
Article:
Field, K.J., Leake, J.R., Tille, S. et al. (5 more authors) (2015) From mycoheterotrophy to mutualism: Mycorrhizal specificity and functioning in Ophioglossum vulgatum sporophytes. New Phytologist, 205 (4). 1492 - 1502. ISSN 0028-646X

https://doi.org/10.1111/nph.13263

From mycoheterotrophy to mutualism: mycorrhizal specificity and functioning in *Ophioglossum vulgatum* sporophytes

Katie J. Field[1*], Jonathan R. Leake[1], Stefanie Tille[1], Kate E. Allinson[1], William R. Rimington[2,3,4], Martin I. Bidartondo[2,3], David J. Beerling[1] and Duncan D. Cameron[1]

[1] *Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, UK*

[2]*Department of Life Sciences, Imperial College London, London SW7 2AZ, UK*

[3]*Jodrell Laboratory, Royal Botanic Gardens, Kew, TW9 3DS, UK*

[4]*Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK*

* Corresponding author:

Katie J. Field (k.field@sheffield.ac.uk)

Tel: +44-(0)114 2220093

Fax: +44-(0)114 2220002

Summary

- Mycorrhizal functioning in the fern *Ophioglossum* is complex and poorly understood. It is unknown whether mature *O. vulgatum* sporophytes form mutualistic associations with fungi of the Glomeromycota and with what specificity. Are green sporophytes able to 'repay' fungal carbon invested in them by mycorrhizal partners during the initially heterotrophic gametophyte and early sporophyte stages of the lifecycle?
- We identified fungal partners of *O. vulgatum* sporophytes using molecular techniques and supplied them with ^{33}P-orthophosphate and *O. vulgatum* sporophytes with $^{14}CO_2$. We traced the movement of fungal-acquired nutrients and plant-fixed carbon between symbionts and analysed natural abundance ^{13}C and ^{15}N isotope signatures to assess nutritional interactions.
- We found fungal specificity of *O. vulgatum* sporophytes towards a mycorrhizal fungus closely related to *Glomus macrocarpum.* Our radioisotope tracers revealed reciprocal carbon-for-phosphorus exchange between fern sporophytes and fungal partners, despite competition from surrounding vegetation. Monocultures of *O. vulgatum* were enriched in ^{13}C and ^{15}N, providing inconclusive evidence of mycoheterotrophy when experiencing competition from the surrounding plant community.
- We show mutualistic and specific symbiosis between a eusporangiate fern and fungi of the Glomeromycota. Our findings suggest a 'take now, pay later' strategy of mycorrhizal functioning through the lifecycle *O. vulgatum,* from mycoheterotrophic gametophyte to mutualistic above-ground sporophyte.

Key words: Competition, mycoheterotrophy, mycorrhiza, Ophioglossaceae*, Ophioglossum vulgatum*, pteridophyte, specificity, symbiosis

Introduction

The symbiosis between plants and arbuscular mycorrhizal fungi dates back more than 450 million years to the colonisation of the land by plants (Read *et al.,* 2000; Bonfante & Selosse, 2010). To better understand the role of mycorrhizal fungi in land plant evolution, there is increasing interest in resolving relationships between plants and their fungal partners to determine how these associations may have changed both across the land plant phylogeny and functionally through coevolution (Bidartondo *et al.,* 2004; Merckx & Bidartondo 2008; Arnold *et al.,* 2010; Merckx *et al.,* 2012; Merckx *et al.,* 2013). Advances in molecular and physiological ecology have provided new insights into the evolutionary history of the symbiosis in the major clades of plants and fungi (Wang *et al.,* 2010; Bidartondo *et al.,* 2011, Field *et al.,* 2012, 2014). However, important knowledge-gaps regarding the fungal partners of plants in key nodes of the land plant phylogeny remain (Fig. 1). In particular, neither the identity of mycorrhizal fungi nor their functional roles have been determined in the widely distributed (Singh *et al.,* 2009) basal euphyllophyte ("true-leaved plant") genus *Ophioglossum*, thought to have evolved prior to the break-up of Gondwana (Parris, 2001).

In common with >1,000 species of lycophytes and ferns, the subterranean gametophyte generations of *Ophioglossum* are achlorophyllous, nourished with organic carbon and nutrients via mycorrhizal fungi (Boullard, 1979; Leake, 1994; Winther & Friedman, 2007). This form of nutrition, termed mycoheterotrophy, has evolved many times in land plants (Leake, 1994; Bidartondo, 2005; Merckx & Freudenstein, 2010) with examples ranging from a liverwort, to lycopods, ferns and angiosperms (Fig. 1). The initial developmental stages of *Ophioglossum* sporophytes are also achlorophyllous (Bruchmann, 1904) and mycoheterotrophic. However, mature sporophytes consist of a characteristic blade-like green photosynthetic shoot (the trophophore) (Fig. S1a), often accompanied by an epiphyllous fertile sporophore, in which the functional role of mycorrhiza has not been investigated. Because of the life-stage changes from heterotrophy to autotrophy mycorrhizal functioning in *Ophioglossum* is both complex and poorly understood.

Ophioglossum vulgatum L. (Fig. S1a), is one of the most widespread and abundant grassland species in the Ophioglossaceae (GRIN taxonomic database). Sporophytes of *O. vulgatum* are initially subterranean, achlorophyllous and colonised by aseptate fungi that form irregular hyphal swellings in the plant tissues (Bruchmann, 1904; Boullard, 1979). These fungi must provide the main carbon and nutrient supplies required to enable development of the underground root axis from which shoots develop (Bruchmann, 1904).

Recent studies of achlorophyllous gametophyte and photosynthetic sporophyte generations of lycopods (*Lycopodium* and *Huperzia*) and ophioglossoid ferns (*Botrychium*) have revealed specificity and intergenerational fidelity in their arbuscular mycorrhizal fungal associates (AMF) (Winther & Friedman, 2007, 2008, 2009). This suggests carbon invested by AMF partners in supporting a mycoheterotrophic gametophyte and early subterranean sporophyte may be repaid by established green sporophytes. Such 'take now, pay later' mycorrhizal functioning has been suggested as the basis of fungal specificity and overall mutualism through the mycoheterotrophic-to-autotrophic life stages of many green-leaved orchids (Cameron *et al.,* 2008). However, experimental evidence for photosynthate 'pay back' to fungal symbionts of any of the lower tracheophytes is currently lacking. Studies of mycorrhiza specificity and functioning in these plants are of particular interest for species like *O. vulgatum* that often inhabit plant species-rich permanent grasslands that host a highly diverse community of AMF ranging from 24 to more than 70 phylotypes (Vandenkoornhuyse *et al.,* 2002; Johnson *et al.,* 2004; Dumbrell *et al.,* 2011).

Intergenerational fungal specificity opens the possibility of intergenerational carbon subsidy from green-leaved sporophytes to achlorophyllous gametophytes via a shared fungal partner, a form of 'parental nurture' suggested by Leake *et al*. (2008). This contrasts with the suggestion that stabilization of mutualistic interactions in AM symbioses with autotrophs involves the plants providing organic carbon strictly in proportion to the nutrients delivered by the fungus (Fitter, 2006; Kiers *et al.,* 2011). Under the latter model of mutualism in the chlorophyllous sporophyte, any fungal 'reward' for carbon investment in the gametophyte and early stages of sporophyte establishment would be conditional upon ongoing nutrient demand by the plant and its supply from the fungus. However, tight regulation of carbon-for-nutrient exchange is not universal but represents only one position along the mutualism-parasitism continuum model of mycorrhizal symbiosis proposed by Johnson *et al*. (1997; Johnson & Graham, 2013).

There has been convergent evolution of mycoheterotrophic protocorm structures in lower tracheophyte gametophytes and the mycoheterotrophic seedlings of higher plants like orchids (Leake *et al.,* 2008). In the case of *O. vulgatum,* the underground early stages of sporophyte ontogeny and anatomy closely parallel those of the mycoheterotrophic seedling stages of members of the angiosperm genera *Monotropa* (Leake *et al.,* 2004) and *Pyrola* (Hashimoto *et al.,* 2012; Hynson *et al.,* 2013a) along with those of the family Orchidaceae (see Fig. S1a-b), in which germination leads to the formation of a simple branched root system from which

shoots later arise by adventitious buds, often only after several growing seasons underground. Sprouting from detached roots has been reported in *O. vulgatum* (Bruchmann, 1904; Wardlaw, 1953), providing a means of asexual reproduction and possible reversion to mycoheterotrophy when roots are detached from photosynthetic shoots, a frequent trait of mycoheterotrophic plants.

It may be possible to assess the extent to which green-leaved plants, such as trophophore-bearing *O. vulgatum,* are partially mycoheterotrophic through measurements of their tissue ^{13}C and ^{15}N natural abundance as these isotopes are often enriched in fully mycoheterotrophic plants (Gebauer & Meyer, 2003; Cameron & Bolin, 2010; Hynson *et al.,* 2013b). However, the extent of relative ^{13}C and ^{15}N enrichment of AM plants with putative and established mycoheterotrophy has proved inconsistent (Courty *et al.,* 2011; Hynson *et al.,* 2013b).

Mycorrhizal colonization of *O. vulgatum* sporophytes has been reported many times, with exquisite line drawings of hyphae in roots by Bruchmann (1904) and degenerated arbuscule-like structures by Boullard (1979), as well as detailed light and electron micrographs of vesicles and coils by Schmidt and Oberwinkler (1996), all of which are consistent with Paris-type AMF (Zhang *et al*., 2004). However, the fungal symbiont(s) in *O. vulgatum* have not been identified and their functional roles are unknown for the green trophophore-bearing fern sporophytes.

This study aims to address the following questions:

1. Do *O. vulgatum* sporophytes form mycorrhizal associations with AMF, and if so, with what specificity?
2. Are mycorrhizal associations in the photosynthetic sporophyte generation mutualistic, i.e. based on exchange of plant-carbon for fungal-acquired nutrients?
3. To what degree are fern shoots supported (via mycoheterotrophy) or out-competed by surrounding vegetation within mixed communities?

Materials and methods

Plant material and fungal identification

Sporophytes of a natural population of *O. vulgatum* were collected as turf monoliths 30 cm (w) x 40 cm (l) x 20 cm (d) sampled from across an area of base-rich grassland of

144 approximately 100 m x 20 m at the margins of mature deciduous woodland at Treborth
145 Botanic Gardens, Bangor, UK in July 2009 (Fig. S2). The vegetation community consisted
146 of grasses and forbs, dominated by *Plantago lanceolata, Ranunculus repens, Trifolium*
147 *pratense, Agrostis capillaris, Festuca rubra,* and *Rubus fruticosus*. Weeding created
148 experimental mesocosms comprising three community types: *O. vulgatum* in monoculture, *O.*
149 *vulgatum* within a mixed community of grasses and forbs, and the mixed community
150 vegetation only. Care was taken to ensure each turf contained ten *O. vulgatum* shoots and
151 regular weeding maintained the composition of each community. Each mesocosm was
152 replicated four times, giving a total of 12 experimental turfs. We created a further four plant-
153 free soil monoliths in which all plants were removed to provide controls for diffusion rates in
154 our ^{33}P and ^{14}C isotope tracer studies described later.

155 We inserted soil-filled, cylindrical plastic cores (20 mm diameter, 90 mm length) with two
156 windows cut out from 30 mm below the top of the tube in a position below the soil surface
157 when the cores are inserted vertically, with 25 mm remaining above ground, covered by 35
158 μm pore-size nylon mesh (Johnson *et al*., 2001). The mesh was fine enough to exclude roots
159 while being coarse enough to allow fungal hyphal ingrowth (Fig. S3). The cores were filled
160 with native soil from the turfs mixed with 5 % v/v of sand-sized basalt grains added to
161 increase AM hyphal proliferation (Field *et al*., 2012). We inserted ten cores vertically into
162 each turf regularly spaced with one further, glass wool-filled core inserted centrally for
163 below-ground gas sampling.

164 The mesocosms were maintained under controlled glasshouse conditions designed to provide
165 conditions similar to early summer (see SI) at the Arthur Willis Environment Centre,
166 University of Sheffield, UK. Turfs were maintained for three months to allow plant and
167 fungal acclimation, community stabilisation and colonisation of the mesh cores by fungi. In
168 late October 2009, the mesocosms were moved outdoors for vernalization for three months
169 over the winter. At the beginning of February 2010, the turfs were returned to the glasshouse
170 to induce early emergence of the *O. vulgatum* sporophytes, which commenced in the first
171 weeks of March 2010 (Fig. S2).

172 Roots were randomly sampled from individual *O. vulgatum* plants from six different
173 mesocosms and cleared and stained for quantification of mycorrhizal colonisation following
174 Trouvelot *et al*. (1986) (details in SI). In parallel, fungal DNA was extracted and sequenced
175 for a further six *O. vulgatum* root samples taken from plants removed from separate turfs in
176 July 2009 (Fig. S4) following the protocols of Desirò *et al*., 2013 (details in SI).

Representative DNA sequences have been deposited in GenBank (accession numbers KJ952225-6, KM065416-20).

Quantification of reciprocal plant-fungal ^{14}C for ^{33}P exchange

Plant-to-fungus C transfer and fungus-to-plant ^{33}P assimilation was quantified using methods adapted from Johnson *et al*. (2001, 2002) and Field *et al*. (2012, 2014). Following emergence of *O. vulgatum* shoots in March/April 2010, 1.4 MBq of ^{33}P (specific activity 148 GBq $mmol^{-1}$) as 38 μl of $H_3{}^{33}PO_4$, comprising 312 ng ^{33}P (Perkin Elmer, UK) in total were added to each turf, including plant-free soil monoliths, via a perforated central capillary tube in each of the ten mesh-windowed cores established the previous year.

After 28 d the turfs were prepared for ^{14}C labelling. Anhydrous lanolin was applied to the rim of each mesh windowed core and the top sealed with a plastic cap. The glass wool-filled core in each turf was sealed with a rubber septum (SubaSeal, Sigma) to allow non-destructive monitoring of below-ground $^{14}CO_2$ respiration. The turfs remained in the greenhouse but were each enclosed in a gas-tight transparent Perspex chamber into which 2 MBq of $^{14}CO_2$, comprising 13.7 μg ^{14}C, was released from $NaH^{14}CO_3$ by addition of 2 ml 10% lactic acid soon after sunrise (07:00 h). After 1 h, and every 4 h thereafter, we sampled 1 ml of the headspace air in the Perspex chambers above each turf through a rubber septum (SubaSeal, Sigma) using a syringe. Similarly, a sample of 1 ml of air from the glass-wool filled mesh cores was taken after 1 h and every 2 h thereafter. We ceased air sampling and removed turfs from the labelling chambers following a peak in $^{14}CO_2$ detected in below-ground gas samples occurring between 14 and 16 h post-$^{14}CO_2$ release. This time-point indicates maximum flux of ^{14}C-labelled photosynthates to symbionts and other soil micro-organisms through inclusion of the isotope in below-ground respired CO_2. The air samples were injected through a SubaSeal into gas-evacuated vials containing 10 ml CarboSorb (Perkin Elmer, Beaconsfield, UK). Permafluor scintillation cocktail (Perkin Elmer, Beaconsfield, UK) was then added (10 ml) and ^{14}C measured by liquid scintillation counting (Packard Tri-Carb 3100TR, Isotec, UK).

Once the ^{14}C detected in the glass-wool filled mesh windowed cores had peaked, we removed the soil-filled cores and placed them into individual airtight containers with 2 ml 2M KOH to trap respired $^{14}CO_2$. Cores were incubated with fresh 2M KOH every 2 h for a further 6 h. We transferred 1 ml of each KOH sample used to trap $^{14}CO_2$ into a scintillation vial containing 10

ml Ultima Gold scintillation cocktail and radioactivity was measured by liquid scintillation (Packard Tri-carb 3100TR, Isotech).

We removed above-ground vegetation from each turf and divided the samples into *O. vulgatum* and 'plant community' tissue samples. We sieved the soil to extract and separate the *O. vulgatum* and plant community roots. For the plant-free soil monoliths the bulk soil outside the mesh cores was sieved, homogenized and subsampled for ^{33}P determination to test for diffusion and non-plant-mycorrhiza mediated transfer of isotope from the cores to the surrounding soil.

All plant and soil samples were freeze-dried, weighed and homogenised into a powder using a Yellowline A10 Analytical Grinder (IKA, Germany). Samples underwent acid-digestion before mixing with Emulsify-Safe (Perkin Elmer, Beaconsfield, UK) and ^{33}P activity measured by liquid scintillation counting (details in SI). The ^{33}P content in plant tissues was calculated by following the equation in Cameron *et al.* (2007) after decay correction and scaled to plant biomass to give total plant ^{33}P (see SI).

To quantify ^{14}C present within each sample, between 10 and 100 mg of freeze-dried homogenized sample were placed into individual Combusto-Cones (Perkin Elmer), oxidised in a Model 307 Packard Sample Oxidiser, (Isotech, UK) with the $^{14}CO_2$ trapped in 1:1 v/v Carbosorb: Permafluor scintillation cocktail and counted (Packard Tri-Carb 3100TR, Isotech). Total C fixed by the plants and made available to AM fungal networks within soil cores (hereafter termed 'fungal carbon') was determined by calculating the difference in ^{14}C found in the soil cores in vegetated turfs compared to those in the plant-free soil monoliths. The calculated quantity of ^{14}C allocated from the plants into the mesh-windowed cores as fungal carbon, after correction using the plant-free controls, was expressed as a function of total volume of atmospheric CO_2 in the labeling chamber and the proportion of the supplied $^{14}CO_2$ label fixed by the plants (Cameron *et al.,* 2008) (see SI).

Natural abundance ^{13}C and ^{15}N enrichment and total % N

Relative abundances of ^{13}C and ^{15}N and tissue %N were determined using Isotope Ratio Mass Spectrometry. Between 2 and 5 mg of freeze-dried homogenised root and shoot tissues of *O. vulgatum* and community plants from all experimental turfs ($n = 3$ for each tissue type in each turf) were weighed out into 6 x 4 mm tin capsules (Sercon Ltd, UK) and analysed using a continuous flow Isotope Ratio Mass Spectrometer (PDZ 2020 IRMS, Sercon Ltd, UK). Data are expressed as δ values, which were calculated using the following equation:

240 $\delta^{13}C$ or $\delta^{15}N = 1000[(R_{sample} / R_{standard}) - 1]$

241 Where R = molar ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ of the sample or standard (Hynson *et al.,*
242 2013b). Air was used as the reference standard for ^{15}N and commercially available poplar
243 leaf standards for ^{13}C after every 10 samples analysed. The detector was regularly calibrated
244 to commercially available reference gases.

245

246 *Statistical analyses*

247 The effect of monoculture of *O. vulgatum* versus growth in grassland community, and the
248 effects on the community of the presence or absence of *O. vulgatum* on biomass, $\delta^{13}C$ and
249 $\delta^{15}N$, %N, ^{33}P uptake and ^{14}C allocation were tested using ANOVA with post-hoc Tukey
250 testing where appropriate. Data were checked for normality and homogeneity of variance.
251 Where assumptions for ANOVA were not met, data were transformed using Log_{10} and
252 arcsine-square-root transformations as indicated in Table 1. All statistics were calculated in
253 Minitab v12.21 (Minitab Inc., US). Different letters in figures denote statistical significance
254 (following transformation where necessary, see Table 1) where $P < 0.05$ and error bars show
255 ± 1 standard error.

256

257 **Results**

258 *Mycorrhizal colonisation and fungal identification*.

259 *Ophioglossum vulgatum* roots (n = 6 plants) showed a 100% frequency of mycorrhizal
260 colonisation (Table S1) with both coils and arbuscules being present (Fig. S5). The mean
261 colonisation intensity was just over 40% of root segments examined. Arbuscule abundance
262 was low, with a mean of 7% of root segments examined. The six *O. vulgatum* sporophytes
263 contained a fungal associate from the Glomeraceae that corresponded closely with the
264 published sequences for *Glomus macrocarpum*, one also harboured a member of
265 Diversisporaceae (Fig. 2; GenBank accession numbers KJ952225-6, KM065416-20).

266 *Biomass*

267 *Ophioglossum vulgatum* roots and shoots had 4 and 8.5 times greater biomass respectively
268 when grown in monoculture than in the plant community turfs (Fig. 3a, Table 1) even though
269 the mesocosms contained identical numbers of *O. vulgatum* shoots (Fig. S4). The

surrounding plant community showed no difference in root or shoot biomass whether *O. vulgatum* sporophytes were present or had been removed from the turf (Fig. 3a).

The root:shoot biomass ratio of *O. vulgatum* more than doubled when the ferns were grown within mixed community turfs compared to monocultures, and in the community it was 22 times higher than the surrounding vegetation (Fig. 3b). There was no significant difference in root:shoot biomass ratio of the community vegetation whether the ferns were present or removed.

Plant-to-fungus carbon transfer into soil compartments.

Ophioglossum vulgatum monocultures supplied the fungal networks within soil cores with just over half the amount of carbon provided by the grassland community turfs, although the shoot biomass of monoculture *O. vulgatum* was only $1/8^{th}$ of that of the community (Fig. 3a and 4a). Removal of *O. vulgatum* had no effects on total fungal C allocated into the soil cores by the plant community.

Carbon allocation to fungus in the mesh-windowed cores per unit of shoot biomass, was more than four times higher for monocultures of *O. vulgatum* than for the plant community turfs, (Fig. 4b, Table 1) irrespective of whether the latter contained *O. vulgatum* or not.

Shoots of the grassland community retained just over double the amount of new photosynthate (^{14}C + $^{12/13}C$) found in *O. vulgatum* shoots grown in monoculture. The suppressed shoots of *O. vulgatum* grown in the community turfs contained negligible amounts of new photosynthate (Fig. 4c). In monoculture, *O. vulgatum* allocated three-fold more photosynthate to its roots than the grassland community, and the community-suppressed ferns showed negligible photosynthate allocation to roots (Fig. 4c, Table 1).

The concentration of new photosynthate in *O. vulgatum* monoculture shoots was between three and four-fold higher than in the shoots of the plant community [two-sample t (4 df) = -6.47, P = 0.0029], and 17-fold higher than in the fern when grown in the community turfs [two-sample t (3 df) = 9.39, P = 0.0026] (Fig. 4d, Table 1). Similarly, new photosynthate concentration in roots of monocultures of the fern exceeded substantially those of the turfs [two-sample t (3 df) = 6.89, P = 0.0063] and the suppressed ferns in the community turfs [two-sample t (3 df) = 7.31, P = 0.0053] (Fig. 4d).

Fungus ^{33}P uptake from mesh-windowed soil cores and transfer to plants

We were unable to detect any ^{33}P transfer from mesh-walled cores to bulk-soil in the plant-free mesocosms, indicating that transfer of ^{33}P to the plants in the vegetated mesocosms was driven by plant-fungal associations. The uptake of ^{33}P into *O. vulgatum* shoots was strongly affected by competition from other plants, falling from 4 ng when grown in monoculture to less than 0.06 ng when grown in the grassland community (Fig. 5a, Table 1). In monoculture *O. vulgatum* took up ~30% of the total ^{33}P that was assimilated by the grassland community on its own, whereas when grown in direct competition with the community, the fern took up < 0.7% of the ^{33}P gained by neighbouring plants. Removal of *O. vulgatum* had no effect on ^{33}P uptake into shoots of the community plants (Fig. 5a).

^{33}P concentration in shoots of *O. vulgatum* grown in monoculture was 2.4 times higher than in the shoots of the plant community without the fern. In the plant community-grown *O. vulgatum* shoot ^{33}P concentrations declined by nearly an order of magnitude (8.6) to become no longer significantly different to that of shoots of the surrounding plants (Fig. 5b).

Plant N concentration and natural abundance ^{15}N and ^{13}C

Overall, *O. vulgatum* shoots had higher N concentrations than community shoots, being 43% higher than the community shoot concentrations when grown separately, and 83% higher than the community values when grown together [two-sample t (10 df) = 3.35, P = 0.012]. Root %N in *O. vulgatum* was consistently higher than in the both root and shoots of the community plants, but this was not statistically significant (Fig. 6a, Table 1). Monoculture and community-grown *O. vulgatum* showed no significant differences in shoot or root %N, and there was no effect of removal of the fern on the %N in the community plant shoots or roots (Fig. 6a).

Ophioglossum vulgatum shoots and roots had greater ^{15}N abundance than the grassland community plants (Fig. 6b). The mean ^{15}N abundance in *O. vulgatum* shoots grown in monoculture was higher than that of community plants by $\delta^{15}N$ values of 3.2 ‰ (two-sample t (10 df) = 5.53, P = 0.0003), although there was no significant difference between fern $\delta^{15}N$ values when it was grown in monoculture or within a mixed community. Overall, *O. vulgatum* root tissues had greater abundance of ^{15}N than the grassland community plant roots $\delta^{15}N$ values by 1.6 ‰, (two sample t (13 df) = 2.52, P = 0.026). There was no significant

difference in root ^{15}N abundance between ferns grown in a mixed community or those grown in monoculture (Fig. 6b, Table 1).

Ophioglossum vulgatum shoots contained a greater abundance of ^{13}C when grown in monoculture compared to the shoots of the community turfs [two-sample t (7 df) = 4.04, P = 0.0049], and compared to the fern grown in the plant community (Fig. 6c, Table 1). ^{13}C was more abundant in *O. vulgatum* grown within mixed community turfs than in the community with which it was growing, but it was not different from the shoots of the community from which it had been excluded the previous year. There was no significant difference between the $\delta^{13}C$ values in the plant communities with, and without, *O. vulgatum* present (Table 1). Root tissue ^{13}C abundance showed similar responses to the shoots, but the differences were not significant (Fig. 6c).

Discussion

Do O. vulgatum sporophytes form mycorrhizal associations with AMF, and if so, with what specificity?

Our study provides the first definitive evidence that photosynthetic sporophytes of a eusporangiate fern in the genus *Ophioglossum* form mutualistic mycorrhizal associations with a fungus in the Glomeromycota. Field-collected roots of *Ophioglossum vulgatum* were all colonised by a mycorrhizal symbiont closely related to *Glomus macrocarpum* (Fig. 2 and Fig. S5). The fungus formed hyphal coils and arbuscules throughout the root cortex, consistent with previous reports indicating 'Paris-type' AMF colonization in the sporophyte of *O. vulgatum* (Russow, 1872; Bruchmann, 1904; Boullard, 1976; Schmidt and Oberwinkler 1996; Zhang *et al.,* 2004).

Glomus macrocarpum is widely distributed throughout grassland, herbaceous and woodland habitats, being especially abundant in association with *Acer* tree species (Klironomos & Kendrick 1996). While there are a number of reports of this fungal species increasing host plant biomass production and phosphorus uptake (Nandini & Tholkappian, 2012), it is also the causal agent of tobacco stunt disease (Guo *et al*. 1994). Large reproductive structures formed by *G. macrocarpum* and stunting of some host plants suggest that colonisation by this fungus can place a high carbon demand on host, potentially representing a substantial carbon source for the mycoheterotrophic gametophytes of *O. vulgatum*. Moreover, *G. macrocarpum*

364 is a derived species of *Glomus* (Glomeraceae) (Fig. 2), a feature shared with the fungal
365 symbionts of members of the sister Ophioglossaceae genus *Botrychium* (Winther &
366 Friedman, 2007). Both *B. lanceolatum* and *B. crenulatum* have specialised on a narrow range
367 of Glomeromycota fungi (Merckx *et al.,* 2012). The specialisation of some ferns on these
368 closely related fungal taxa suggests that plant and fungal partners have co-evolved through
369 phylogenetic tracking of the partnership over millions of years, as has been shown in the
370 highly AM-specific mycoheterotroph genus *Afrothismia* (Burmanniaceae) in which more
371 recently diverged members of this genus associate with Glomeromycete fungi that have
372 diverged from a specific fungal partner of the ancestral plants (Merckx & Bidartondo, 2008).
373
374 The apparent specificity of *O. vulgatum* is consistent with the high fungal specificity in the
375 sister genus *Botrychium* where it is linked to intergenerational gametophyte-sporophyte
376 fidelity (Winther & Friedman, 2007), and contrasts to many other fern species which
377 typically host several species of Glomeromycota fungi (West *et al.,* 2009). Fungal specificity
378 and intergenerational fidelity is documented in several lycopods (see Fig. 1) including
379 *Lycopodium clavatum* and *Huperzia* spp. (Winther & Friedman, 2008). In these cases,
380 fidelity is maintained, even though there is no fungal colonisation across the gametophyte-
381 sporophyte tissue junctions (Ligrone *et al.,* 1993; Winther & Friedman, 2007, 2008). In the
382 *Botrychium* species studied by Winther & Friedman (2007), and in *Psilotum nudum* (Winther
383 & Friedman, 2009; Fig. 1), the mycoheterotrophic gametophytes show extreme fungal
384 specificity, each plant typically only associated with a single fungal partner. However, the
385 green shoot stage of sporophyte generations often host several mycorrhizal fungi in its roots
386 in addition to retaining the specific partner of the gametophytes. The single *O. vulgatum* root
387 that contained a fungus from the Diversisporaceae in addition to *G. macrocarpum*, may be an
388 example of this, but a larger number of plants across a number of sites would need to be
389 sampled to resolve this issue.
390
391 We were unable to find or raise any *O. vulgatum* gametophytes to test for intergenerational
392 fidelity in its AMF. Assuming this specificity occurs at all developmental stages, we concur
393 with the previously published notion that parental nurture may occur from photosynthetic
394 sporophyte-to-mycoheterotrophic gametophyte via interconnecting fungal partners, as
395 suggested for members of the Lycopodiaceae and Ophioglossaceae (Winther & Friedman,
396 2008; Leake *et al.,* 2008).
397

Are mycorrhizal associations in the photosynthetic sporophyte generation mutualistic, i.e. based on exchange of plant-carbon for fungal-acquired nutrients?

Using isotope tracers, we clearly demonstrate the reciprocal exchange of plant-carbon for fungal-acquired nutrients (N and P) between the green sporophyte of *O. vulgatum* and its fungal partner, *G. macrocarpum*. This life-stage dependent trophic switch from heterotrophic juvenile to autotrophic adult is not uncommon and has been observed across a diverse array of tracheophytes including most orchids (Leake and Cameron 2010). The substantial carbon allocation to the external mycorrhizal mycelium in our *O. vulgatum* monocultures provides the first experimental evidence that carbon invested by a fungus in supporting the heterotrophic juvenile life stage of a eusporangiate fern may be repaid with photosynthates later.

In this 'take now, pay later' form of mutualism, fungal partners may have to wait many years before they are repaid for their investment of carbohydrate in the plants (Cameron et al. 2008; 2006). This contrasts with the view that mycorrhizal mutualism in plant communities is stabilized by a 'biological market' in which plant photosynthate is exchanged for fungal-acquired nutrients in a tightly coupled pay and reward system. This is conceptualized as involving either preferential photosynthate supply to fungal partners that are delivering the greatest amounts of growth-limiting nutrients (Fitter, 2006; Kiers *et al.,* 2011), or fungal partners preferentially allocating nutrients to host plants supplying the greatest amount of photosynthate (Fellbaum *et al.,* 2012; Fellbaum *et al.,* 2014). Clearly, in plants that for part of their lifecycles do not photosynthesise, during which time they are mycoheterotrophic, the biological market model of mutualism cannot be operational (Selosse & Rousset, 2011). We hypothesise that high fungal specificity and fidelity through mycoheterotroph-to-autotroph plant lifecycles, stabilizes the symbiosis through selection for net overall fitness benefits for both partners over their lifetimes. However, such a symbiosis does leave the fungi vulnerable to exploitation in cases where the plants do not repay them in full as in complete mycoheterotrophs (Leake & Cameron 2010).

To what degree are fern shoots supported (via partial mycoheterotrophy) or out-competed by surrounding vegetation within mixed communities?

The major impact of the plant community is to partially supress both trophophore and root biomass, together with reducing ^{33}P uptake by *O. vulgatum*. Moreover, under competition

from the grassland community the root:shoot ratio of *O. vulgatum* increased, likely reflecting reduced shoot growth in response to light, water or most likely, nutrient limitation. Together, these observations demonstrate the superior competitive ability of the community vegetation compared to this fern.

Previous work has shown the efficiency of phosphorus exchange for photosynthates between plants and AM fungi increases with the evolution of increasing plant complexity from liverworts to a fern and angiosperm (Field *et al.,* 2012). This is consistent with the results of the present study. In nature, *O. vulgatum* often occurs in mixed communities adjacent to woodlands where there is likely to be at least some grazing pressure. We did not simulate grazing or mowing of the community, consequently the herbaceous plant community grew taller, and probably produced denser shade than in the woodland margin site from which the *O. vulgatum* turfs originated. The fern trophophore has a small photosynthetic area, simple vasculature and limited root surface area, so is poorly adapted to compete for light, water and nutrients with the more structurally advanced angiosperm community. Although we did not measure it directly in this investigation, low rates of photosynthesis are reported in *O. vulgatum* (Löhr, 1968). We expect this to also be the case in the shaded examples within mixed community turfs presented here.

Partial mycoheterotrophy is typically accompanied by a suite of distinguishing physiological characteristics, including elevated tissue ^{13}C and ^{15}N contents. Such traits have been seen in green-leaved orchids as well as in mycoheterotrophic orchid protocorms and fully mycoheterotrophic adult orchids (Gebauer & Meyer, 2003; Leake & Cameron, 2010; Hynson *et al.,* 2013b; Stöckel *et al.,* 2014;) along with some mycoheterotrophic plants associated with AM fungi (Cameron & Bolin 2010; Merckx *et al*. 2010; Courty *et al.* 2011). Under suppression by the plant community, we expected that if the sporophyte were able to revert to partial mycoheterotrophy, this would be reflected in the stable isotope abundances of ^{15}N and ^{13}C compared to *O. vulgatum* grown in monoculture. In contrast to our expectations, the ^{13}C abundance of fern shoots was higher when grown in monoculture and there were no differences in ^{13}C in roots or ^{15}N in both roots and shoots of fern monocultures versus the ferns grown in the grassland community (Fig. 6d). However, the fern consistently contained higher concentrations of these isotopes than the mixed-species community, most likely resulting from high rates of carbon allocation belowground to their mycorrhizal partner and to storage, both causing preferential respiratory losses of ^{12}C, and storage of N resulting in

enrichment in ^{15}N. When grown in the turf, *O. vulgatum* trophophores have $\delta^{13}C$ values closer to those of the community plants, either as a result of reduced allocation of C to below-ground storage (Fig. 4c-d) or mycoheterotrophic carbon gain from surrounding vegetation. Unfortunately, our experimental design does not allow us to resolve the extent to which either (or both) processes are involved and this issue warrants further investigation.

Conclusions and perspectives:

This study represents the first assessment of mycorrhizal functioning in a eusporangiate fern revealing the association is mutualistic in mature sporophytes. The symbiosis appears to operate on a 'take-now, pay-later' basis (Cameron *et al.,* 2008) with fully mycoheterotrophic subterranean gametophytes and early achlorophyllous sporophyte stages (Bruchmann, 1904; Boullard, 1979; see Fig. 1), followed by the photosynthetic trophophore of the sporophyte participating in bidirectional exchange of organic carbon-for-nutrients with a fungus closely related to *Glomus macrocarpum*. Whether the sporophyte then reverts to mycoheterotrophy during its below-ground dormancy period from mid-summer until early spring of the following year requires further investigation.

Our findings support the hypothesis that other early-diverging trachaeophytes with mycoheterotrophic gametophytes that retain the same fungal partners as in their sporophytes, are also likely to operate this form of mutualism. This strengthens the view that specificity and fidelity may be important in stabilizing the benefits of mutualistic interactions for mycoheterotroph-to-autotrophic lifecycle transitions in plants. Our findings provide clear evidence of the strength of competitive interactions and suppression of the growth of *O. vulgatum* sporophytes by angiosperms, but we were unable to establish the extent to which sporophytes are mycoheterotrophic when the trophophore is not present. Further experimental work is required to resolve the relative carbon contributions of ophioglossoid ferns to shared mycorrhizal networks and whether this carbon contributes to the establishment of the subterranean gametophyte and initial stages of the sporophyte generations.

Acknowledgments

We thank Nigel Brown, Treborth Botanic Gardens, Bangor for field logistics, Dr Heather Walker for stable isotope analyses and Irene Johnson for technical support. The work was

498 funded though a NERC award (NE/F019033/1). DDC is supported by a Royal Society
499 University Research Fellowship. We thank the anonymous referees and the editor for
500 comments that have improved the manuscript.

501

502 **Tables**

503 **Table 1. Summary of differences in resource allocation and acquisition (*F* ratio from**
504 **ANOVA) in root and shoot tissues of *Ophioglossum vulgatum* and community**
505 **vegetation.** All two-way ANOVA have 3, 24 d.f., and 3, 15 d.f. for one-way ANOVA. **P* <
506 0.05, ***P*<0.01, ****P*<0.001; post-hoc Tukey test (*n* = 4). Where assumptions for ANOVA
507 were not met, data were transformed as indicated in the table.

	Turf treatment	**Tissue type**	**Turf x tissue**
Dry mass (g) *(log(10) transformed)*	37.59 ***	0.04	12.51 ***
Root : shoot (g) *(log(10) transformed)*	13.05 ***		
Fungal C / above-ground plant biomass (ng g^{-1})	7.70 *		
Fungal C (ng)	4.43 *		
Total ^{14}C in plant tissues (ng) *(log(10) transformed)*	41.72 ***	28.72 ***	9.60 ***
[^{14}C] in plant tissues (ng g^{-1}) *(arcsine square root transformed)*	268.36 ***	127.20 ***	19.24 ***
^{33}P in shoot tissues (ng) (*arcsine square root transformed)*	58.98 ***		
[^{33}P] in plant tissue (ng g^{-1}) *(arcsine square root transformed)*	11.51 **		
Plant tissue % N	7.48 **	12.19 **	3.78 *
$\delta^{15}N$ in shoots	11.20 ***	1.93	1.42
$\delta^{13}C$ in shoots	10.63 ***	22.95 ***	4.10 *

508

Figure legends

Figure 1. Evolution of mycoheterotrophy in land plants. Lineages where myco-heterotrophy is present indicated with bold branches, dashed branches show clades with myco-heterotrophic gametophytes. Text in brackets indicates genus/families within clades displaying myco-heterotrophic properties. Clades with arbuscular mycorrhizas are indicated with an asterisk (adapted from Bidartondo, 2005 and Field *et al*., 2012)

Figure 2. Molecular identification of fungal symbionts within root tissues of *O. vulgatum* sporophytes showing phylogenetic placement within AM fungi. Taxonomy follows Schüßler *et al*. (2011). Maximum likelihood tree produced in MEGA v.5, evolutionary model T92 + G + I with 10,000 bootstrap replicates.

Figure 3 (a) Total dry mass of above-ground and below-ground plant tissues in experimental turfs (g). ($n = 4$) **(b)** Root:shoot dry mass ratios of plant tissues in experimental turfs ($n = 4$), ± 1 S.E. Letters show statistical significance, where $P < 0.05$ (ANOVA, Tukey post-hoc, data log(10) transformed to meet assumptions for ANOVA). Note that the letter codes in (a) compare both root and shoot biomass.

Figure 4 (a) Total carbon allocated by plant assemblages to below-ground mycorrhizal networks per gram of above-ground plant biomass (ng g^{-1}) **(b)** Total carbon allocated by plant assemblages to mycorrhizal fungal networks within turfs (ng) **(c)** Total new photosynthate (ng) within plant tissues following the ^{14}C labelling period **(d)** Tissue concentration of new photosynthate following the ^{14}C labelling period (ng g^{-1}). Error bars show ± 1 S.E. Different letters denote statistical significance, where $P < 0.05$ (ANOVA, Tukey post-hoc, data transformed using log(10) and arcsine-square-root transformations where assumptions of ANOVA were not met) $n = 4$.

Figure 5 (a) Total ^{33}P assimilation into above-ground tissues of plants in experimental turfs (ng) **(b)** ^{33}P concentration (ng g^{-1}) of above-ground *O. vulgatum* and mixed community plant tissues ($n = 4$). Error bars show ± 1 S.E. Different letters indicate where $P < 0.05$ (ANOVA, Tukey post-hoc. Data arcsine-square-root transformed where assumptions for ANOVA not met).

Figure 6 (a) Total tissue % N of plants within turfs **(b)** $\delta^{15}N$ enrichment of plant tissues within turfs **(c)** $\boldsymbol{\delta}^{13}C$ enrichment in above-ground biomass for *O. vulgatum* and mixed community plants in experimental turfs **(d)** $\boldsymbol{\delta}^{13}C$ vs. $\delta^{15}N$ enrichment of plant tissues ($n = 4$).

In all panels, error bars show ± 1 S.E. Different letters denote statistical significance, where $P < 0.05$ (ANOVA, Tukey post-hoc).

References

Arnold AE, Lamit LJ, Gehring CA, Bidartondo MI, Callahan H. 2010. Interwoven branches of the plant and fungal trees of life. *New Phytologist* **185:** 874-878.

Bidartondo MI. 2005. The evolutionary ecology of myco-heterotrophy. *New Phytologist* **167**: 335-352.

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

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **271**: 1799-1806.

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

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

Bonfante P, Selosse MA. 2010. A glimpse into the past of land plants and of their mycorrhizal affairs: from fossils to evo-devo. *New Phytologist* **186**: 267-270.

Boullard B. 1979. Considerations sur la symbiose fongique chez les pteridophytes. *Syllogeus* **19**: 1-64

Bruchmann H. 1904. Über das Prothallium und die Keimpflanze von *Ophioglossum vulgatum* L. *Botanische Zeitung* **62**: 227-248.

Cameron DD, Bolin JF. 2010. Isotopic evidence of partial mycoheterotrophy in the Gentianaceae: *Bartonia virginica* and *Obolaria virginica* as case studies. *American Journal of Botany* **97**: 1272-1277.

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

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

Courty E-P, Walder F, Boller T, Ineichen K, Wiemken A, Rousteau A, Selosse M-A. 2011. Carbon and nitrogen metabolism in mycorrhizal networks and mycoheterotrophic plants of tropical forests: a stable isotope analysis. *Plant Physiology* **156:** 952-961.

Desirò A, Duckett JG, Pressel S, Villarreal JC, Bidartondo MI. 2013. Fungal symbioses in hornworts: a chequered history. *Proceedings of the Royal Society B-Biological Sciences* **280**:1-8.

Doyle GJ. 1985. A woodland habitat for *Botrychium lunaria (*L.) Swartz in Ireland. *Irish Naturalist Journal*. **21:** 403-406.

Duff RJ, Nickrent DL. 1999. Phylogenetic relationships of land plants using mitochondrial small-subunit rDNA sequences. *American Journal of Botany* **86**: 372-386.

Dumbrell AJ, Ashton PD, Aziz N, Feng G, Nelson M, Dytham C, Fitter AH, Helgason T. 2011. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytologist* **190:** 794-804

Fellbaum CR, Gachomo EW, Beesetty Y, Choudhari S, Strahan GD, Pfeffer PE, Kiers ET, Bücking H. 2012. Carbon availability triggers fungal nutrient uptake and transport in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences* **109:** 2666-2671.

Fellbaum CR, Mensah JA, Cloos AJ, Strahan GD, Pfeffer PE, Kiers ET, Bücking H. 2014. Fungal nutrient allocation in common mycelial networks is regulated by the carbon source
strength of individual host plants. *New Phytologist* **203:** 646-656.

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, Rimington WR, Bidartondo MI, Allinson KE, Beerling DJ, Cameron DD, Duckett JG, Leake JR, Pressel S. 2014. 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* **doi:** 10.1111/nph.13024

Fitter AH. 2006. What is the link between carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. *New Phytologist* **172:** 3-6.

Gebauer G, Meyer M. 2003. ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist* **160**: 209-223.

Guo BZ, An ZQ, Hendrix JW. 1994. A mycorrhizal pathogen (*Glomus macrocarpum* Tul. & Tul.) of tobacco: effects of long- and short-term cropping on the mycorrhizal fungal community and stunt disease. *Applied Soil Ecology* **1:** 269-276.

Hashimoto Y, Fukukawal S, Kunishil A, Suga H, Richard F, Sauve M, Selosse M-A. 2012. Mycoheterotrophic germination of *Pyrola asarifolia* dust seeds reveals convergences with germination in orchids. *New Phytologist* **195:** 620-630.

Hauk WD, Parks CR, Chase MW. 2003. Phylogenetic studies of Ophioglossaceae: evidence from *rbcL* and *trnL-F* plastic DNA sequences and morphology. *Molecular Phylogenetics and Evolution* **28:** 131-151.

Hynson NA, Preiss K, Gebauer G. 2009a. Is it better to give than to receive? A stable isotope perspective on orchid–fungal carbon transport in the green orchid species *Goodyera repens* and *Goodyera oblongifolia*. *New Phytologist* **182**: 8-11.

Hynson NA, Preiss K, Gebauer G, Bruns TD. 2009b. Isotopic evidence of full and partial myco-heterotrophy in the plant tribe Pyroleae (Ericaceae). *New Phytologist* **182**: 719-726.

Hynson NA, Bruns, TD. 2010. Fungal hosts for mycoheterotrophic plants: a non-exclusive but highly selective club. *New Phytologist* **185**: 598-601.

Hynson NA, Weiss M, Preiss K, Gebauer G, Treseder KK. 2013a. Fungal host specificity is not a bottleneck for the germination of Pyroleae species (Ericaceae) in a Bavarian forest. *Molecular Ecology* **22:** 1473-1481

Hynson N, Madsen T, Selosse M-A, Adam IU, Ogura-Tsujita Y, Roy M, Gebauer G 2013b. The Physiological Ecology of Mycoheterotrophy. In: Merckx V ed. *Mycoheterotrophy*: *The Biology of Plants Living on Fungi.* Springer, New York, 297-342

Johnson D, Leake JR, Read DJ. 2001. Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. *New Phytologist* **152**: 555-562.

Johnson D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ. 2004. Plant communities affect arbuscular mycorrhizal fungal

638 diversity and community composition in grassland microcosms. *New Phytologist* **161:**
639 503-515
640 **Johnson NC, Graham JH, Smith FA. 1997.** Functioning of mycorrhizal associations along
641 the mutualism-parasitism continuum. *New Phytologist* **135:** 575-585.
642 **Johnson NC, Graham JH. 2013.** The continuum concept reamins a useful framework for
643 studying mycorrhizal functioning. *Plant & Soil* **363**: 411-419.
644 **Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse M-A. 2005.**
645 Mixotrophy in orchids: insights from a comparative study of green individuals and
646 nonphotosynthetic individuals of *Cephalanthera damasonium*. *New Phytologist* **166:**
647 639-653.
648 **Kenrick P, Crane PR. 1997.** The origin and early evolution of plants on land. *Nature* **389:**
649 33-39.
650 **Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum**
651 **CR, Kowalchuk GA, Hart MM, Bago A, Palmer TM, West SA,**
652 **Vandenkoornhuyse P, Jansa J, Bucking H. 2011.** Reciprocal Rewards Stabilize
653 Cooperation in the Mycorrhizal Symbiosis. *Science* **333**: 880-882.
654 **Klironomos JN, Kendrick WB. 1996.** Palatability of microfungi to soil arthropods in
655 relation to the functioning of arbuscular mycorrhizae. *Biology and Fertility of Soils*
656 **21**: 43-52
657 **Krings M, Taylor TN, Taylor EL, Dotzler N, Walker C. 2011.** Arbuscular mycorrhizal-
658 like fungi in Carboniferous arborescent lycopsids. *New Phytologist* **191**: 311-314.
659 **Leake JR. 1994.** The biology of myco-heterotrophic (saprophytic) plants. *New Phytologist*
660 **127**: 171-216.
661 **Leake JR, Cameron DD. 2010.** Physiological ecology of mycoheterotrophy. *New*
662 *Phytologist* **185**: 601-605.
663 **Leake JR, Cameron DD, Beerling DJ. 2008.** Fungal fidelity in the myco-heterotroph-to-
664 autotroph life cycle of Lycopodiaceae: a case of parental nurture? *New Phytologist*
665 **177**: 576-579.
666 **Leake JR, McKendrick SL, Bidartondo MI, Read DJ. 2004.** Symbiotic germination and
667 development of the myco-heterotroph *Monotropa hypopitys* in nature and its
668 requirement for locally distributed *Tricholoma* spp. *New Phytologist* **163:** 405-423.
669 **Löhr E. 1968.** Trehalose, respiration und photosynthese in dem farn *Ophioglossum*, ein neuer
670 schattenblattypus. *Physiologia Plantarum* **21**: 668-672.

671 **MacDougal DT, Dufrenoy J. 1944.** Mycorrhizal symbiosis in *Aplectrum*, *Corallorhiza* and
672 *Pinus*. *Plant Physiology* **19**: 440-465.
673 **Merckx V, Bidartondo MI. 2008.** Breakdown and delayed cospeciation in the arbuscular
674 mycorrhizal mutualism. *Proceedings of the Royal Society B: Biological Sciences* **275**:
675 1029-1035.
676 **Merckx V, Freudenstein JV. 2010.** Evolution of mycoheterotrophy in plants: a phylogenetic
677 perspective. *New Phytologist* **185**: 605-609.
678 **Merckx V, Stöckel M, Fleischmann A, Bruns TD, Gebauer G. 2010.** ^{15}N and ^{13}C natural
679 abundance of two mycoheterotrophic and a putative partially mycoheterotrophic
680 species associated with arbuscular mycorrhizal fungi. *New Phytologist* **188**: 590-596.
681 **Merckx VS, Janssens SB, Hynson NA, Specht CD, Bruns TD, Smets EF. 2012.**
682 Mycoheterotrophic interactions are not limited to a narrow phylogenetic range of
683 arbuscular mycorrhizal fungi. *Molecular Ecology* **21**: 1524-1532.
684 **Merckx VSFT.** 2013 Mycoheterotrophy: An Introduction. In: Merckx V ed.
685 *Mycoheterotrophy*: *The Biology of Plants Living on Fungi.* Springer, New York, 1-18.
686 **Nandini RTS, Tholkappian P. 2012.** Co-inoculation of AM fungi *Glomus macrocarpum*
687 and *Acaulospora laevis* and *Bacillus megaterium* var. *phosphaticum* on the growth
688 and nutrient contents of *Solanum xanthocarpum* Schrad. and Wendl. *International*
689 *Journal of Pharmaceutical & Biological Archives* **3: 1137-1141**
690 **Parkash V, Aggarwal A. 2009.** Diversity of endomycorrhizal fungi and their synergistic
691 effect on the growth of *Acacia catechu* Willd. *Journal of Forest Science.* **55:** 461-468
692 **Parris BS. 2001.** Circum-Antarctic continental distribution patterns in pteridophyte species.
693 *Brittonia* **53**: 270-283.
694 **Read DJ, Duckett JG, Francis R, Ligrone R, Russell A. 2000.** Symbiotic fungal
695 associations in 'lower' land plants. *Philosophical Transactions of the Royal Society of*
696 *London Series B-Biological Sciences* **355**: 815-830.
697 **Redecker D, Kodner R, Graham LE. 2000.** Glomalean fungi from the Ordovician. *Science*
698 **289**: 1920-1921.
699 **Richburg, JA.** (2004) *Aplectrum hyemale* (Muhl. Ex Willd.) Nutt. (Puttyroot) Conservation
700 and Research Plan for New England. *New England Wild Flower Society,*
701 *Framingham, Massachusetts, USA.*
702 **Russow E. 1872.** Vergleichende Untersuchungen betreffend die Hitologie der vegetativen
703 und sporenblidenden Organe und die Entwickelung der Sporen der Leitbündel-
704 kryptogamen. In: *Mémoires de l'Acadame Impériale des Sciences de St-Pétersbourg,*

VII. Sené. Tome 19 (1). St. Petersbourg: Commissionnaires de l'Académie Impériale des sciences 1809-1830.

Schmidt E, Oberwinkler F. 1996. Light and electron microscopy of a distinctive VA mycorrhiza in mature sporophytes of *Ophioglossum reticulatum*. *Mycological Research* **100:** 843-849.

Selosse M-A, Rousset F. 2011. The plant-fungal marketplace. *Science* **333:** 828-829

Shinohara W, Nakato N, Yatabe-Kakugawa Y, Oka T, Kun Kim J, Murakami N, Noda H, Sahashi N. 2013. The use of matK in Ophioglossaceae phylogeny and the determination of *Mankyua* chromosome number shed light on chromosome number evolution in Ophioglossaceae. *Systematic Botany* **38**: 564-570.

Singh AP, Mishra S, Gupta S, Behera S, Khare P. 2009. Studies on the Genus *Ophioglossum* L. in Pachmarhi Biosphere Reserve, Madhya Pradesh-India. *Taiwania* **54**: 353-364.

Smith SE, Read DJ. 2008. *Mycorrhizal symbiosis, 3rd edition*. Cambridge, UK: Academic Press.

Stöckel M, Těšitelová T, Jersáková J, Bidartondo, MI, Gebauer G. 2014. Carbon and nitrogen during the growth of orchid seedlings in nature. *New Phytologist* **202:** 606-615

Trouvelot A, Kough J, Gianinazzi-Pearson V. 1986. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In: *Physiological and Genetical Aspects of Mycorrhizae*. Proceedings of the 1st European Symposium on mycorrhizae (Ed. V. Ginaninazzi-Pearson & S. Ginaninazzi): 217-221. Paris: INRA Press.

Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, Young JPW. 2002. Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Molecular Ecology* **11:** 1555-1564

Wang B, Yeun LH, Xue J-Y, Liu Y, Ane´ 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.

Wardlaw CW. 1953. Experimental and analytical studies of pteridophytes: XXIII. The induction of buds in *Ophioglossum vulgatum* L. *Annals of Botany* **17**: 513-528.

West B, Brandt J, Holstien K, Hill A, Hill M. 2009. Fern-associated arbuscular mycorrhizal fungi are represented by multiple *Glomus* spp.: do environmental factors influence partner identity? *Mycorrhiza* **19:** 295-304.

Wikström N, Kenrick P. 2000. Relationships of *Lycopodium* and *Lycopodiella* based on combined plastid rbcL gene and trnL intron sequence data. *Systematic Botany* **25**: 495-510.

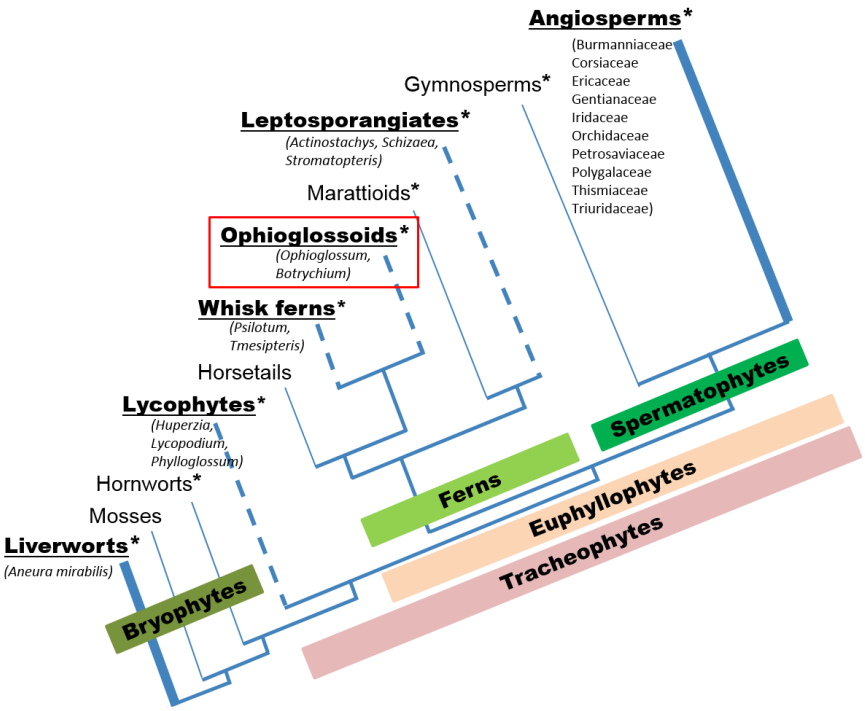
Winther JL, Friedman WE. 2007. Arbuscular mycorrhizal symbionts in *Botrychium* (Ophioglossaceae). *American Journal of Botany* **94**: 1248-1255.

Winther JL, Friedman WE. 2008. Arbuscular mycorrhizal associations in Lycopodiaceae. *New Phytologist* **177**: 790-801.

Winther J, Friedman W. 2009. Phylogenetic affinity of arbuscular mycorrhizal symbionts in *Psilotum nudum*. *Journal of Plant Research* **122**: 485-496.

Zhang Y, Guo L-D, Liu R-J. 2004. Arbuscular mycorrhizal fungi associated with common pteridophytes in Dujiangyan, southwest China. *Mycorrhiza* **14:** 25-30.

(a)


Angiosperms*
(Burmanniaceae
Corsiaceae
Ericaceae
Gentianaceae
Iridaceae
Orchidaceae
Petrosaviaceae
Polygalaceae
Thismiaceae
Triuridaceae)
Gymnosperms*
Leptosporangiates*
(Actinostachys, Schizaea, Stromatopteris)
Marattioids*
Ophioglossoids*
(Ophioglossum, Botrychium)
Whisk ferns*
(Psilotum, Tmesipteris)
Horsetails
Lycophytes*
(Huperzia, Lycopodium, Phylloglossum)
Hornworts*
Mosses
Liverworts*
(Aneura mirabilis)
Bryophytes
Ferns
Spermatophytes
Euphyllophytes
Tracheophytes

(b)

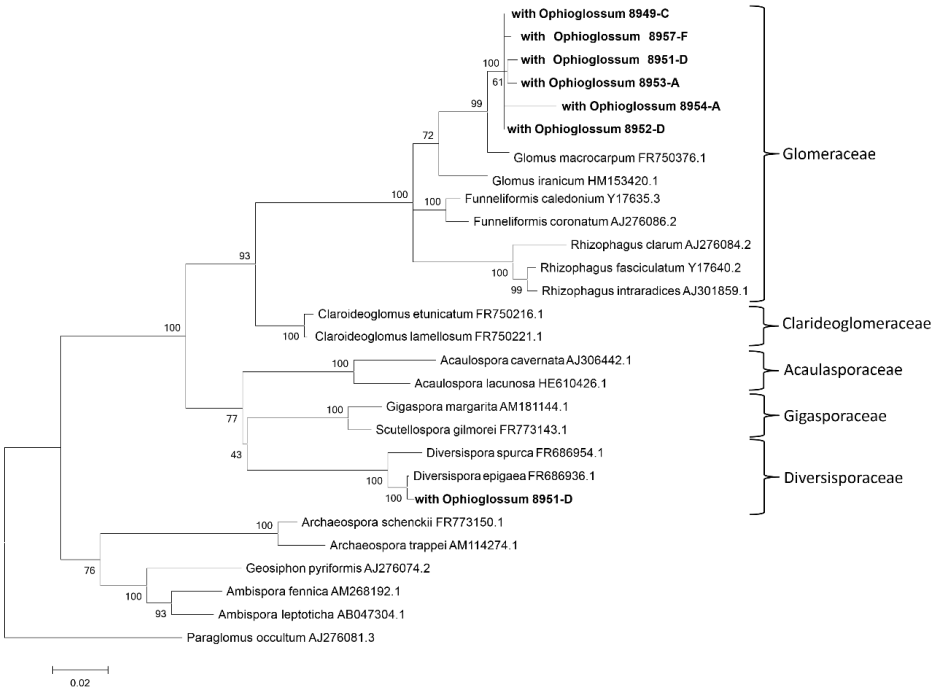

with Ophioglossum 8949-C
with Ophioglossum 8957-F
with Ophioglossum 8951-D
with Ophioglossum 8953-A
with Ophioglossum 8954-A
with Ophioglossum 8952-D
Glomus macrocarpum FR750376.1
Glomus iranicum HM153420.1
Funneliformis caledonium Y17635.3
Funneliformis coronatum AJ276086.2
Rhizophagus clarum AJ276084.2
Rhizophagus fasciculatum Y17640.2
Rhizophagus intraradices AJ301859.1
Claroideoglomus etunicatum FR750216.1
Claroideoglomus lamellosum FR750221.1
Acaulospora cavernata AJ306442.1
Acaulospora lacunosa HE610426.1
Gigaspora margarita AM181144.1
Scutellospora gilmorei FR773143.1
Diversispora spurca FR686954.1
Diversispora epigaea FR686936.1
with Ophioglossum 8951-D
Archaeospora schenckii FR773150.1
Archaeospora trappei AM114274.1
Geosiphon pyriformis AJ276074.2
Ambispora fennica AM268192.1
Ambispora leptoticha AB047304.1
Paraglomus occultum AJ276081.3
0.02
Glomeraceae
Claroideoglomeraceae
Acaulasporaceae
Gigasporaceae
Diversisporaceae

Figure 1

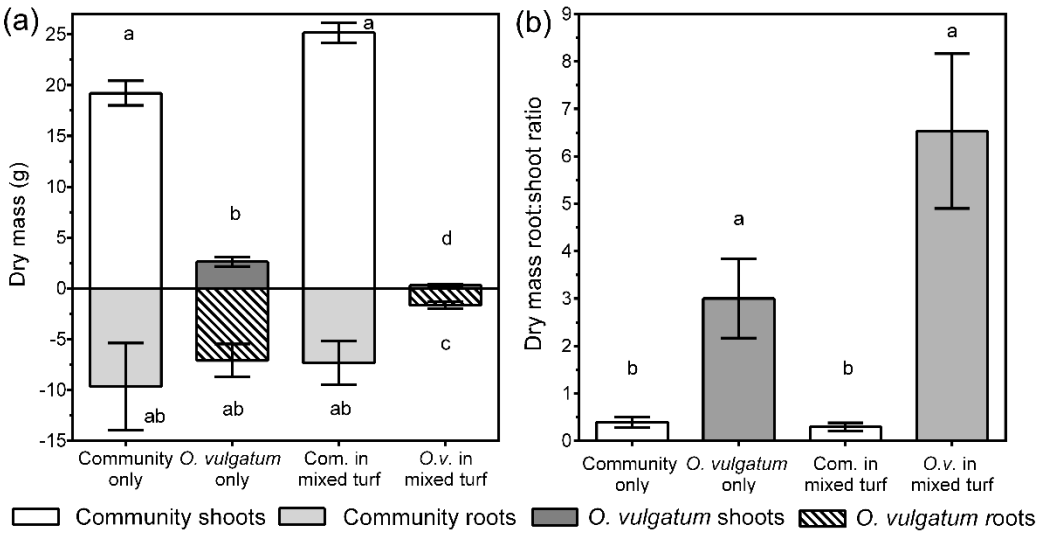

(a)
Dry mass (g)
25
20
15
10
5
0
-5
-10
-15
a
ab
b
ab
a
ab
d
c
Community only
O. vulgatum only
Com. in mixed turf
O.v. in mixed turf
(b)
Dry mass root:shoot ratio
9
8
7
6
5
4
3
2
1
0
b
a
b
a
Community only
O. vulgatum only
Com. in mixed turf
O.v. in mixed turf
Community shoots
Community roots
O. vulgatum shoots
O. vulgatum roots

Figure 2

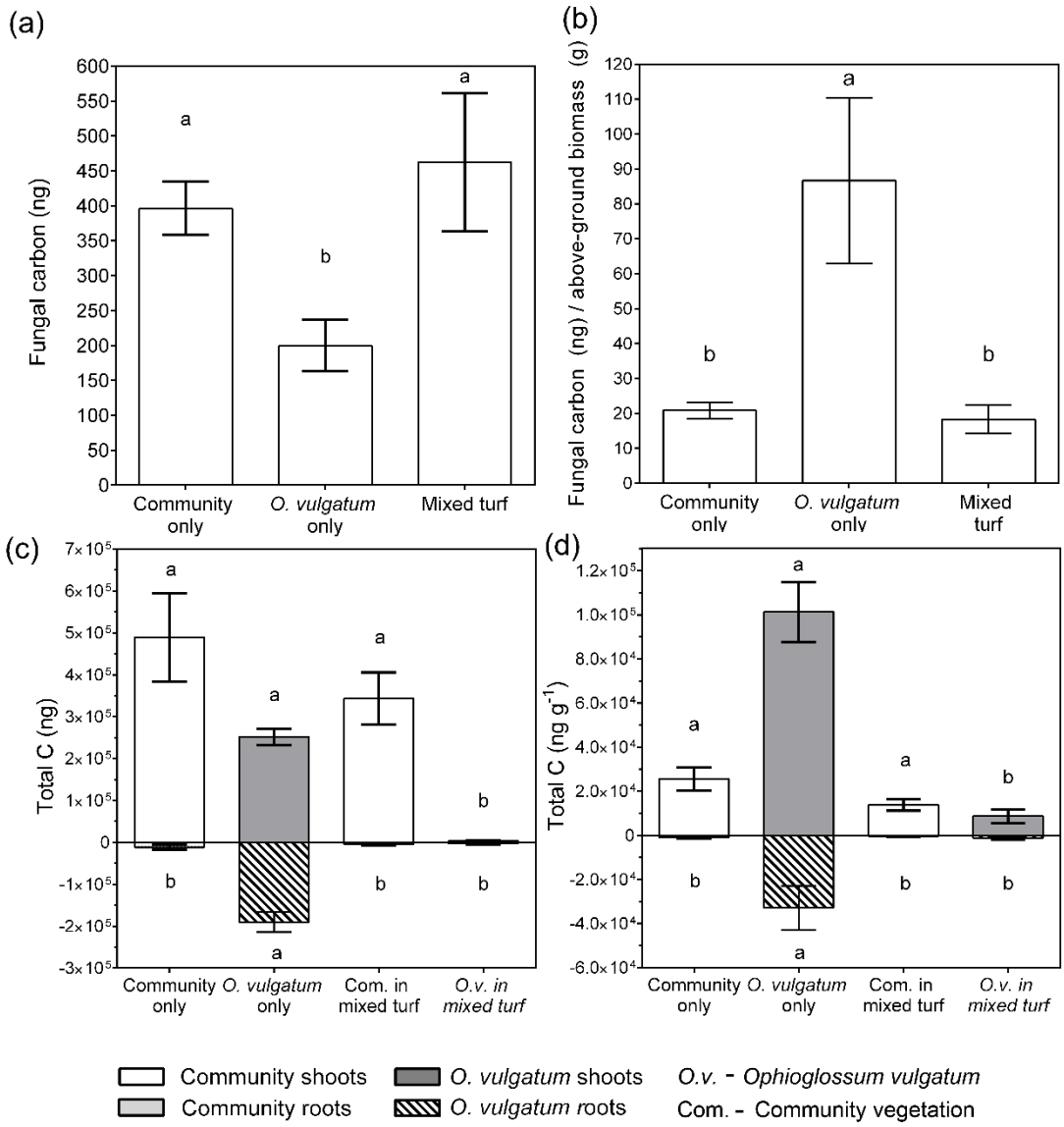

(a)
Fungal carbon (ng)
Community only
O. vulgatum only
Mixed turf
(b)
Fungal carbon (ng) / above-ground biomass (g)
Community only
O. vulgatum only
Mixed turf
(c)
Total C (ng)
Community only
O. vulgatum only
Com. in mixed turf
O.v. in mixed turf
(d)
Total C (ng g-1)
Community only
O. vulgatum only
Com. in mixed turf
O.v. in mixed turf
Community shoots
Community roots
O. vulgatum shoots
O. vulgatum roots
O.v. - Ophioglossum vulgatum
Com. - Community vegetation

Figure 3

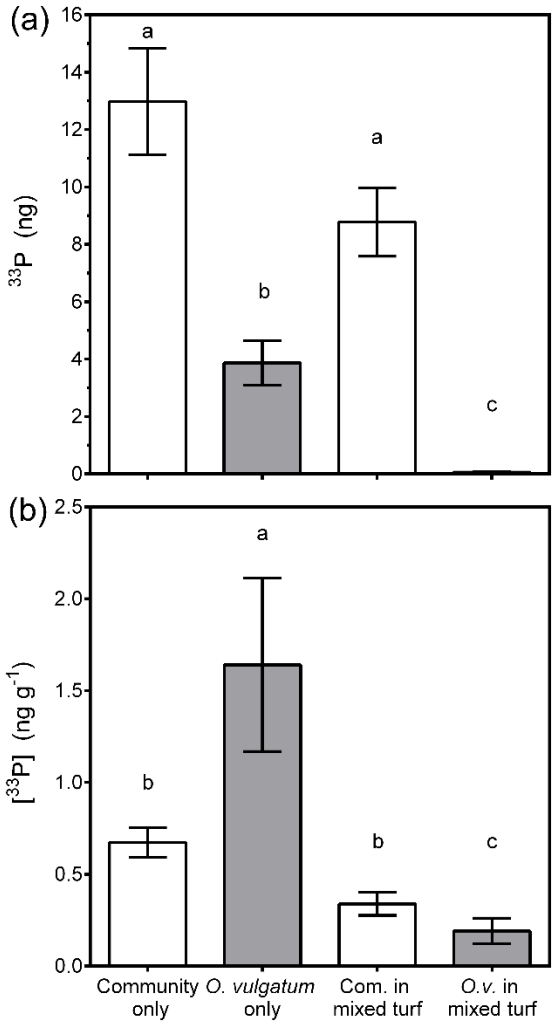
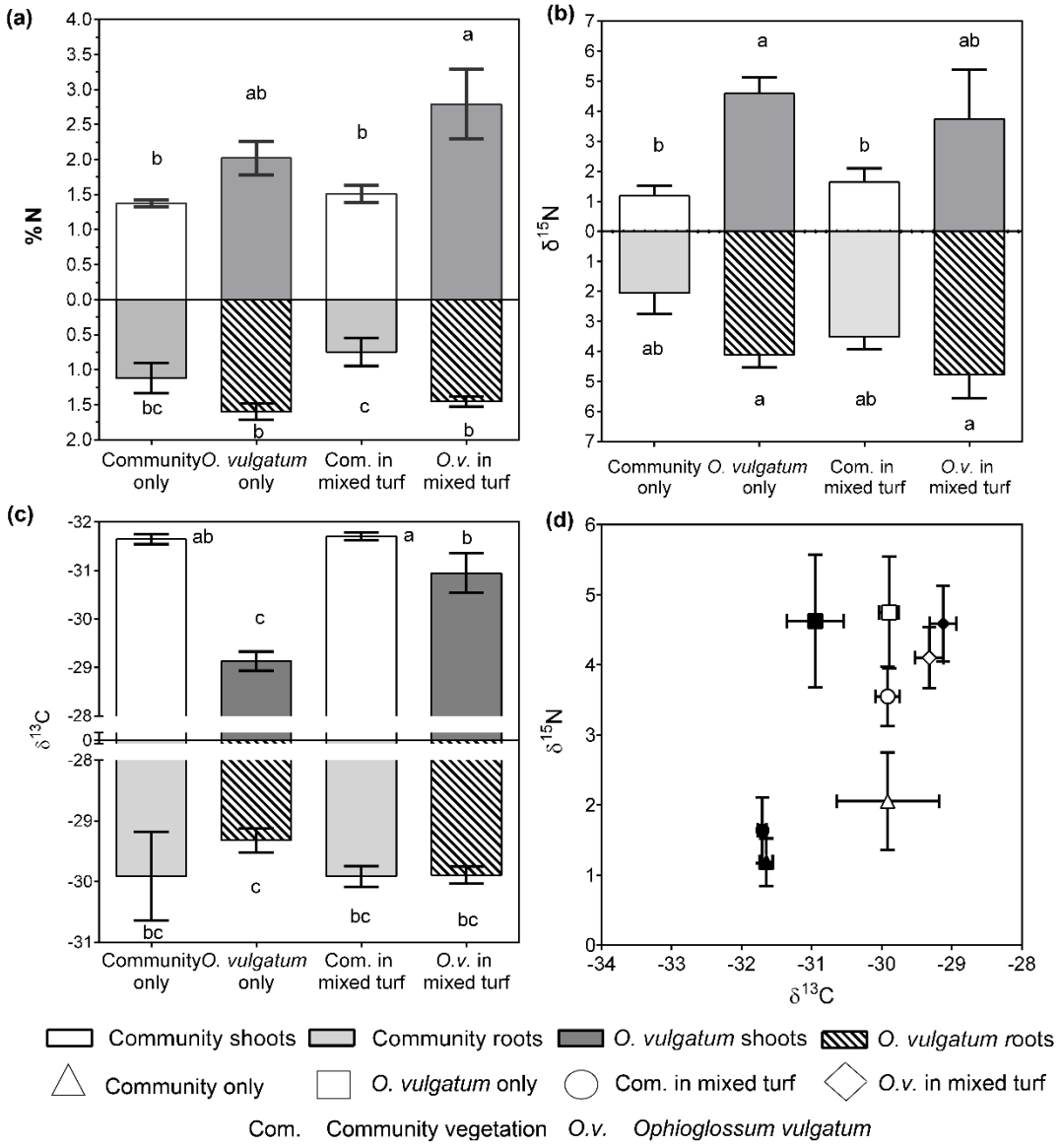

(a)
^{33}P (ng)
16
14
12
10
8
6
4
2
0
a
b
a
c
(b)
[^{33}P] (ng g^{-1})
2.5
2.0
1.5
1.0
0.5
0.0
b
a
b
c
Community only
O. vulgatum only
Com. in mixed turf
O.v. in mixed turf

Figure 4



804

805

806

807 **Figure 5**

808

809