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Symbiotic options for the conquest of land

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The domination of Earth's landmasses by plants starting in the Ordovician Period drastically altered the development of the biosphere and the composition of the atmosphere, with far-reaching consequences for all life ever since. It is widely thought that symbiotic soil fungi facilitated the colonisation of the terrestrial environment by plants. However, recent discoveries in molecular ecology, physiology, cytology and palaeontology have brought into question the hitherto-assumed identity and biology of the fungi engaged in symbiosis with the earliest diverging lineages of extant land plants. We reconsider the existing paradigm and show that the symbiotic options available to the first plants emerging onto the land were more varied than previously thought.

The current paradigm

The colonisation of the terrestrial environment by plants was a major turning point in Earth's evolutionary history. In conquering the land, plants have sculpted the biosphere and geosphere through their influence on global carbon, nutrient and water cycles [1], leading to the development of climates and habitats essential for supporting the diverse array of life that now exists in terrestrial environments [2,3]. It is estimated that more than 80% of living plant species form symbioses with horizontally-transmitted filamentous fungi, representing 92% of plant families worldwide [4] (Figure 1). These intimate associations are known as 'mycorrhizas', or 'mycorrhiza-like' when referring to plants lacking true roots such as non-vascular plants. Through mycorrhizas and mycorrhiza-like associations, plants assimilate fungus-acquired mineral nutrients far beyond their roots' soil depletion zones and from soil pores too narrow for their root hairs. In return, plants supply their fungal symbionts with carbohydrates fixed from atmospheric carbon dioxide through photosynthesis [5].

Plant life diversified on land some 70 MY after the diversification of most major animal lineages in the seas during the Cambrian explosion (Fig. 1) [6]. Classic palaeoclimate modelling shows that land plants diversified against a backdrop of falling atmospheric CO_2 [7] (Figure 2), likely driven by growing demand and evolving capacity for carbon assimilation of the burgeoning Earth flora [8,9]. Long before land plants emerged, however, the terrestrial environment had been colonised by fungi [10,11]. Among the early-branching fungal lineages were those that today form mutualistic associations with most plants (Figure 1). The macrofossil record for non-vascular plants is even more fragmentary than that for vascular plants, though the study of microfossils is now providing key new data [12,13], and molecular studies that date land plant origins and the divergence of the non-vascular groups (i.e. liverworts, mosses and hornworts [14]), remain imprecise compared to those on flowering plant evolution [15]. This has resulted in some doubt over the precise evolutionary relationships both among non-vascular plants themselves and between non-vascular and vascular plants [16,17]. Despite these lacunae, it has been proposed by plant and fungal biologists [31,32] that initial plant colonisation of the terrestrial environment was facilitated through interactions with symbiotic fungi. Such hypotheses are supported by data spanning five decades of research and three scientific disciplines. Firstly, the incredibly well preserved early-Devonian Rhynie chert macrofossils show individual vascular plant cells harbouring fungal structures that are strikingly similar to those formed by arbuscular mycorrhizal (AM) fungi of the phylum Glomeromycota within living cells of modern vascular plants [18,19] (see Box 1). Secondly, molecular evidence indicates that genes and biochemical signalling pathways initiating and regulating the arbuscular mycorrhizal symbiosis are ubiquitous across all living land plant lineages [20-23]. Thirdly, physiological data demonstrate nutritional mutualisms between Glomeromycota fungi and living members of the earliest branching lineages of land plants [24,25].

The resulting paradigm is that the earliest, rootless, terrestrial plants co-evolved with Glomeromycota fungi [4,26-30] that in exchange for plant photosynthates, enhanced access to soil mineral nutrients (Box 2). This hypothesis was elaborated in classic papers by T. H. Nicolson, K. A. Pirozynski and D. W. Malloch [31,32]. All other mycorrhizal symbioses (i.e. ectomycorrhizal, ericoid, orchid) are thought to have evolved through later switches to other fungal lineages [33]. Even symbioses between flowering plants and nitrogen-fixing bacteria, such as Rhizobium, must have evolved from the ancient AM symbiosis [21]. These diverse plant-microbe partnerships continue to drive the establishment and development of terrestrial ecosystems [33,34]. Recent discoveries showing that the earliest diverging groups of land plants form mutualistic associations with fungi other than Glomeromycota mean it is now timely to challenge the existing paradigm for plant and fungal colonisation of land. In this review we bring together the most recent advances and emerging perspectives in the field, leading to the development of a new paradigm for the conquest of the terrestrial environment.

Early interactions between plants and fungi

In early land plants associated with symbiotic fungi, movement of carbohydrates from photosynthetic tissues would likely extend into the associated fungal hyphae [35]. In turn, symbiotic fungi might have used those plant carbohydrates to power resource extraction and plant provisioning from surrounding soil, greatly increasing the surface area for nutrient capture and uptake in the earliest rootless plants - one of the most important but least discussed adaptations to life on land. Compared to unicellular non-vascular plant rhizoids and vascular plant root hairs, the multicellular filaments of fungi are much thinner, far longer and able to branch and fuse, creating complex underground networks. The resulting enhancement of biological mineral weathering by mycorrhizal and mycorrhiza-like associations has been linked to the dramatic 90% decline in atmospheric CO_2 concentration [36] and to pedogenesis in the Palaeozoic [2,37,38] (Figure 2). The acceleration of mineral weathering by the first rooted vascular plants during the Devonian could have been preceded by an earlier peak driven by rootless non-vascular plants during the Ordovician as suggested by an experiment with living non-vascular plants [39]. Non-vascular plants are still major contributors to global nitrogen and carbon cycling [40].

Some of the first plants to emerge onto land were morphologically similar to modern-day liverworts [3,41], while others were unlike any extant species (Figure 1). As non-vascular plants are the closest living relatives of the first land plants [42,43], they are the most appropriate organisms for inferring experimentally the ecology and evolution of partnerships between early land plants and fungi. Until recently this opportunity remained untapped; the existing models (the moss Physcomitrella patens and the liverwort Marchantia polymorpha) both lack symbiotic fungi (see Box 3) and no efforts had been made to experimentally test the functioning of mycorrhiza-like associations in non-vascular plants. Thus, evidence for homology in terms of both fungal identity and function relied solely on morphological congruence [27] and the ecological nature of the intimate interactions between non-vascular plants and fungi remained pure speculation [5].

Fungi in extant early-diverging land plants

Some commonalities in fungal symbiont morphologies between non-vascular and vascular plants supported the long-held assumption that fungi observed in non-vascular plants belong to the Glomeromycota [19] (see Box 1 and Figure 3c,d). Molecular identification of the symbionts of some complex and simple thalloid liverworts supported this view [24,25]. Symbioses - of still untested function - of the more recently evolved fungal phyla Basidiomycota (e.g., Sebacina and Tulasnella) and Ascomycota (e.g., Pezoloma) with derived liverwort clades [19,44] demonstrate that fungal partners in modern liverworts are not always conserved among groups. Nonetheless, given the early branching of liverworts during land plant evolution (Figure 1), it was generally assumed that their fungal symbioses predated those of vascular plants [4]. The unexpected identification of the fungal symbionts of some liverworts as only being members of derived Glomeromycota (order Glomerales) that are widespread in vascular plants, raised the alternative possibility that the earliest lineages of symbiotic fungi were replaced by more derived ones through secondary shifts from vascular to non-vascular plants [45,46].

However, this Glomeromycota-centred understanding of the nature of fungal symbioses in land plants is now being revised. Through the application of inclusive molecular detection methods, in 2011 Bidartondo et al. [47] discovered that the earliest branching lineage of liverworts – the class Haplomitriopsida – do not harbour Glomeromycota fungal partners, but instead associate with another group of early-diverging fungi, the Mucoromycotina, considered either basal or sister to the Glomeromycota [48,49] (Figure 1 and Figure 3b). Further investigation has revealed that symbiotic associations in many other early divergent liverworts, most hornworts, and some early branching vascular plants include unexpectedly diverse members of the Glomeromycota and/or diverse Mucoromycotina fungi [47,50-52]. These findings raise the novel hypothesis that plant-Mucoromycotina associations represent an ancestral land plant-fungal symbiosis. It is possible that facultatively biotrophic and sexual Mucoromycotina fungi were gradually replaced by the now strictly biotrophic and asexual Glomeromycota fungi as land plants grew in stature, soil organic matter accumulated and atmospheric CO_2 levels dropped. This could have resulted in shifts in the dynamics and trade-offs of early plant-fungal joint ventures [53].

Plant-Glomeromycota symbiosis

The vast majority of modern mycorrhizas are formed between plants and the arbuscule-forming fungi of the Glomeromycota (Figure 1) (see Box 1). Glomeromycota fungi are strictly biotrophic, relying entirely on their plant partners for organic carbon and to complete their strictly asexual life cycle. The routes by which plants and fungi exchange carbon for nutrients have been studied extensively in a wide range of flowering plants, by far the largest group of extant plants. Flowering plants rapidly diversified and dominated the land during the last 100 million years, outcompeting most earlier plant lineages in the majority of terrestrial ecosystems - Darwin's "abominable mystery". The classic 2006 compilation by Wang & Qiu [4] of mycorrhizal and mycorrhiza-like associations in 3,617 plant species from 659 publications confirmed the wide host range of AM fungi, indicating that the evolution of Glomeromycota likely coincided with that of the earliest diverging land plants. Therefore, it was firmly concluded that AM fungi formed the ancestral mycorrhizas, a view consistent with the hitherto most widely-held hypothesis on the origin and evolution of land plant-fungus symbiosis [54,55]. However, the functional significance of mycorrhiza-like associations in non-vascular plants - although key to unravelling the role of plant-fungal interactions in the evolution and diversification of land plants through the Phanerozoic - was conjectural [27], having been bypassed by physiological research. Further, at that time it was unknown that a wide range of non-vascular and early divergent vascular plant lineages regularly form symbiotic associations with fungi of the Mucoromycotina [47,52,53,56].

We now know that liverwort-Glomeromycota symbioses can be mutualistic, involving reciprocal exchange of organic carbon and nutrients between partners [25] with measureable fitness benefits to both plant and fungus [24]. Similarly, the first studies on mycorrhizal functioning in early branching lineages of vascular plants show their root fungal symbionts are also mutualistic [25,53]. However, whilst vascular plants harbouring Glomeromycota benefit from lower, near modern day atmospheric CO_2 in terms of reduced carbon costs per nutrients gained from their fungal partners, the symbiotic efficiency of liverwort-Glomeromycota associations is severely compromised by simulated Palaeozoic drops in CO_2 [25]. Given recent evidence indicating that the transporter proteins characteristic of vascular plant-Glomeromycota symbioses are not operational in non-vascular plants [22], it is likely that alternative nutrient exchange and translocation pathways are at work in these more ancient partnerships thus resulting in their different responses to atmospheric CO_2 concentrations.

Ancestral plant-Mucoromycotina symbiosis?

The still-unfolding awareness that partnerships with Mucoromycotina fungi in non-vascular and early branching lineages of vascular plants are widespread has major ramifications throughout modern mycorrhizal research. The Mucoromycotina encompasses species that, until the rise of molecular phylogenetics in the 1990s, were classified together with Glomeromycota [30]. They include saprotrophic and sexual lineages such as Endogone, Sphaerocreas and at least ten unnamed lineages [47,50-52,57] largely neglected by biologists since the 1980s as the study of Glomeromycota arbuscular mycorrhizas blossomed. Both Glomeromycota and Mucoromycotina have coenocytic hyphae occupied by specific Mollicutes-related endobacteria [51]. Some Mucoromycotina have recently been cultured axenically from host plants (Figure 3a) [53], so they should prove considerably more orthodox and tractable in experiments than the obligate biotrophic Glomeromycota fungi.

Within hornworts, either fungal group might have been ancestral, the fungi occupy varied locations within plants in close association with cyanobacteria, and symbiotic function remains to be tested [50]. Within liverworts, so far only the Haplomitriopsida genera Haplomitrium and Treubia are known to associate exclusively with Mucoromycotina. In Treubia the fungus has distinct inter- and intracellular colonization zones in the thallus midrib whilst in Haplomitrium it is confined to the epidermal layers in mucilage-secreting subterranean axes. In both genera, the fungus produces abundant thin-walled intracellular hyphal coils and unique short-lived swellings, which might serve as sites of carbon-for-nutrient exchange and storage, potentially functioning like mycorrhizal arbuscules. Studies thus far show unequivocally that Haplomitriopsida-Mucoromycotina associations are mutualistic in terms of carbon-for-nutrient exchange, on a par with liverwort-Glomeromycota partnerships and vascular plant arbuscular mycorrhizas [53]. These findings lend support to the notion that associations with Mucoromycotina could be an ancestral state, giving way to plant-Glomeromycota fungal associations as the atmosphere and soils developed and plants diversified through the Palaeozoic.

The occurrence of Mucoromycotina and/or Glomeromycota symbioses across extant early lineages of non-vascular and vascular plants challenges the hitherto monolithic paradigm of arbuscular mycorrhizas as the ancestral land plant-fungal symbiosis, and it raises the probability that early plant lineages, through their long evolutionary history, variously became independent of fungi and then recruited them again. Similarly, some Mucoromycotina and Glomeromycota fungi appear to have switched symbioses, e.g. some Endogone form ectomycorrhizas with trees and Geosiphon harbour cyanobacteria, respectively. The current, albeit fragmentary due to limited plant and fungal sampling, understanding of the phylogenetic distribution of fungal symbioses in liverworts points to the potential loss of ancestral Mucoromycotina symbiosis and the subsequent reacquisition of the same plus Glomeromycota. Thus, Mucoromycotina fungi occur in the earliest diverging Haplomitriopsida, are lacking in the early Marchantiopsida orders Sphaerocarpales and Blasiales [19] and co-occur with Glomeromycota across complex and simple thalloid lineages. Exclusive associations with Glomero-, Basidio- and Ascomycota are restricted to the more derived groups [19,47].

The first functional study of liverwort-Mucoromycotina symbiosis [53] showed that nutritional benefits to the host plant remained the same or even increased under modern day atmospheric CO_2 concentrations, in sharp contrast to the response observed in liverwort-

Glomeromycota associations [25]. If liverwort-Mucoromycotina symbioses perform much better than glomeromycotean symbioses under modern atmospheric scenarios, why did the first apparently lose out to the latter? With latest research indicating that a wide range of extant early divergent land plant clades engage in partnerships with diverse members of both the Mucoromycotina and Glomeromycota, sometimes simultaneously [50,52], an emerging evolutionary scenario would envisage that dual fungal partnerships might have been a prevailing strategy among land pioneers. It is possible that Haplomitriopsida liverworts never evolved the capacity to form symbioses with Glomeromycota fungi and remained Mucoromycotina-specific through their 400+ million year evolutionary history. Conversely, liverworts known to harbour exclusively Glomeromycota fungi, including some species in the genera Marchantia, Conocephalum and Preissia, most likely diverged during the Cretaceous, a period of rapid flowering plant and fern radiation [58]. We speculate that during this period, major changes in both abiotic and biotic, below- and above-ground dynamics led to the predominance of strictly biotrophic Glomeromycota fungi in plant roots. It is possible that Glomeromycota-specific liverworts evolved in Glomeromycota-dominated environments and subsequently never engaged in associations with Mucoromycotina fungi. Thus, it is possible that these later diverging, Glomeromycota-specific liverworts either lack or have lost the ability to engage with Mucoromycotina fungi.

Varied options to conquer land

Did early land colonists rely on more symbiotic strategies than hitherto assumed? The phylogenetic distribution of partnerships involving one, both or neither of the early diverging fungal lineages Mucoromycotina and Glomeromycota, in early diverging lineages of extant plants suggests that this was indeed the case. Additional support for the possible antiquity of dual fungal symbioses comes from the fossil record. Whilst it seems likely that all Rhynie chert plants interacted with Glomeromycota fungi [11,18], recent palaeontological research points to more diverse early symbiotic plant-fungus encounters. A re-examination of the early Devonian fossil plant Horneophyton lignieri reveals that its fungal endophytes have characteristic features of Glomeromycota and Mucoromycotina [59], including intercellular fungal proliferation, a distinct character of Mucoromycotina colonization in extant Haplomitriopsida liverworts, hornworts and lycopods. This observation places Mucoromycotina-only and dual Mucoromycotina and Glomeromycota-plant associations within the timeframe of early plant terrestrialization and diversification, pointing to more versatile and shifting evolutionary scenarios in plant-fungal symbioses than previously assumed.

Whilst the potential nutritional benefits of dual partnerships with both Glomeromycota and Mucoromycotina fungi in living plants remain to be tested, it is tempting to speculate that for small plants occupying highly heterogeneous and dynamic environments, the ability to associate with more than one fungal partner might be a winning strategy. For the earliest land colonizing plants, and similarly for many modern plants occupying equivalent wide ranges of stable and disturbed habitats, a bet-hedging strategy whereby a plant can ‘pick and choose’ a facultative symbiont (i.e. Mucoromycotina), an obligate one (i.e. Glomeromycota), both, or neither, can yield significant fitness advantages and additional plasticity. Parallel functional studies to those on Glomeromycota- [25] and Mucoromycotina-specific [53] land plant symbioses are needed to determine the potential benefits of contrasting strategies across the carbon dioxide gradient that accompanied the evolution and diversification of land plants. Axenic isolation and symbiotic resynthesis experiments coupled with cytological investigation can reveal subtle diagnostic differences in the plant-fungus intracellular interface between Glomeromycota and Mucoromycotina colonizations in plants. Given that structures close in morphology to arbuscules, the diagnostic feature of glomeromycotean colonization (see Box 1), can be produced by Mucoromycotina, we can no longer be certain that the presence of these structures in plant cells is diagnostic of exclusive partnerships with Glomeromycota fungi unless inclusive identification methods are applied.

Widening views of plant-fungal symbiosis

The biochemical and genetic mechanisms regulating and promoting the interactions between plants and Glomeromycota fungi appear largely conserved throughout the plant kingdom [21]. The mechanisms by which Mucoromycotina fungi engage with plants remain to be investigated. Recently identified diffusible plant and fungal signalling molecules engage plant roots and AM fungi in dialogue. The plant molecular SYM pathway, or "symbiotic toolkit", guides entry and accommodation of fungi (and in legumes, of nitrogen-fixing bacteria) within plant root tissues [23]. The release of biochemical signals into soil by vascular plants engages AM fungi in the processes of contacting and entering roots. The production of plant signals, including strigolactones, has been detected in some green algae, non-vascular and flowering plants. However, in algae and non-vascular plants they do not engage fungi; instead they behave as hormones stimulating plant rhizoid elongation [60-62]. Despite this, there is conservation of the ‘toolkit’ of proteins, microRNAs and other small molecules involved in initiation and regulation of symbiosis across the land plant phylogeny, even in non-symbiotic lineages such as mosses (see Box 3). Together with the ubiquitous presence of SYM genes across all plant lineages, intriguingly including the Mucoromycotina-specific Haplomitriopsida [20], these findings suggest that the ability to form mycorrhizas or mycorrhiza-like associations has been conserved from symbiotic gametophytes of non-vascular plants through to symbiotic sporophytes of vascular plants. It remains to be determined, however, which plant genes originally permitted the accommodation of which fungi within plant tissues. An alternative but untested hypothesis is that their evolution was linked to harbouring an overlooked or extinct microbial symbiont of green algae [22].

The assumption that function of mycorrhizal and mycorrhiza-like fungal associations is also conserved across land plant evolution has, until recently, been based on striking homology between fossilised arbuscule-like structures in early Devonian plants and the Glomeromycota arbuscular structures of most living plants [63] (see Box 1). Based on shared arbuscule morphology it is assumed that any nutritional gains in well-studied vascular plants would be echoed in the earliest branching lineages of non-vascular plants and, by extension, in the earliest land plants to colonise the inhospitable Ordovician landmasses. However, while we know for instance that functioning in modern vascular plant-Glomeromycota symbiosis changes according to atmospheric CO_2 concentrations [64], the only evidence for similar responses in early-branching lineages of vascular plants is based on a single fern [25] and two liverworts [24,25]. Recent research demonstrates that the exclusively Mucoromycotina symbioses of the Haplomitriopsida are also responsive to changes in CO_2 [53], but the functional significance of dual fungal colonisations across early branching lineages of land plants remains to be elucidated on a comprehensive range of liverworts, hornworts and early vascular plants.

Concluding remarks

Typically asymptomatic microbes are universal symbionts in land plants, as in the microbiomes of other multicellular eukaryotes, but there is accumulating evidence in the few non-vascular plants that have been examined so far that important nutritional mutualisms occur with diverse fungi. Associations with newly discovered Mucoromycotina and/or well-known Glomeromycota fungi occur in several non-vascular and vascular plants. Research is required to assess the extent to which Mucoromycotina colonize land plants, particularly within liverworts and early-branching lineages of both non-flowering and flowering vascular plants; how often Mucoromycotina and Glomeromycota co-colonize plants; and whether the resulting associations are consistently mutualistic. Combined molecular, cytological, physiological and ecological studies are essential to understand the biology of Mucoromycotina, their intimate interactions with plants, the significance of dual fungal colonizations (viz. how carbon gains and nutrient costs are shared by different co-occurring symbionts) and their potential to partition ecosystems. It is possible that, given their shared symbionts, pioneer non-vascular plants facilitated vascular plant colonization and diversification in the past, and might still be doing the same today. Further, it is critical that research prioritizes understanding the physiological and molecular mechanisms by which plant specificity towards Mucoromycotina versus Glomeromycota fungi, and viceversa, arises and is regulated. An unprecedented approach merging palaeontology, eco-physiology, biogeochemistry, developmental biology, and -omics disciplines including neglected non-vascular plants, early-branching vascular plants, and fungi promises fascinating discoveries into the intertwined past, present and future of plants, microbes, soils and the biosphere.

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Boxes

Glossary

Arbuscular mycorrhizal fungi: members of the phylum Glomeromycota that form cell-to-cell nutritional associations with plant roots and extend filaments into soil to forage for minerals, colonize other roots and form spores. They often produce characteristic highly branched tree-like structures known as arbuscules within colonised plant cells.

Biotroph: an organism nutritionally dependent on another living organism.

Coenocytic: refers to a multinucleate mass of cytoplasm, characteristic of non-septate filamentous fungi.

Depletion zone: the volume of soil surrounding a root that becomes depleted of nutrients.

Embryophyte: plants in which the embryo is retained within maternal tissues, includes all clades of living land plants.

Endophyte: typically cryptic and asymptomatic microbe living within a plant, or any microbe within or between intact fossilized plant cells.

Gametophyte: haploid stage in the alternation of generations during the life cycle of plants, it is dominant in non-vascular plants.

Glomeromycota: a fungal phylum composed of biotrophs including all known arbuscular mycorrhizal fungi and a mutualistic fungus of photosynthetic bacteria (i.e. Geosiphon). It includes the orders Archaeosporales, Paraglomerales, Diversisporales and Glomerales.

Hypha: a fungal filament made up of tubular cells.

Mucoromycotina: a fungal subphylum of early diverging fungal lineages composed of saprotrophs, parasites and mutualists. It includes the orders Endogonales (pea truffles) and Mucorales (pin moulds).

Mutualism: a symbiosis that increases the fitness of all partners.

Mycorrhiza: "fungus-root", a typically mutualistic symbiosis between plant roots and fungi. “Mycorrhiza-like” is used to refer to mutualistic fungal symbiosis in plants without true roots.

Non-vascular plants: land plants without true (i.e. lignin-containing) vascular tissue, also known as bryophytes. They include three phyla: Marchantiophyta (liverworts), Anthocerotophyta (hornworts) and Bryophyta (mosses).

Rhizoid: unicellular tubular extension of an epidermal plant cell into the substrate functioning in attachment and resource uptake.

Saprotroph: an organism that fulfils its nutritional needs from dead organic matter, a decomposer.

Sporophyte: diploid stage in the alternation of generations during the life cycle of plants. It is dominant in vascular plants.

Symbiosis: the living together of unlike organisms, sometimes restricted to persistent mutualisms.

Thallus: body of some plants and fungi that is not differentiated into organs.

Vascular plants: land plants with lignified vascular tissue (i.e. xylem), also known as tracheophytes. They include the phyla Lycopodiophyta (lycopods or clubmosses), Pteridophyta (ferns and allies), Pinophyta (conifers), Cycadophyta (cycads), Ginkgophyta (Ginkgo), Gnetophyta (Gnetum, Welwitschia and Ephedra), and Magnoliophyta (flowering plants or angiosperms).

Box 1. Structure of arbuscular mycorrhizal associations

Mycorrhizal fungi of the Glomeromycota in flowering plants preferentially colonize root cortical cells. The principal sites for nutrient exchange through phosphorus, nitrogen and carbon transporter proteins between the symbiotic partners are intracellular tree-like fungal structures known as arbuscules. Depending on the plant they colonise, the fungi take two main forms inside plant root tissues. In '*Arum*' colonisation, fungi grow through plant intercellular spaces before penetrating root cells and branching to form characteristic arbuscules and/or vesicles. '*Paris*'-type colonisation is common in many non-vascular plants including liverworts and hornworts. The latter is typical of many plants with minimal intercellular spaces where colonisation is characterised by extensive intracellular growth producing fungal coils, sometimes without producing arbuscules or vesicles [5].

Box 2. Evolutionary stability of plant-fungal symbioses

It has been postulated that for mutualistic interactions between plants and fungi to persist across evolutionary timescales they require stabilisation in the form of reciprocal rewards, i.e. 'generous' plant partners are rewarded with more nutrient returns from fungal partners in exchange for enhanced plant carbon investment on fungi. Kiers et al. [65] carried out one of the few investigations to take into account the fact that most plants have multiple simultaneous Glomeromycota fungal partners and that likewise, most symbiotic fungi simultaneously associate with multiple plants. Accordingly, it is hypothesised that the plant-mycorrhizal mutualism is stabilised because both plants and fungi are able to detect variation in phosphorus and carbon resources exchanged, allowing them to adjust resource allocation - individuals are able to withhold nutrients until maximum "reward" for their "investment" is achieved. The reciprocity of the exchange, with partners able to regulate allocation of their resources to each other, should generate evolutionary stability. It has been shown that this could also apply to nitrogen and carbon resources, with carbon supply by host plants resulting in increased nitrogen allocation by fungal partners [66] and that fungi might be able to discriminate between plant partners based on their carbon wealth [67]. This in turn corroborates a previous study suggesting the symbiosis operates on a "tit-for-tat" basis [68]. However, stability does not mean invincibility. Mutualisms are open to the evolution of "cheating" whereby a partner fails to "repay" a carbon or nutrient "debt", becoming parasitic. For example, narrowly specialized non-photosynthetic angiosperms that depend on an arbuscular mycorrhizal fungus and the non-photosynthetic liverwort Aneura mirabilis that parasitizes a basidiomycete fungus [69]. Plants that have evolved mycoheterotrophic-to-autotrophic lifecycles are also not taken into account in the model of evolutionary stability. For example, the arbuscular mycorrhizal symbiosis operates on a 'take now, pay later' basis in an early-diverging fern lineage [56]. Here, a subterranean gametophyte is supported by fungi until commencement of autotrophy, at which point the fern 'repays' the Glomeromycota fungus with photosynthates [56]; specificity and intergenerational fidelity could be important in stabilizing benefits of mutualisms in some plants.

Box 3. Mosses are asymbiotic

Given the widespread occurrence of fungal symbioses in liverworts and hornworts, their absence in mosses stands out as anomalous; there are some 10,000 moss species in the same range of habitats as liverworts and hornworts, and mosses possess the genetic toolkit to form fungal symbioses [22]. Though fungal fruiting bodies, including species-specific ones, are often found growing on mosses [70], the fungi are either saprophytes or parasites. There is no physiological evidence for nutritional interdependence or any image showing fungi in symbiosis with healthy cells. Indeed, the extensive incidence of pegs of host cell wall encasing invading hyphae is indicative of an anti-fungus immune response apparatus [19].

A likely answer to the absence of symbiotic fungi in mosses relates both to their early evolutionary history and to the acquisition of highly differentiated multicellular rhizoids subsequent to the divergence of the early Sphagnales, Andreaeales and Takakiales. The peat moss Sphagnum has limited contact with mineral substrates and obtains nutrients principally from atmospheric sources whilst the same is likely in Andreaea which mainly grows on hard siliceous rocks. The ill-differentiated thallus-like structures at the stem bases function solely as organs of attachment [71]. The thickened epidermal cell walls in the rhizomes of Takakia are an unlikely fungal interface.

The mosses' multicellular rhizoids, found in all later moss lineages with their tip-growing filaments, subapical side branching and differentiated cells differ from the unicellular rhizoids and root hairs of all other land plants. Whereas the latter typically have diameters ca. 20µm, branching of moss rhizoids sees a gradual reduction in size to 3-5 µm (Figure Ia,b), i.e. the same as those of soil-growing fungi. Moreover, unlike unicellular rhizoids and root hairs which are highly vacuolated, the cytology of moss rhizoids mirrors that in the photosynthate-conducting leptoids of large moss stems. Their differentiation involves mixing of the vacuolar and cytoplasmic contents (a striking parallel with phloem sieve elements), and the alignment of mitochondria and plastids along longitudinal arrays of endoplasmic microtubules [72]. The comparative efficiency of nutrient uptake between moss rhizoids and symbiotic rhizoids remains untested.

Figure I. The two rhizoid types in non-vascular plants. **(a)** Multicellular rhizoids of the moss Funaria hygrometrica, arrow points to main axis. **(b,c)** Unicellular rhizoids of the liverwort Dumortiera hirsuta, arrows point to fungi. Main moss axes have diameters like liverwort rhizoids, but ultimate moss ramifications are as narrow as fungi. Scale bars: 50µm.

Outstanding questions

The recent molecular, cytological and physiological studies on plant-fungal symbioses have given unique insights on their biology and evolution. However, several areas remain open to study and should form the focus of future lines of investigation. Many of these questions could be addressed through genomic, metabolomic and eco-physiological investigation of early-branching plant lineages and Mucoromycotina fungi using monoxenic cultures.

1) The evolutionary relationships between fungi of the Glomeromycota and Mucoromycotina remain under investigation; studies are now required to establish whether these fungal lineages are sisters or one is older and to reveal the full molecular diversity of symbiotic Mucoromycotina. In resolving the phylogenomic relationships between these key fungal groups, we could be able to infer the ancestral symbiotic type and how it evolved to the present day.

2) In terms of symbiotic engagement, what were the first signals between fungi and newly evolved land-plants? For example, are comparable fungal and non-vascular plant responses observed upon exposure to plant strigolactones and fungal lipochitooligosaccharides, respectively, as in vascular plants?

3) Regarding nutritional exchange between partners, are similar or different phosphorus, nitrogen and carbon transporter proteins operating in arbuscular mycorrhizas and plant-Mucoromycotina symbioses? Are the fungal symbioses of hornworts and lycopods nutritionally mutualistic? How do asymbiotic plants compete with symbiotic plants? What is the significance of plant-fungal-cyanobacterial symbioses known from some hornworts, liverworts and cycads?

4) It remains unclear whether and how declining CO_2 through the Palaeozoic favoured the retention of dual fungal associations in many non-vascular plants to the present day. Methods allowing discrimination between fungal partners in vivo will provide clues as to why dual fungal colonisation occurs in some liverworts, hornworts, lycopods and ferns.

Figure legends

Figure 1. Land plant phylogeny showing estimated dates of divergence, symbiotic fungi and key features of major land plant clades. A + indicates presence of fungal group within extant members of plant clade. The numbers of living species in each plant group are shown between parentheses and fossilised plant groups are shown at the bottom with approximate dates of fossilisation. Liverworts are the earliest diverging group of extant plants and are shown to associate with Mucoromycotina, Glomeromycota, Basidiomycota and Ascomycota fungi. The only other clade known to associate with all mycorrhizal fungal groups are the conifers. Mosses are asymbiotic, while hornworts, lycopods and ferns are only known to associate with Mucoromycotina and Glomeromycota fungi. The Rhynie chert fossils provide the only detailed snapshot of early vascular plants and their associated fungi, some of which bear resemblance to extant examples of plant-Glomeromycota and/or plant-Mucoromycotina partnerships.

Figure 2. Key abiotic and biotic characteristics of the environment, plants and fungi during colonisation of the land by plants. The first emergent plants were likely liverwort-like, lacking in roots and vasculature. Fossil and molecular evidence indicates that the earliest plants were associated variously with obligately biotrophic Glomeromycota, partially saprotrophic Mucoromycotina or even both fungal groups simultaneously. The fungi likely aided plants in their conquest of the land masses through provision of mineral nutrients in return for photosynthetically-fixed carbohydrates through enhanced biological mineral weathering. With the evolution of increasingly complex plant vasculature and organs and the buildup of organic matter in the soil, atmospheric CO_2 dramatically decreased in line with increasing plant demand. Ultimately, flowering plants rose to supremacy on Earth. The majority of extant land plants now form arbuscular mycorrhizal (or mycorrhiza-like in plants

without roots) associations with fungi of the Glomeromycota. However, recent discoveries show Mucoromycotina and dual Mucoromycotina-Glomeromycota fungal associations occur throughout non-vascular plants. Atmospheric carbon dioxide concentrations from GEOCARB III [73]. The first land plants were possibly Ordovician or Silurian cryptophytes [13], though body form and rhizoids are unknown. Drawings are adapted and reproduced with permission from [74-76].

Figure 3. Scanning electron micrographs illustrating key features of the fungi that form intimate symbioses with the most ancient lineages of land plants. **(a)** Mucoromycotina in pure culture; these fungi were recently isolated from a host plant for the first time [53]; **(b)** Mucoromycotina in symbiosis within cells of the Haplomitriopsida liverwort Treubia lacunosa. **(b)** Within plant cells, Mucoromycotina produce coils (asterisk), and short-lived swellings (arrow). **(c,d)** Glomeromycota in symbiosis with the complex thalloid liverwort Marchantia pappeana, showing fungal structures typical of arbuscular mycorrhizas in flowering plants: an intracellular arbuscule and trunk hyphae (c) and a large vesicle (d). **(e)** Arbuscules are not a constant feature of Glomeromycota in early diverging land plants; for example, in the rhizome of the fern Psilotum nudum, Glomeromycota fungi only form coils and terminal vesicles. **(f)** Except for Haplomitriopsida liverworts, fungal entry into liverworts is via rhizoids, shown here in the complex thalloid liverwort Neohodgsonia mirabilis. Note the abundance of fungi within a rhizoid (insert). The arrow points to the fungal colonization zone in the centre of the liverwort. Scale bars: (c, d) 10µm; (b, e) 50µm; (a) 100µm; (f) 1000µm.

(d, e) reproduced from Fig. 4f, l in Strullu-Derrien et al., 2014.