BDR16, Conviron, Canada) using the following chamber settings: half light-saturating conditions for bryophytes of 50 μmol m^{-2} s^{-1} irradiance (Nobel, 1999; Fletcher *et al.,* 2006), 70% relative humidity, 12 h, 15 °C day, 12 °C night.

Shortly after collection, the liverworts were planted directly into microcosms (120 mm diameter, 100 mm depth pots) containing 95% pot volume acid-washed silica sand and 5% pot volume Irish moss-peat to aid water retention of the substrate. Native soil surrounding liverwort rhizoids was left in place to prevent damage and to act as a natural inoculum, including mycorrhizal fungi, associated microorganisms and saprotrophs.

Based on the methods of Field *et al.* (2012), we constructed cylindrical plastic cores (85 mm length, 15 mm diameter) with two 20 mm x 50 mm windows that, together with the base, were covered with nylon mesh of 10 μm pore size to prevent ingrowth of liverwort rhizoids while allowing penetration by fungal hyphae (Fig. 1a).

Three cores were inserted into each of the microcosms, two of these having a perforated, fine-bore capillary tube (100 mm length, 1.02 mm internal diameter; Portex, UK) placed vertically in the centre and secured to the basal mesh fast-setting cement. Both cores were filled with a mixture of acid-washed silica sand (89% core volume), finely ground tertiary basalt mineral grains (1% core volume) to encourage growth of fungal hyphae into the core (Quirk *et al.,* 2012) and native soil gathered from around rhizoids (*Marchantia* and *Neohodgsonia*) and underground axes (*Haplomitrium*) of wild plants (10% core volume) to act as a natural microbial inoculum containing fungi, hyphal-associated and saprotrophic microorganisms. The third mesh-covered windowed core was inserted and filled with glass wool to allow below-ground gas sampling during ^{14}C labelling.

A total of 40 microcosms were prepared for each of the liverwort species tested, 20 to be maintained under ambient atmospheric CO_2 concentrations (440 ppm) and 20 under an elevated CO_2 atmosphere of 1,500 ppm. Microcosms were regularly weeded to remove any seedlings or mosses, and maintained for 12 weeks to allow plant acclimation to cabinets and fungal mycelial networks to develop within the pots. Microcosms were rotated regularly within cabinets to avoid positional effects, and the chamber settings and contents swapped over every two weeks to avoid cabinet effects. Across the three plant species, microcosms were arranged to share a common drip tray in each cabinet, thereby providing a common pool of saprotrophic and hyphal-associated microorganisms to all the plant species.

After the acclimation period, microcosms were moved to individual drip-trays immediately prior to isotope labelling to avoid label-cross contamination. Upon commencement of isotope labelling, one core in each microcosm was left static ("static core"), ensuring hyphal connections between liverworts, fungi and core

contents remained intact. A second core in each pot was rotated ("rotated core"), thereby severing the fungal hyphal connections between the liverwort and the core contents (Fig. 1b, c). This acts as a control wherein fungal symbionts were prevented from forming continuous hyphal connections between the liverwort host and the contents of the core.

Radio- and stable isotope tracer additions to cores in microcosms (Fig. 1)

In half of the microcosms for each CO_2 treatment (n = 10 in each CO_2 condition), an internal plastic core was inserted into one of the soil-filled cores with the space between the outer and inner cores filled with the sand/basalt/soil substrate. The inner core was filled with a ^{15}N-labelled algal necromass-sand mixture (1.25% algae; 0.05 g algae and 2800 μg ^{15}N per pot). To prepare the necromass-sand mixture, cultures of *Chlorella vulgaris* var. *viridis* Chodat (CCAP 211/12) were obtained from CCAP (Argyll, UK) and cultured in 3N-BBM +V medium (see SI), where $NaNO_3$ was supplied as $Na^{15}NO_3$ (≥ 98 atom % ^{15}N; Sigma Aldrich, UK). After growth in the labelled medium, the liquid cultures were filtered, rinsed with water, re-suspended and mixed well with 200 g acid-washed sand before drying in an oven (80 °C) for three hours. This generated algae-covered dry sand particles, 4 g of which were then introduced into the inner core (see Fig. 1b) using a small funnel. The solid-walled plastic inner core remained *in situ* until immediately before ^{33}P labelling, ensuring the algal necromass was only in contact with the rest of the core contents, and therefore accessible to the symbiotic fungi, for the same time period as the fungi had access to the ^{33}P tracer (see Fig. 1a).

Molecular identification of fungal associates

DNA analyses were conducted on five samples (thalli of *Marchantia* and *Neohodgsonia*, underground axes of *Haplomitrium*) to confirm that the three liverworts maintained their previously identified fungal associates (Bidartondo *et al.,* 2011; Field *et al.,* 2016; Humphreys *et al.,* 2010) throughout the experimental periods. Wild *Haplomitrium* and *Neohodgsonia* plants (please refer to SI for details of collections and sampling) were prepared for DNA analyses within one day of collection to establish their native fungal partners. Similarly, we took samples of laboratory-grown *Marchantia* plants that have previously been confirmed to be colonised by Glomeromycotina fungi (Humphreys *et al.,* 2010), immediately before the experiments started. Additional representative samples of five individual plants across each of the three liverwort species used in our experiments were also taken immediately following our isotope labelling experiments. Sequencing of the fungal symbionts was performed using universal fungal primers and molecular cloning (Rimington *et al.,* 2018). Firstly, a full genomic DNA extraction was performed on a section of liverwort thallus using chloroform extraction (Gardes & Bruns, 1993) and the GeneClean II kit (QBioGene). The fungal DNA in the extract was then amplified using the Sigma JumpStart kit and the universal fungal primers NS1 (White *et al.,* 1990) and EF3 (Smit *et al.,* 1999). PCR products from all samples were cloned using the TOPO TA cloning kit (Invitrogen) and reamplification of fungal DNA was performed on four colonies per sample using the same primers and the Sigma JumpStart kit. The fungal DNA from reamplification was sequenced using the primers NS1, NS3 and NS5 (White *et al.,* 1990) and BigDye v. 3.1 (Applied Biosystems). Sequences were assembled into contigs using Geneious v. 7 (Kearse *et al.,* 2012) and identified to the level of fungal subphylum using BLAST searches of

GenBank (Altschul *et al.,* 1997). Further detail of the fungal sequencing method can be found in Rimington *et al.* (2018). This method found Mucoromycotina colonisation in *Haplomitrium,* Glomeromycotina in *Marchantia,* and both fungal lineages in *Neohodgsonia* – in line with previous molecular and microscopy investigations (Field et al., 2015, 2016). New sequences for *Haplomitrium gibbsiae* (accession number MF621059) and *Neohodgsonia mirabilis* (MF621060 and MF621061) have been uploaded to GenBank while Glomeromycotina sequences from *Marchantia paleacea* were identical to those previously found in these plants by Humphreys *et al.* (2010) (accession number FR690120).

Microscopy and cytology to confirm mycorrhizal status of the plants

Wild-collected plants were processed for scanning electron microscopy (SEM) as described previously (Duckett *et al.,* 2006). Briefly, underground axes of *Haplomitrium gibbsiae* and thalli of *Marchantia paleacea* and *Neohodgsonia mirabilis* (10 samples of each plant species) were fixed in 3% glutaraldehyde, dehydrated through an ethanol series, critical-point dried using CO_2 as a transfusion fluid, sputter coated with 390 nm palladium-gold and viewed under a FEI Quanta scanning electron microscope at 10 Kv.

Quantification of carbon-for-nutrient exchange between liverworts and fungi

After 12 weeks of growth, we introduced 100 µl, carrier-free ^{33}P-labelled H_2PO_4 orthophosphate solution (^{33}P specific activity 148 GBq mmol^{-1}, total 223 ng ^{33}P; Perkin Elmer, Beaconsfield, UK) into one soil core in each microcosm (Fig. 1b, c). At the same time as introducing the ^{33}P tracer, the plastic inner core separating the ^{15}N-labelled algal necromass in pots with this treatment was removed to allow the

symbiotic fungi to grow into this part of the soil-filled core. Half of the isotope-labelled cores and half of the non-labelled cores were then rotated to sever the symbiotic fungal hyphal connections between host liverwort and the soil in the cores to serve as a control for the effects of microbial nutrient cycling and saprotrophic fungal action (Fig. 1c). For each liverwort species there were 5 replicates pots for each treatment combination at ambient (440 ppm a[CO_2]) and simulated Paleozoic (1,500 ppm a[CO_2]): **1**. Fungal access to ^{33}P and ^{15}N-labelled algae; **2.** Fungal access to ^{33}P and no algae; **3.** No fungal access to ^{33}P or ^{15}N-labelled algae; **4**. No fungal access to ^{33}P and no algae.

Sub-samples of cores from 18 pots where algal necromass was included (Treatments 1 and 3) and 18 pots where algae were not included (Treatments 2 and 4) were digested using concentrated sulphuric acid to determine total phosphorus pools and potential for pool dilution effects. Total phosphorus was determined using a colorimetric method adapted from Leake, (1988) (see SI for details). There were no significant differences in total P content between cores where algae were included compared to cores where algal necromass was not included (Fig. S1), therefore P pool dilution effects were not included in the calculations for ^{33}P tracer uptake.

Twenty-one days after introducing the ^{33}P and exposing the algal necromass patches to mycorrhizal fungal colonization, the tops of all the sand and soil filled cores in all microcosms were sealed with anhydrous lanolin and plastic caps. Glass wool-filled cores were sealed with a rubber septum (SubaSeal, Sigma) to allow regular below-ground gas samples to be taken. We then placed each pot into a three-litre gas-tight chamber and added 2 ml 10% lactic acid to 15 µl of $Na^{14}CO_3$ (specific activity 2.04 GBq $mmol^{-1}$) in a cuvette within the chamber. This resulted in

1.1 MBq $^{14}CO_2$ being released within the chambers (Fig. 1d). To monitor below-ground respiration of $^{14}CO_2$, 1 ml of soil air was sampled via the glass wool-filled core after 1 hour and then every two hours thereafter for ca. 17 hours. Gas samples were injected into gas-evacuated scintillation vials containing 10 ml Carbosorb (Perkin Elmer, Beaconsfield, UK) and then mixed with 10 ml Permafluor (Perkin Elmer, Beaconsfield, UK) before radioactivity was measured via scintillation counting (Packard Tri-Carb 3100TR, Isotech, Chesterfield, UK). At the point at which the ^{14}C flux detected in below-ground gas samples stopped increasing, 2 ml 2M KOH was introduced into vials within the labelling chambers to trap any remaining $^{14}CO_2$ over a subsequent period of 6 hours. An aliquot (1 ml) of the KOH was then transferred to 10 ml Ultima Gold (Perkin Elmer, Beaconsfield, UK) and the radioactivity determined using liquid scintillation (Packard Tri-Carb 3100TR, Isotech, Chesterfield, UK).

Plant harvest and tissue analyses

Fungus-to-plant ^{15}N and ^{33}P transfer

All plant tissues and soils were separated, freeze-dried and weighed. Plant tissues were homogenised in a Yellowline A10 Analytical Grinder (IKA, Germany). The ^{15}N abundance (ng) in plant tissues was determined using isotope ratio mass spectrometry. Between 2 and 5 mg of homogenised, freeze-dried plant tissue was weighed out into 6 x 4 mm ultra-clean tin capsules (Sercon, Ltd., UK) and analysed using a continuous flow IRMS (PDZ 2020, Sercon Ltd., UK). Air was used as the reference standard and the detector was regularly calibrated to commercially available reference gases. To calculate ^{15}N content of samples, previously published equations were used (see SI) (Cameron *et al.,* 2006).

To determine the ^{33}P content of liverworts and the effect of the algal additions on ^{33}P uptake via the fungal partners, between 10-30 mg of liverwort biomass was charred in 1 ml conc. H_2SO_4 for two hours. Charred samples were then heated to 365 °C in a block heater (Grant Instruments, BT5D-26L, Cambridge UK) for 15 minutes before cooling and clearing with H_2O_2 to give complete digestion. Clear digests were diluted up to 10 ml total volume using distilled H_2O. A 2 ml aliquot of this solution was then added to 10 ml of Emulsify Safe (Perkin Elmer, Beaconsfield, UK) to measure the radioactivity via liquid scintillation counting (Packard Tri-Carb 3100TR, Isotech, Chesterfield, UK). Total ^{33}P transferred from fungi within the cores to the liverworts was determined using previously published equations (see SI) (Cameron *et al.,* 2006). To determine total symbiotic fungal-acquired ^{33}P in plant tissue where algal necromass was not added the ^{33}P values in plant tissues in algae-free microcosms where fungal access to the isotope was restricted by core rotation (Treatment 4) were subtracted from the ^{33}P values in plant tissues where fungal hyphal connections between liverworts and core contents remained intact (Treatment 2). This controls for diffusion of isotopes and microbial nutrient cycling in pots, ensuring only ^{33}P gained by the plant via intact fungal hyphal connections is accounted for. The equivalent analysis when algae was present were obtained by subtracting the ^{33}P values calculated from plant tissues in algae-containing microcosms where fungal access to the isotope was restricted by core rotation (Treatment 3) from the values from plant tissues where fungal hyphal connections between liverworts and core contents remained intact (Treatment 1).

Plant-to-fungus carbon transfer into mesh cores

The activity of the ^{14}C fixed by the liverworts and transferred via the external fungal hyphae into the mesh core substrate and remaining in this substrate was determined through sample oxidation (Packard 307 Sample Oxidiser, Isotech, UK) and liquid scintillation counting of $^{14}CO_2$ trapped in 10 ml Carbosorb and 10 ml Permafluor as described previously. Total carbon was then calculated as a function of the volume and CO_2 content of the labelling chamber and the proportion of the supplied $^{14}CO_2$ label that was fixed by the liverworts during the labelling period using previously published equations (Cameron *et al.,* 2008). To determine total plant-fixed carbon due to symbiotic fungi within soil cores in each pot, the values calculated from cores where symbiotic fungal hyphae were severed from connections to plants by core rotation were subtracted from the values in cores where fungal hyphal connections between liverworts and core contents remained intact.

Statistics

All data were checked for homogeneity of variance and normality prior to analysis of variance. Analysis was conducted with three-way ANOVA, General Linear Model (GLM), where core rotation, a[CO_2], and symbiont identity were the variables for plant ^{15}N uptake; presence of algae, a[CO_2], and symbiont identity were variables for plant ^{33}P uptake; and presence of algae, a[CO_2], and fungal symbiont identity were the variables for fungal C allocation. Where required, data were log10 transformed to meet the assumptions for ANOVA. Where there were significant interaction terms within the 3-way ANOVA's, additional 2-way ANOVAs were conducted to understand the nature of these interactions. All statistics were calculated using Minitab v.17 (Minitab, PA, USA).

Data accessibility

Should this manuscript be accepted, all data will be archived in the appropriate public repository and the data DOI will be included at the end of the article.

Results

Confirmation of fungal partner identity using molecular and cytological methods (Fig. 2)

Microscopy analyses confirmed the DNA-based results and showed colonisation patterns of the underground axes of *H. gibbsiae* by Mucoromycotina fungi consistent with previous observations (Carafa *et al*., 2003), including intracellular fungal coils with terminal lumps (Fig. 2b, c). Fungal colonisation of *M. paleacea* was typical of plant-Glomeromycotina associations, comprising relatively coarse intracellular hyphae, arbuscules and vesicles (Fig. 2e, f). Dual fungal colonisations in *N. mirabilis* had features of both fungal symbionts, particularly in the presence of coarse (>3 μm in diameter) and finer (ca. 1.5 μm in diameter) intracellular hyphae forming arbuscules and coils (Fig. 2h, i).

Biomass (Fig S2)

Most of the plant biomass was developed before the experiments commenced, and it was not feasible to measure initial biomass in each microcosm at the commencement of the experiments. Unsurprisingly therefore, we did not detect significant effects of access to algal necromass on final biomass ($F_{1, 48}$ = 1.18, P = 0.283; three-way ANOVA; Table S1), for any of the liverwort-fungal partnerships studied; Fig. S2, Table S1). The liverwort associated only with Mucoromycotina (*Neohodgsonia*) had significantly greater biomass than the Glomeromycotina-and

dual symbiont liverworts ($F_{2, 48}$ = 8.20, P=0.01, three-way ANOVA; Table S1). *Neohodgsonia* also showed a significant interaction effect with lower final biomass for the elevated compared to ambient CO_2, treatments ($F_{2, 48}$ = 4.41, P = 0.018, three-way ANOVA; Table S1) but there was no overall effect of $_a[CO_2]$ across all the plant species ($F_{1, 48}$ = 2.83, P = 0.099, three-way ANOVA; Table S1).

Nitrogen uptake in plants via symbiotic fungi (Fig. 3)

Significant fungus uptake and transfer to plants from algal necromass determined by total ^{15}N in the liverworts, was limited to plants whose fungal partners had access to the algae within static cores (Fig. 3 and Table 1; effect of static versus rotated cores: $F_{1,48}$ = 23.10, P<0.001, three-way ANOVA). This resulted in significantly higher ^{15}N concentrations in liverwort thalli grown with static cores (Table 1: $F_{1,48}$ = 49.26, P<0.001, three-way ANOVA). The uptake of ^{15}N differed significantly by symbiont type (Table 1: $F_{1,48}$ = 4.79, P=0.018). Single and dual partnerships with Mucoromycotina fungi (i.e. *Neohodgsonia* and *Haplomitrium*) had mean ^{15}N contents more than 2.4 times those of the Glomeromycotina-only colonised *M. paleacea* at 440 ppm a[CO_2] (Fig. 3a), rising to >90-fold times more at 1,500 ppm a[CO_2], however there were no significant effects of a[CO_2], and no significant two or three-way interaction effects of core rotation, a[CO_2] or symbiont type (Table 1). The concentration of ^{15}N in the liverwort thalli (Fig. 3c,d), was 35-90-fold higher in plants associated with Mucoromycotina fungi compared to those associated only with Glomeromycotina fungi, this effect of symbionts being significant ($F_{2, 48}$ = 4.37, P = 0.027; three-way ANOVA; Table 1).

Phosphorus uptake by plants via symbiotic fungi (Fig. 4)

Total ^{33}P uptake by the liverworts grown at 1500 ppm a[CO_2] was nearly twice as high in the Glomeromycotina-only *M. paleacea* than in the liverworts in single or dual association with Mucoromycotina, (Fig. 4a- note the log scale), but for the liverworts grown at 440 ppm a[CO_2] there was no significant difference between the symbionts (Fig. 4b; Table S2). This resulted in a significant interaction between symbionts and CO_2 treatment on total ^{33}P uptake by the liverworts ($F_{2, 48} = 5.39$, $P = 0.008$, three-way ANOVA; Table 2), but there were no other significant main or interactive effects of symbiont identity, a[CO_2] and availability of algae (Table 2).

The ^{33}P concentrations in liverwort biomass was significantly affected by symbiont identity (Table 2, $F_{2,48} = 8.99$, $P = 0.001$), being highest in the Glomeromycotina-only *M. paleacea.* In addition, there was a significant symbiont x a[CO_2] interaction (Table 2, $F_{2, 48} = 5.24$, $P = 0.009$), as well as a symbiont x a[CO_2] x algae interaction (Table 2, $F_{2, 48} = 4.60$, $P = 0.015$) but no other significant effects. The significant interactions arose from the dual-symbiotic liverwort showing no effects of algal additions (Fig. 4c,d), whereas the Glomeromycotina-only and Mucoromycotina-only associated liverworts showed responses to algae that differed in magnitude or direction depending on the a[CO_2], but there was no overall effect of algal additions on ^{33}P concentrations (Table 2).

Algal additions to cores also did not have a significant effect on total (non-radioactive) P content or P concentrations of cores (Fig. S1). Phosphorus pool dilution effects in treatments where the ^{15}N labelled algal necromass was included would appear to be negligible, and therefore unlikely to affect plant ^{33}P tracer uptake.

Liverwort carbon allocation to external mycelium of symbiotic fungi (Fig. 5)

The proportion of the liverwort photosynthate that was detected by ^{14}C tracing into the unrotated mesh cores was significantly affected by the type of symbiosis (Table 2), and was <2% of the total C fixed (Fig. 5a,b- note that the data are on a log scale in Fig. 5b). External mycelium of dual partnerships in the mesh cores received a greater proportion of photosynthate than mycelia of liverworts associating with Glomeromycotina only or Mucoromycotina only (Table 2; $F_{2, 48} = 4.96$, $P = 0.011$, three-way ANOVA), but there was no significant effect of a[CO_2], algae, or interactions between the symbionts or other variables.

The total ^{14}C traced into static cores via fungal partners was highly significantly affected by the symbiosis (Fig. 5 c,d), with the liverworts with dual partnerships receiving on average more than 5 times that of the single-fungal type liverworts (Table 2; $F_{2, 48} = 49.84$, $P < 0.001$; three-way ANOVA). In addition, there was a significant effect of a[CO_2], with an average 4-fold increase in carbon allocation to the fungi across symbioses and algal treatments at 1,500 ppm compared to 440 ppm a[CO_2] (Compare Fig. 5c and d; Table 2: effect of CO_2 $F_{1, 48} = 6.87$, $P = 0.012$, three-way ANOVA). The addition of algae significantly increased total photosynthate allocated into the mesh-walled cores (Table 2: effect of algae $F_{1, 48} = 10.57$, $P = 0.002$, three-way ANOVA), and there were no significant interactions between the symbiont types and the effects of algae, or between algae and a[CO_2] treatments, and no three-way interactions.

Discussion

Our results show that there are important differences in functionality between plant-fungal symbioses involving Mucoromycotina, Glomeromycotina, and dual symbioses with both groups of fungi in the effectiveness of N transfer from organic matter and inorganic P to host plants in non-sterile soil. The data from our microcosm experiments suggest that variation in atmospheric carbon dioxide concentration and the form in which nutrients are available affects the nutrient exchange dynamics between the liverwort hosts tested and their fungal symbiont(s) and that these responses are distinct according to the type of fungal partner present.

Mucoromycotina fungi enhance transfer of N from algal necromass to liverwort partners.

Although limited in the number of plant species tested and quantification of fungal biomass not performed, our experiments demonstrated that Mucoromycotina fungal partners, both when occurring singly and in dual partnerships with Glomeromycotina assimilated and transferred significant amounts of ^{15}N supplied as algal necromass in non-sterile soil to their liverwort hosts. In contrast, we detected negligible fungal ^{15}N transfer to the plant in the *Marchantia*-Glomeromycotina symbiosis. The detection of small amounts of ^{15}N in liverworts where the hyphae of symbiotic fungi growing into the mesh cores were severed by rotation are likely to be due to mass flow and diffusion of ^{15}N-containing molecules resulting from mineralization by soil microorganisms (Thirkell *et al.,* 2016). Our data support the hypothesis that Mucoromycotina fungi are more successful in competing with soil microbes for organic N resources than Glomeromycotina fungi. The higher rates of N uptake via the Mucoromycotina fungi compared to only Glomeromycotina might be due to these

fungi (a) competing better with soil saprotrophs for organic N from the algal necromass, (b) competing better for N mineralized by the saprotrophs, or (c) supporting a mycorrhizosphere-microbial community that better accelerates depolymerisation and / or mineralization of N. It is well-established that the role of mycorrhizal fungi in nutrient uptake arises from their interactions with other soil microorganisms, as most of the N in soil is naturally present in organic forms originating from plant and microbial litter that require microbial depolymerisation and potentially mineralization before it can be utilized (Vitousek & Howarth, 1991). For example, AM fungi develop specific "mycorrhizosphere" microbial populations that may be involved in increasing mobilization and mineralization of nutrients, as plant-growth promoting and P-solubilizing bacteria *Pseudomonas* and *Burkholderia* can be supported by organic C supplied by AM fungal mycelium (Zhang *et al.,* 2014, 2016). Consequently, the overall functioning of mycorrhizas in N uptake by plants is the result of complex interactions between the symbiotic fungi and other soil organisms in mutualistic and competitive/antagonistic activities in the processes of depolymerisation, mineralization and immobilization. Given this complexity for interpreting the role of mycorrhizal fungi in nutrient uptake, further studies are now required to resolve the mechanistic basis of the overall functioning of the mycorrhizal partnerships in interacting with soil microorganisms. It remains unclear whether the Mucoromycotina fungi are facultative saprotrophs and able to depolymerise the main constituents of algal necromass N such as proteins, lipids and nucleic acids, and structural carbohydrates such as cellulose cell walls.

Nonetheless, our findings provide new insights into the functional biology and ecology of these two fungal groups in symbiosis with extant members of early-divergent land plant groups in non-sterile soil. This is particularly pertinent to pioneer

plants like liverworts, given the low N status of disturbed, primary successional habitats (Ollivier *et al.,* 2011) in which soil crusts containing fast-turning over green algae and cyanobacteria are likely to provide important N rich microsites, a situation not dissimilar to that assumed to have been experienced by early land plants colonising poorly-developed soils as part of cryptogamic ground covers (Edwards *et al.,* 2015). Where organic N is available in such systems, there is likely strong competition between plants and soil microbes (Hodge *et al.,* 2000; Kuzyakov & Xu, 2013).

In the Glomeromycotina association of *Marchantia,* the limited fungal transfer of ^{15}N to the host liverwort and corresponding plant-to-fungus C allocation patterns observed here are consistent with previous studies that have found especially under elevated a[CO_2] and higher C fixation by plants, AM fungi become major sinks of plant photosynthate (Hodge, 1996; Johnson *et al.,* 2002; Herman *et al.,* 2012). Increased C efflux from mycorrhizal fungal mycelium into soil may help the priming of microbial decomposers – a common soil-biotic response to increased organic matter availability (de Graaff *et al.,* 2010; Drigo *et al.,* 2010; Verbruggen *et al.,* 2013). Thus, AM fungi may also rely on members of the saprotrophic microbial community to decompose complex organic sources and release N in inorganic form (Govindarajulu *et al.,* 2005; Leigh *et al.,* 2011) for hyphal capture. While several studies (Leigh *et al.,* 2009; Hodge & Fitter, 2010) have demonstrated that AM fungi transfer ^{15}N from ^{15}N-labelled organic patches to their host angiosperm plants, and a plant ammonium transporter that is mycorrhiza-specific and preferentially activated in cells containing arbuscules has been discovered (Guether *et al.,* 2009a; 2009b), others have reported no or negligible transport of ^{15}N from fungus to host (Hodge & Fitter, 2010). Hodge and Fitter (2010) showed that whilst AM fungal hyphae proliferated on and

acquired N from a ^{15}N-labelled organic patch the fungus retained most of the ^{15}N rather than transferring it to the host plant. Our results point toward similar N dynamics in the *Marchantia*-Glomeromycotina symbiosis as for angiosperm-Glomeromycotina AM, given the major increase in plant C allocation to the fungus with access to an organic patch at elevated a[CO_2] (Fig. 5c) for little to no ^{15}N return (Fig. 3c). Thus, it is plausible that under elevated a[CO_2] the Glomeromycotina symbiont contributes to soil N immobilization, limiting ^{15}N transfer to the liverwort host rather than contributing to liverwort host N nutrition. It should also be noted that in previous studies using non-sterile soil or root pieces from soil as fungal inocula that both Glomeromycotina and FRE may have colonized test plant roots, so that nutritional effects that have been attributed in the past exclusively to effects of Glomeromycotina AM fungi, may sometimes have unknowingly involved both groups.

Whether the Mucoromycotina fungal symbionts use the organic nutrient patch as a source of C or remains entirely dependent on the host liverwort as its main source of metabolic C is yet to be elucidated. In this regard, it is interesting to note that the general idea that putatively facultative saprotrophic ectomycorrhizal fungi behave like free-living saprotrophs in gaining C compounds from organic matter decomposition (Buée *et al.,* 2005; Courty *et al.,* 2007) has recently been challenged. Lindahl and Tunlid (2015) proposed that ectomycorrhizal fungi perform co-metabolic oxidation of organic matter with the main benefit being not the acquisition of metabolic C but the mobilization of N locked up in non-hydrolysable, recalcitrant organic matter complexes (Lindahl *et al.,* 2007). Thus, most ectomycorrhizal fungi remain dependent on their host plants as their principal source of metabolic C,

behaving as decomposers rather than facultative saprotrophs (Lindahl & Tunlid, 2015). Our results, showing stable or increased host C allocation to Mucoromycotina symbionts with access to an organic nutrient patch (Fig. 5), point to similar C dynamics in the liverwort-Mucoromycotina partnership.

Fundamental functional differences among symbioses

Overall, our results indicate functional differences among the three types of plant-fungal symbioses investigated (Table 3):

1) Associations involving Mucoromycotina-only partners provide substantial access to N supplied as algal necromass, and increase P returns to the liverwort host in exchange for relatively little plant-fixed C, especially under modern a[CO_2]. However, the amount of ^{33}P gained by a plant host per unit of C invested in the fungal partner was reduced in patches containing algal necromass at 440 ppm but not at 1500 ppm a[CO_2], possibly as a result of less effective P uptake or retaining more P in its own biomass and passing a smaller proportion on to the host plant compared to Glomeromycotina fungi.

2) In Glomeromycotina-only associations, the fungal symbionts of *M. paleacea* do not appear to facilitate significant plant N acquisition from an organic N source. This symbiosis is very effective in ^{33}P transfer to the host plant and this is largely independent of whether fresh algal necromass is added to soil – consistent with the obligately biotrophic nature of these fungi and their highly evolved role in supplying P from soil to plants.

3) Dual symbioses involving simultaneous Mucoromycotina and Glomeromycotina fungal partners combine the nutritional benefits afforded to the host plants by each of the single-fungus partnerships i.e. ^{15}N transfer from available

organic nutrient sources by the Mucoromycotina symbiosis together with the more constant ^{33}P returns from the Glomeromycotina symbiosis, whether or not organic nutrient sources are available. However, these nutritional benefits come at a considerably higher carbon 'cost' to the host than single-fungus symbioses (Table 3). The relative host carbon allocation to, and nutrient gains from each fungal partner within dual colonisations remain to be established.

Our findings support our hypothesis that the ability to engage with both Mucoromycotina and Glomeromycotina fungal partners, whether singly or in simultaneous dual symbiosis, offers additional plasticity in terms of nutrient uptake to liverworts and other early-divergent land plant clades known to engage in dual symbioses (Field *et al.,* 2015a; Desirò *et al.,* 2013; Rimington *et al.,* 2015). Our results indicate that the outcome from these multiple symbiotic options is influenced by a complex interplay of biotic and abiotic factors, including a[CO_2] and hence plant photosynthetic capacity, fungal symbiont type, localized resource-patches (such as provided by our ^{15}N algal necromass), as well as interactions with other soil microbiota (which we have not specifically investigated). The apparent superior ability of Mucoromycotina fungi over the Glomeromycotina fungi tested in supplying liverwort partners with organically-derived N may provide a competitive advantage in habitats where N is limiting and/or where the distribution of organic nutrients is patchy, whether spatially or temporally. The apparent complementarity of the fungal partners with respect to provisioning of host plants with N and P may help to explain the apparent presence and persistence of both fungal lineages in partnerships with plants from the early stages of terrestrial ecosystems (Strullu-Derrien *et al.,* 2014) through to contemporary agro-ecosystems (Orchard *et al.,* 2017a; 2017b).

Latest evidence indicates that fine root endophyte fungi (FRE), which are widespread ecologically and across vascular plants, are members of the Mucoromycotina and closely related to liverwort and lycophyte Mucoromycotina symbionts (Bidartondo *et al.,* 2011; Rimington *et al.,* 2015; 2016; Field *et al.,* 2015b; Orchard *et al.,* 2017a). Furthermore, the same Mucoromycotina fungi can enter into symbiosis with liverworts, early vascular plants and angiosperms (Hoysted *et al.,* 2019). This finding greatly expands the potential significance of Mucoromycotina fungi in modern ecosystems (Field *et al.,* 2015a; Hoysted *et al.,* 2018; Field and Pressel, 2018). Thus, it is now critical to explore the functioning of Mucoromycotina fungal symbionts in later-divergent groups of land plants such as lycophytes, ferns and angiosperms. Such investigations together with a better appreciation of the roles of Glomeromycotina fungi in plant host N nutrition, are key to understanding the consequences of diverse nutritional mutualisms among plants and fungi for biogeochemistry and ecosystems in the past, present and future.

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Author contributions

KJF, JRL, DDC, DJB, MIB, JGD and SP conceived and designed the research. KJF conducted the isotope tracer work and GAH conducted P analyses. MIB and WRR conducted the molecular analyses. SP and JGD conducted the cytological analyses. SP, KJF, MIB and JRL led the writing; all authors provided comments on the manuscript.

References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402

Ames RN, Reid CPP, Porter LK, Cambardella C. 1983. Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources of *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus. *New Phytologist* **95**: 381–396.

Azcón R, Ambrosano E, Charest C. 2003. Nutrient acquisition in mycorrhizal lettuce plants under different phosphorous and nitrogen concentration. *Plant Science* **165**: 1137–1145.

Berner, R.A., 2006. GEOCARBSULF: a combined model for Phanerozoic atmospheric O_2 and CO_2 *Geochimica et Cosmochimica Acta* **70**(23): 5653-5664.

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.

Bücking H, Kafle A. 2015. Role of arbuscular mycorrhizal fungi in nitrogen uptake of plants: current knowledge and research gaps. *Agronomy* **5**: 587-612.

Buee M, Vairelles D, Garbaye J. 2005. Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus sylvatica*) forest subjected to two thinning regimes. *Mycorrhiza* **15**: 235–245.

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, 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.

Carafa A, Duckett JG, Ligrone R. 2003. Subterranean gametophytic axes in the primitive liverwort Haplomitrium harbour a unique type of endophytic association with aseptate fungi. *New Phytologist* **160**: 185-197.

Courty PE, Breda N, Garbaye J. 2007. Relation between oak tree phenology and the secretion of organic matter degrading enzymes by *Lactarius quietus* ectomycorrhizas before and during bud break. *Soil Biology and Biochem*istry **39**: 1655–1663.

de Graaff MA, Classen AT, Castro HF, Schadt CW. 2010. Labile soil carbon inputs mediate the soil microbial community composition and plant residue decomposition rates. *New Phytologist* **188**: 1055–1064

Desirò A, Duckett JG, Pressel S, Villarreal JC, Bidartondo, MI. 2013. Fungal symbioses in hornworts: a chequered history. *Proceedings of the Royal Society: B* **280:** 20130207.

Drigo B, Pijl AS, Duyts H, Kielak AM, Gamper HA, Houtekamer MJ, Boschker HTS, Bodelier PLE, Whiteley AS, van Veen JA, Kowalchuk GA. 2010. Shifting carbon flow from roots into associated microbial communities in response to elevated

atmospheric CO_2. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 10938–10942.

Duckett, Carafa A, Ligrone R. 2006. A highly differentiated glomeromycotean association with the mucilage-secreting, primitive antipodean liverwort *Treubia* (Treubiaceae): clues to the origins of mycorrhizas. *American Journal of Botany* **93**: 797–813.

Edwards E, Cherns L, Raven JA. 2015 Could land-based early photosynthesizing ecosystems have bioengineered the planet in mid-Palaeozoic times? *Palaeontology* **58**: 803–837.

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 Communications* **3**: 835.

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

Field KJ, Rimington WR, Bidartondo MI, Allison KE, Beerling DJ, Cameron DD, Duckett JG, Leake JR, 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* **154**: 743–756.

Field KJ, Rimington WR, Bidartondo MI, Allison 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.

Field KJ**,** Pressel S. 2018. Tansley Review - Unity in diversity: structural and functional insights into an ancient partnership between plants and fungi. *New Phytologist* **220:** 996-1011.

Fletcher BJ, Brentnall SJ, Quick WP, Beerling DJ. 2006. BRYOCARB: a process-based model of thallose liverwort isotope fractionation in response to CO_2, O_2, light and temperature. *Geochimica et Cosmochimica Acta* **70**: 5676–5691.

Gardes M, Bruns TD. (1993). ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118

Govindarajulu M, Pfeffer PE, Jin HR, Abubaker J, Douds DD, Allen JW, Bücking H, Lammers PJ, Shachar-Hill Y. 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* **435**: 819–823.

Guether M, Neuhaüser B, Balestrini R, Dynowski M, Ludewig U, Bonfante P. 2009a. A mycorrhizal-specific ammonium transporter rom *Lotus japonicus* acquires nitrogen released by arbuscular mycorrhizal fungi. *Plant Physiology* **150**: 73–83.

Guether M, Balestrini R, Hannah M, He J, Udvardi MK, Bonfante P. 2009b. Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in *Lotus japonicus*. *New Phytologist* **182**: 200–212.

Herman DJ, Firestone MK, Nuccio E, Hodge A. 2012. Interactions between an arbuscular mycorrhizal fungus and a soil microbial community mediating litter decomposition. *FEMS Microbiology Ecol*ogy **80**: 236–247.

Hodge A. 1996. Impact of elevated CO_2 on mycorrhizal associations and implications for plant growth*. Biology and Fertility of Soils* **23**, 388–398.

Hodge A, Robinson D, Fitter A. 2000. Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science* **5**: 304-308

Hodge A, Fitter AH. 2010. Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *Proceedings of the National Academy of Science of the United States of America* **107**: 13754–13759.

Hodge A, Storer K. 2015. Arbuscular mycorrhiza and nitrogen: implications from individual plants through to ecosystems. *Plant and Soil* **386**: 1-19.

Hoysted GA**,** Kowal J, Jacob A, Rimington WR, Duckett JG, Pressel S, Orchard S, Ryan M, Field KJ, Bidartondo MI. 2018. A mycorrhizal revolution. *Current Opinions in Plant Biology* **44**: 1-6.

Hoysted GA, Jacob AS, Kowal J, Giesemann P, Bidartondo MI, Duckett JG, Gebauer G, Rimington WR, Schornack S, Pressel S, Field KJ. 2019. Mucoromycotina fine root endophyte fungi form nutritional mutualisms with vascular plants. *bioRxiv*, https://doi.org/10.1101/531103

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 Communications* **1**: 7.

Johnson D, Leake JR, Ostle N, Ineson P, Read DJ. 2002. In situ CO_2-C^{13} pulse labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. *New Phytol*ogist **153**: 327–334.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647-1649.

Kuzyakov Y, Xu X. 2013. Competition between roots and microorganisms for nitrogen: mechanisms and ecological reference. *New Phytologist* **198**: 656-669

Leake, JR. 1988. *Causes and Effects of Soil Acidification by Calluna vulgaris (L.) Hull: With Special Reference to the Role of Mycorrhizas* (Doctoral dissertation, University of Sheffield, Department of Plant Sciences).

Leigh J, Hodge A, Fitter AH. 2009. Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. *New Phytologist* **181**: 199–207.

Leigh J, Fitter AH, Hodge A. 2011. Growth and symbiotic effectiveness of an arbuscular mycorrhizal fungus in organic matter in competition with soil bacteria. *FEMS Microbiology Ecology* **76**: 428–438.

Lenton TM, Daines SJ, Mills BJW. 2018. COPSE reloaded: An improved model of biogeochemical cycling over Phanerozoic time. Earth Science Reviews **178**: 1-28

Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD. 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytologist* **173**(3): 611-620.

Lindahl BD, Tunlid A. 2015. Ectomycorrhizal fungi – potential organic matter decomposers, yet not saprotrophs. *New Phytologist* **205**: 1443–1447.

Morris JL, Puttick MN, Clark JW, Edwards D, Kenrick P, Pressel S, Wellman CH, Yang Z, Schneider H, Donoghue PC. 2018. The timescale of early land plant evolution. *Proceedings of the National Academy of Sciences* **115**(10):.E2274-E2283.

Nobel PS. 1999. *Physicochemical and environmental plant physiology*, 2nd edn. Amsterdam, the Netherlands: Academic Press.

Ollivier J, Toewe S, Bannert A, Hai B, Kastl EM, Meyer A, Su MX, Kleineidam K, Schloter M. 2011. Nitrogen turnover in soil and global change. *FEMS Microbiology Ecology* **7**8: 3–16

Orchard, S, Hilton S, Bending GD, Dickie IA, Standish RJ, Gleesons DB, Jeffery RP, Powell JR, Walker C, Bass D, Monk J, Simonin A, Ryan MH. 2017a. Fine endophytes (*Glomus tenue*) are related to Mucoromycotina not Glomeromycota. *New Phytologist* **213**: 481–486.

Orchard S, Standish RJ, Dickie IA, Renton M, Walker C, Moot D, Ryan MH. 2017b. Fine root endophytes under scrutiny: a review of the literature on arbuscule-producing fungi recently suggested to belong to the Mucoromycotina. *Mycorrhiza* **27**: 619-638

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.

Quirk J, Beerling DJ, Banwart SA, Kakonyi G, Romero-Gonzalez ME, Leake JR. 2012. Evolution of trees and mycorrhizal fungi intensifies silicate mineral weathering. *Biology letters* **8**: 1006-1011.

Rimington WR, Pressel S, Duckett JG, Bidartondo MI. 2015. Fungal diversity in early vascular plants: Reopening a closed book? *New Phytologist* **205**: 1394–1398.

Rimington WR, Pressel S, Field KJ, Strullu-Derrien C, Duckett JG, Bidartondo MI. 2016. Reappraising the origin of mycorrhizas. In *Molecular Mycorrhizal Symbiosis* (ed. F Martin). pp. 31-32 Hoboken, NJ, USA: John Wiley & Sons Inc.

Rimington WR, Pressel S, Duckett JG, Field KJ, Read DJ, Bidartondo MI (2018). Ancient plants with ancient fungi: liverworts associate with early-diverging arbuscular

mycorrhizal fungi. *Proceedings of the Royal Society B: Biological Sciences* **285:** 20181600.

Smit E, Leeflang P, Glandorf B, van Elsas JD, Wernars K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR- amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology* **65**: 2614-2621.

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

Smith SE, Anderson IC, Smith FA. 2015. Mycorrhizal associations and phosphorus acquisition: from cells to ecosystems. *Annual Plant Reviews* **48**: 409-440.

Strullu-Derrien C, Kenrick P, Pressel S, Duckett JG, Rioult JP, Strullu DG. 2014. Fungal associations in *Horneophyton ligneri* from the Rhynie Chert (c. 407 million year old) closely resemble those in extant lower land plants: novel insights into ancestral plant–fungus symbioses. *New Phytologist* **203**: 964-979.

Thirkell T, Cameron DD, Hodge A. 2016. Resolving the 'nitrogen paradox' of arbuscular mycorrhizas: fertilization with organic matter brings considerable benefits for plant nutrition and growth. *Plant Cell Environment* **39**: 1683–1690.

Verbruggen E, Veresoglou SD, Anderson IC, Caruso T, Hammer EC, Kohler J, Rillig MC. 2013. Arbuscular mycorrhizal fungi – short-term liability but long-term benefits for soil carbon storage? *New Phytologist* **197**: 366–368.

Vitousek PM, Howarth RW. 1991. Nitrogen limitation on land and in the sea; how can it occur? *Biogeochemistry* **13**: 87–115.

Walker C, Gollotte A, Redecker D (2018). A new genus, *Planticonsortium* (Mucoromycotina), and new combination *(P. tenue),* for the fine root endophyte*, Glomus tenue (basionym Rhizophagus tenuis). Mycorrhiza* **28**: 213-219.

Wang B, Qui Y-L. 2006. Phylogenetic distribution and evolution of mycorrhizas. *Mycorrhiza* **16**: 299–363.

Wang B, Yeun L H, Xue J-Y, Liu Y, Ané J-M, Qiu Y-L. 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.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: a Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds. (Orlando: Academic Press), pp. 315-322.

Zhang L, Fan J, Ding X, He X, Zhang F, Feng G, 2014. Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. *Soil Biology & Biochemistry* **74**: 177–183.

Zhang L, Xu M, Liu Y, Zhang F, Hodge A, Feng G, 2016. Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium. *New Phytologist* **210**: 1022–1032.

Supporting Information

Figure S1. Mean total phosphorus content and concentration in core substrate where algal necromass was absent (- algae) and where algal necromass was included (+ algae).

Figure S2. Total mean biomass of liverworts in plants grown under ambient atmospheric [CO_2] (440 ppm) and elevated atmospheric [CO_2] (1,500 ppm) with (and without fungal access to algal necromassa.

Notes S1 - Supplementary materials and methods (analysis of total P, biomass, fungus-to-plant ^{15}N and ^{33}P transfer and plant-to-fungus C transfer).

Notes S2 – Supplementary results (total phosphorus content of mesh-walled cores, plant biomass).

Table S1. Three-way ANOVA (GLM) showing effects of fungal symbiont, inclusion of algae, and a[CO_2] on liverwort biomass at end of experiment.

Table S2. Two-way ANOVA (GLM) showing effects of fungal symbiont and access to algae within static core (S) treatments. Data are log10 transformed to conform to ANOVA assumptions.

Figure Legends

Fig. 1. Diagrams of experimental microcosms showing (a) contents of cores containing ^{15}N-labelled algal necromass (right) and cores without algal addition (left). Location of algal necromass indicated by green shading running length of the soil column within the core; (b) experimental microcosm where liverworts have access to ^{15}N-labelled algal necromass and ^{33}P-orthophosphate via intact fungal hyphal connections between host liverwort and core contents (static core treatment); (c) experimental microcosm where liverworts do not have access to ^{15}N-labelled nutrient patch and ^{33}P-orthophosphate via severed fungal hyphal connections between core contents and host plant achieved by rotating the core (blue arrow; rotated core treatment) prior to isotope introduction (red arrow), and (d) ^{14}C labelling experimental set-up showing fixation of $^{14}CO_2$ by liverworts and transfer of labelled carbon compounds to fungi within static cores.

Fig. 2. Liverworts and fungal symbionts studied in the present investigation; wild whole plants (a, d, g) and scanning electron micrographs of respective fungal endophytes (b, c, e, f, h, i). (a-c) The earliest-divergent Haplomitriopsida liverwort *Haplomitrium gibbsiae* (a) associates exclusively with Mucoromycotina-FRE fungi. Mucoromycotina colonise *Haplomitrium* underground axes where their fine hyphae (0.5-1.5 µm) form coils with swellings (arrowed in b and enlarged in c). (d-f) *Marchantia paleacea* harbouring in its thallus only Glomeromycotina fungal symbionts with cytology consisting of coarse hyphae (> 3 µm), arbuscules (e) and large vesicles (arrowed in f), as typical of AM symbioses. (g-i) *Neohodgsonia mirabilis* in dual symbiosis with Glomeromycotina and Mucoromycotina fungi

showing in its thallus structures typical of both fungi: arbuscules on trunk hyphae (Glomeromycotina, h) and fine hyphae with arbuscule-like structures (Mucoromycotina, i). Scale bars: (e) 200 µm; (b) 100 µm; (f, h, i) 50 µm; (c) 20 µm.

Fig. 3. Fungus-to-plant nitrogen transfer between liverworts and fungi at simulated Palaeozoic and modern ambient atmospheric [CO_2]. Mean total fungal-acquired ^{15}N (a, b) and concentration of fungal-acquired ^{15}N detected in plants (c, d) at simulated Palaeozoic a[CO_2] of 1,500 ppm (a, c) and modern ambient a[CO_2] of 440 ppm (b, d) where ^{15}N labelled algal necromass was ("Static"; black bars) or was not ("Rotated"; white bars) accessible to fungal symbionts. Error bars indicate ± S.E., n = 5 (see Table 1 for statistics).

Fig. 4. Fungus-to-plant phosphorus transfer between liverworts and fungi at simulated Palaeozoic and modern ambient atmospheric [CO_2]. Mean total fungal-acquired ^{33}P (a, b) and concentration of fungal-acquired ^{33}P in plants (c, d) at simulated Palaeozoic a[CO_2] of 1,500 ppm (a, c) and modern ambient a[CO_2] of 440 ppm (b, d). "+ algae" values represent fungal-acquired ^{33}P where algal necromass was present, "-algae" representing fungal-acquired ^{33}P where algal necromass was not present. Error bars indicate ± S.E., n = 5 (see Table 2 for statistics).

Fig. 5. Plant-to-fungus carbon transfer between liverworts and fungi at simulated Palaeozoic and modern ambient atmospheric [CO_2]. Percentage allocation (a, b) and total (c, d) liverwort-fixed carbon transferred to fungal partners within soil cores with (black bars) and without (white bars) presence of organic nutrient patch at (a, c) 1,500 ppm [CO_2] and (b, d) 440 ppm [CO_2]. Note difference in

scale of *y*-axis in figures (c) and (d). Error bars indicate ± S.E., $n = 5$ (see Table 2 for statistics).

Tables

Table 1. Summary of three-way ANOVA (GLM) results testing the effects of core rotation on ^{15}N content and [^{15}N] of liverworts (Fig. 3).

Variable	Symbiosis (S) *d.f. 2, 48*		CO_2 (C) *d.f. 1, 48*		Core rotation (R) *d.f. 1, 48*		S x C *d.f. 2, 48*		S x R *d.f. 2, 48*		C x R *d.f. 1, 48*		S x C x R *d.f. 2, 48*	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Plant ^{15}N (ng) (See Fig. 3a,b)	4.79	**0.018**	0.30	n.s.	23.10	**<0.001**	2.45	n.s.	1.33	n.s.	1.31	n.s.	0.75	n.s.
Plant [^{15}N] (ng g^{-1}) (See Fig. 3 c,d)	4.37	**0.027**	0.52	n.s.	49.26	**<0.001**	11.34	n.s.	2.44	n.s.	0.93	n.s.	2.04	n.s.

Data were log10 transformed to meet assumptions of ANOVA. Hyphal connections between liverworts and ^{15}N-labelled algal necromass are preserved in static core treatments whereas rotated core treatment causes hyphal connections between liverwort to be severed. Experiments carried out under 440 ppm a[CO_2] and 1,500 ppm a[CO_2]. Significant results ($P<0.05$) are indicated in **bold**, n.s. = $P>0.05$.

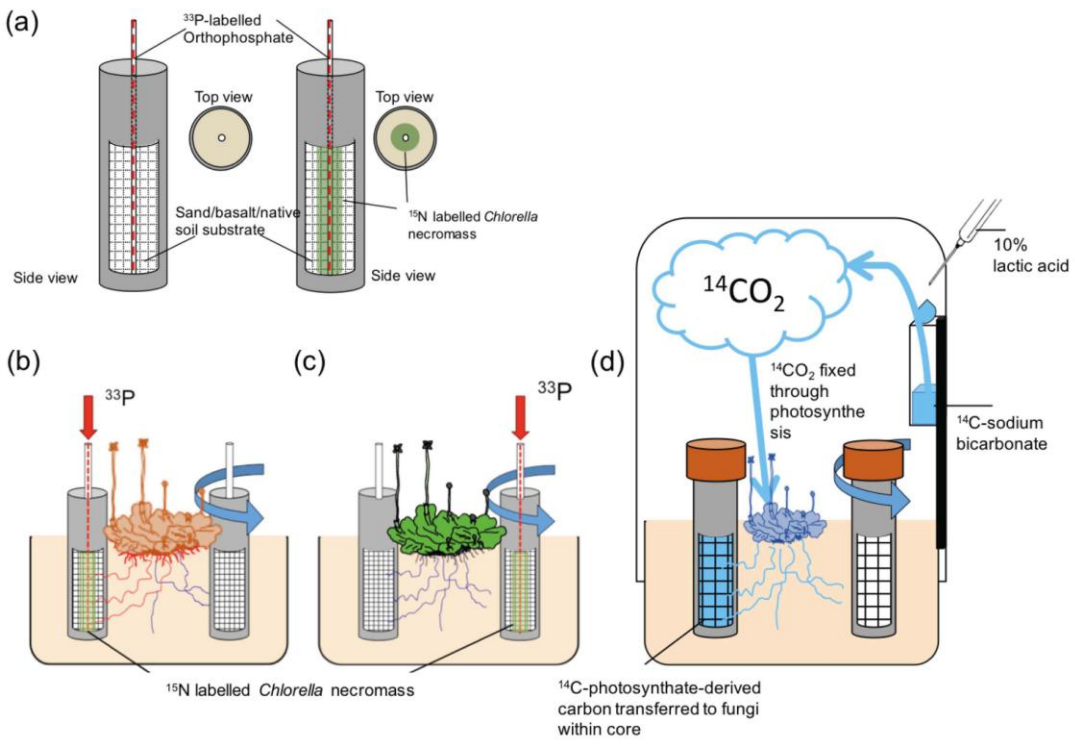
Table 2. Summary of three-way ANOVA (GLM) results testing the effects of algae inclusion and fungal symbiont(s) on exchange of fungal-acquired phosphorus (Fig. 4) for plant carbon (Fig. 5) in experiments conducted under 440 ppm a[CO_2] and 1,500 ppm a[CO_2].

Variable	Symbiosis (S) *d.f. 2, 48*		CO_2 (C) *d.f. 1, 48*		Algae (A) *d.f. 1, 48*		S x C *d.f. 2, 48*		S x A *d.f. 2, 48*		C x A *d.f. 1, 48*		S x C x A *d.f. 2, 48*	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Plant ^{33}P (ng)	0.96	n.s.	1.50	n.s.	0.50	n.s.	5.39	**0.008**	0.54	n.s.	1.84	n.s.	1.13	n.s.
Plant [^{33}P] (ng g^{-1})	8.99	**0.001**	4.42	n.s.	2.23	n.s.	5.24	**0.009**	1.97	n.s.	0.10	n.s.	4.60	**0.015**
% C allocation to cores	4.96	**0.011**	0.08	n.s.	0.04	n.s.	2.87	n.s.	1.39	n.s.	0.98	n.s.	1.53	n.s.
Plant allocation of C to fungi within cores (ng)	49.84	**<0.001**	6.87	**0.012**	10.57	**0.002**	0.59	n.s.	1.49	n.s.	0.15	n.s.	1.77	n.s.

Data were log10 transformed to meet assumptions of ANOVA. Significant results (P<0.05) are indicated in **bold**, n.s. = P>0.05.

Table 3. Summary of key findings of the costs and benefits of single and dual symbiosis with Mucoromycotina and Glomeromycotina fungi in liverworts.

Functions	**Mycorrhiza type**		
	Mucoromycotina fungi	Glomeromycotina fungi	Both Mucoromycotina and Glomeromycotina
Relative carbon costs of external mycelium to host plant	Low	Low	High
^{15}N from algal necromass supplied to host plant	High	Low	High
^{33}P orthophosphate supplied to host plant	Low-High Reduced by algal necromass at ambient CO_2 but not at elevated CO_2.	High Unaffected by algal necromass	Moderate-High Unaffected by algal necromass



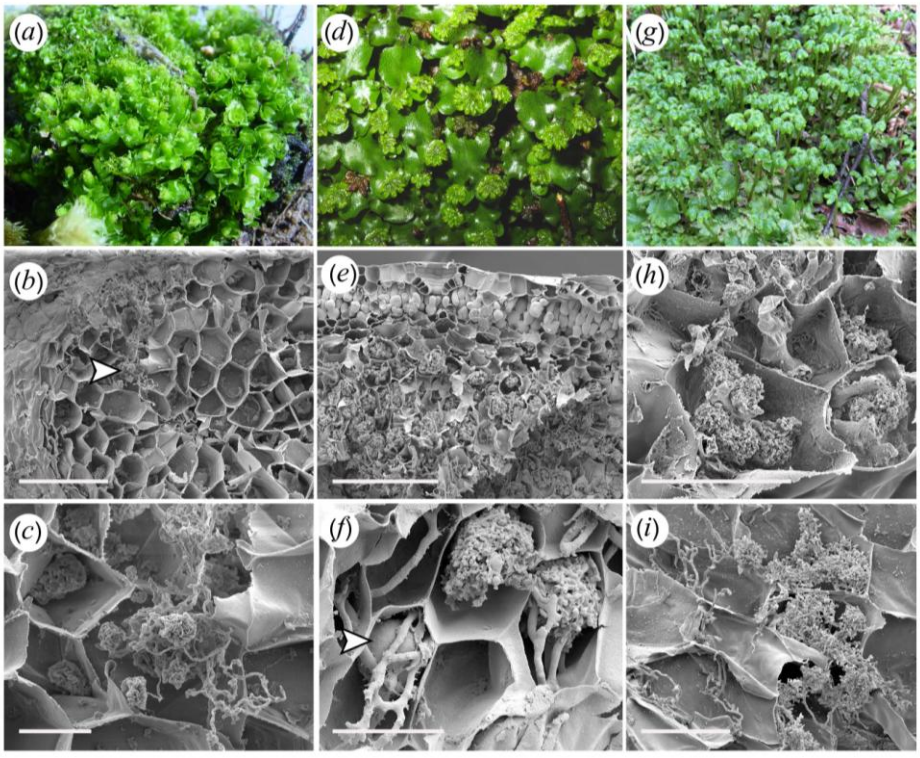


Figure 1