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Research

Photosynthate transfer from an autotrophic orchid to conspecific heterotrophic protocorms through a common mycorrhizal network

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Summary

- The minute 'dust seeds' of some terrestrial orchids preferentially germinate and develop as mycoheterotrophic protocorms near conspecific adult plants. Here we test the hypothesis that mycorrhizal mycelial connections provide a direct pathway for transfer of recent photosynthate from conspecific green orchids to achlorophyllous protocorms.
- Mycelial networks of *Ceratobasidium cornigerum* connecting green *Dactylorhiza fuchsii* plants with developing achlorophyllous protocorms of the same species were established on oatmeal or water agar before the shoots of green plants were exposed to $^{14}CO_2$. After incubation for 48 h, the pattern of distribution of fixed carbon was visualised in intact entire autotrophic/protocorm systems using digital autoradiography and quantified in protocorms by liquid scintillation counting.
- Both methods of analysis revealed accumulation of ^{14}C above background levels in protocorms, confirming that autotrophic plants supply carbon to juveniles via common mycorrhizal networks. Despite some accumulation of plant-fixed carbon in the fungal mycelium grown on oatmeal agar, a greater amount of carbon was transferred to protocorms growing on water agar, indicating that the polarity of transfer may be influenced by sink strength.
- We suggest this transfer pathway may contribute significantly to the pattern and processes determining localised orchid establishment in nature, and that 'parental nurture' via common mycelial networks may be involved in these processes.

Introduction

The Orchidaceae is a diverse, globally distributed plant family, representatives of which are found in nearly all terrestrial ecosystems. As such, it is important to better understand the mechanisms that regulate the structure of its populations. Such knowledge is critical both from the perspective of understanding orchid ecology, but also to aid efforts to conserve these iconic plants. Due to their diminutive size and lack of reserves, orchid seed germination is reliant on both organic carbon and mineral nutrient supplies derived from their orchid mycorrhizal (OM) fungal associates (Arditti & Ghani, 2000; Smith & Read, 2010). In terrestrial orchids, symbiotic germination leads to the formation of an achlorophyllous mycoheterotrophic subterranean tuber-like body called a protocorm, from which shoots and roots subsequently develop (Dearnaley *et al.*, 2016; Fang *et al.*, 2016). The developmental transition from protocorm to emergent plant normally involves a full transition to autotrophy with photosynthetic leaves and shoots, except in a minority of species that remain fully or partially mycoheterotrophic (Leake, 1994; Selosse & Martos, 2014).

Most orchids form partnerships with common and globally distributed basidiomycetes in the polyphyletic genus 'rhizoctonia' especially members of the Tulasnellaceae, Ceratobasidiaceae, and sometimes Serendipitaceae (Jacquemyn *et al.*, 2017). These basidiomycetes mostly comprise endophytes and saprotrophs that are known to have some ability to decompose litter (Veldre *et al.*, 2013; Selosse & Martos, 2014; Rasmussen *et al.*, 2015; Jacquemyn *et al.*, 2017), which is a transient carbon source. One alternative mechanism by which OM fungi could gain access to organic carbon would be if they receive significant amounts of photosynthate directly from autotrophic green orchid partners. However, early investigations found no evidence of plant-to-fungus transfers of photosynthate in the green-leaved orchid *Goodyera repens* (Hadley & Purves, 1974; Purves & Hadley, 1976; Alexander & Hadley, 1985). The results of these studies led to the view that orchids are always a net sink for carbon received from their fungal partners (Rasmussen & Rasmussen, 2007). However, Cameron *et al.* (2006) challenged this generalisation by showing that adult *G. repens* can provide its fungal partner with significant quantities of photosynthate, whilst at the

same time receiving mineral nutrients taken up and transported by the fungus. These authors suggested that carbon invested by orchid mycorrhizal fungi into germinating seedlings and protocorms might therefore be repaid by green-leaved adult plants in a true mutualism. This finding, whilst for only one orchid species, also opened the possibility that many green-leaved orchids provide photosynthate to support their symbiotic mycelial networks, so enabling their proliferation through soil around the plants. However, such a carbon transfer pathway via mycorrhizal hyphae in support of orchid seedling development has not been established. In this paper, we demonstrate the presence of this pathway in the Common Spotted Orchid, *Dactylorhiza fuchsii*.

Orchids produce prodigious quantities of seeds per flower, each seed capsule typically producing between 1000 and 10 000 dust seeds, but up to 4 million has been reported (Arditti & Ghani, 2000). The buoyancy achieved by the inflated testa of orchid seeds (Lee & Yeung, 2023), coupled to their profligacy, is assumed to enable wide dispersal by wind, a feature which has been seen to underpin their successful global colonisation and speciation (Arditti & Ghani, 2000; Roberts & Dixon, 2008). However, in his characteristically perceptive analysis of seed distribution in terrestrial orchids, Darwin (1862) was puzzled by his observation that despite the enormous fecundity of parents and the extremely light weight of the 'dust seeds', the progeny were geographically restricted in their occurrence. He wrote 'What checks this unlimited multiplication cannot be told'. More recently, quantitative analysis of terrestrial orchid seed dispersal (Jersáková & Malinová, 2007) indicates that the highest density of seeds is found close (10–30 cm) to the shoots of adults and declines rapidly by 50 cm radial distance. Germination and successful establishment, leading to aggregation, often occurs preferentially in similarly close proximity to parental or conspecific plants, including *D. fuchsii* (Chung *et al.*, 2005; Pierce *et al.*, 2006; Diez, 2007; Jacquemyn *et al.*, 2007, 2009; McCormick & Jacquemyn, 2014; McCormick *et al.*, 2018; Těšitelová *et al.*, 2022).

Clustering patterns of this kind have generally been ascribed to the likelihood that the availability of the compatible OM fungi that are essential to support seedling development is spatially heterogeneous, and that where adult orchids occur these localities correspond to areas where edaphic and ecological characteristics support the required fungi (McCormick & Jacquemyn, 2014; Těšitelová *et al.*, 2022). Further confirmation of this localisation comes from detailed molecular analysis of roots and soil from individuals of the common perennial orchid species *Anacamptis morio*, *Gymnadenia conopsea*, and *Orchis mascula*. This has revealed that the fine scale abundance of OM fungi is locally enriched and declines steeply over 50 cm radial distances from the plants (Waud *et al.*, 2016b). Similar spatial mapping using quantitative DNA analysis of the specific OM fungal partners of *O. mascula* and *Orchis purpurea* also showed exponential declines of fungal abundance over distances of 5–50 cm from adults (Waud *et al.*, 2016a). These studies revealed that the seedlings themselves followed a similar pattern, with 75–80% of recruits growing within 20 cm or less of adults although some plants lacked visible recruits nearby, and a minority of recruitment occurred at substantial distances from adults (Waud *et al.*, 2016a). The strength of the fine spatial association between adults, OM fungal abundance and seedling recruitment led to the suggestion that the adult plants might provide a nursing effect to seedlings via their mycorrhiza (Waud *et al.*, 2016b).

Crucially, despite strong circumstantial evidence that the mycoheterotrophic progeny of some orchids, ferns and lycophytes may be supported by organic carbon transfer from autotrophic adults and sporophytes, experimental evidence of this process is lacking (Leake *et al.*, 2008). Such a mechanism of carbon distribution would contrast with the view that mycorrhizal associations are stabilised only by immediate reciprocal proportional exchanges of organic carbon for mineral nutrients (Fitter, 2006; Kiers *et al.*, 2011), and that OM is 'a unilateral relationship' in which the plants universally parasitise fungi (Rasmussen & Rasmussen, 2007). In this model, OM access and supply carbon derived primarily from complex organic matter in the soil to developing orchid protocorms, resorting to plant-derived carbon only in 'starvation' scenarios where complex organic carbon sources are scarce (Rasmussen & Rasmussen, 2007). We hypothesise here that photosynthetic orchids may provide carbon to conspecific achlorophyllous protocorms so providing a physiological mechanism underpinning the extreme propensity of seedlings of some green-leaved orchids to develop preferentially near adult plants. Colonisation by a common mycorrhizal network growing from, and supported by, an autotrophic adult orchid may provide not only a source of mycorrhizal inoculum but also the supply of photoassimilate necessary for their development. Such a process would support the 'parental nurture' hypothesis in which C transfer occurs between plants at different generational life stages via a shared mycorrhizal network. The occurrence of this type of pathway has previously been suggested for some Lycopodiaceae and eusporangiate ferns that have mycoheterotrophic protocorm-like gametophytes (Leake *et al.*, 2008). Here, we test the hypothesis that a common and widespread European terrestrial orchid species *D. fuchsii* can supply carbon to achlorophyllous protocorms of the same species via mycelial interconnections formed by the common OM fungus *Ceratobasidium*. We also test the hypothesis that carbon transfer through common mycorrhizal networks occurs independently of the carbon content of substrate. In order to carry out these tests, we developed novel microcosm-based approaches using ^{14}C tracers to both visualise and quantify carbon dynamics in the experimental systems.

Materials and Methods

Establishment and propagation of plant and fungal cultures

Dactylorhiza fuchsii var. *rhodochila* Turner Ettl. seeds (from cultivated plants, originally collected according to local permits by members of the Hardy Orchid Society) were sown thinly onto Modified Malmgren's medium (75 mg l^{-1} each of KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and $Ca_3(PO_4)_2$, 300 mg l^{-1} casein hydrolysate plus 100 mg l^{-1} peptone, vitamin B complex, 8–10 g l^{-1} sucrose, 0.5–1 g l^{-1} activated charcoal, 5–6 g l^{-1} agar, pH 5.7–5.9; Haggar, 2012) with 2% pineapple juice. This medium promotes

asymbiotic germination of *D. fuchsii* seeds. Cultures were maintained in the dark in refrigerated conditions (4°C) for 3 months (January–April 2023) before being moved to room temperature and maintained in the dark for two further months (April–June 2023) after germination of seeds into protocorms.

Symbiotic seedlings were established from protocorms by plating protocorms onto Basic Oats medium (3.5 g finely sieved powdered oats and 0.1 g yeast extract in 1 l of 2% agar made up with purified rainwater; Manuel, 1996; Haggar, 2012) at room temperature and seeded with fungal isolate 'A36' (from the Hardy Orchid Society Seed Bank); and maintained asymbiotically on Basic Oats medium. This fungus, originally isolated from UK grown *O. mascula* by Adrian Blundell in 1995, is routinely used to propagate and maintain symbiotic cultures of green orchids, including *Dactylorhiza*. Representative ITS sequences for this fungus are available in GenBank, accession no. KY014293 (Figura *et al.*, 2021). Direct sequencing of the fungal isolate using the methods of Bidartondo *et al.* (2011) suggested close sequence similarity to the orchid mycorrhizal fungus *Ceratobasidium* sp.

Following inoculation, the rapidly developing protocorms were moved into a temperate glasshouse (12 h light : dark). In June 2023, 10 orchid plantlets (3–4 cm tall) with green shoots were transplanted into experimental microcosms comprising 565 ml micropropagation boxes (kayorchid.com) filled with *c.* 120 ml of medium. In order to test whether the availability of complex organic C and other nutrient sources to OM impacts on transfer and movement of plant photosynthates to and through the OM mycelial network, we used three different types of 2% agar-based media in the microcosms. These included 100% Basic Oats (hereafter referred to as 'oatmeal') medium ($n = 4$) where organic complex carbon and other nutrients are abundant via inclusion of powdered oats and yeast extract in the agar medium, 100% water ($n = 2$) where the medium was made up with water and 2% agar only, or 50 : 50 microcosms ($n = 4$) where half the media comprised oatmeal, and half was water (Fig. 1a).

Asymbiotic *D. fuchsii* protocorms ($n = 80$ in total) were established by transplanting vernalised protocorms onto 50% Phytamax Orchid Maintenance medium (Sigma-Aldrich) with 1% pineapple juice and 2% potato within micropropagation boxes. These were maintained in the dark at room temperature until transplanting into experimental microcosms in June 2023. Eight asymbiotic, achlorophyllous protocorms were planted in each microcosm so that they were evenly spaced around the symbiotic green orchid seedling, each *c.* 2–3 cm away from the autotrophic plant. The symbiotic fungal hyphae grew outwards from the green plant into the media. In so doing, they reached the protocorms, forming a dense, interconnected mycelial network directly connecting protocorms with the autotrophic green plant (Fig. 1a).

Experimental microcosms and $^{14}CO_2$ labelling

After *Ceratobasidium* hyphae reached the protocorms in each microcosm (*c.* 2 wk after planting), but before emergence of green shoots, microcosms were prepared for $^{14}CO_2$ labelling. The shoots of the autotrophic plants were isolated from the protocorms, fungus and growth medium by a gas impermeable Mylar polyester film (Tekra, New Berlin, WI, USA) barrier cut to fit within the microcosm and sealed with anhydrous lanolin (Sigma) where the film met the edges of the container and centrally where the orchid shoot was threaded through (Fig. 1a). This barrier prevented diffusion and dissolution of $^{14}CO_2$ into the growth medium and prevented any direct ^{14}C fixation by achlorophyllous protocorms. A damp 2-cm^2 piece of synthetic sponge was attached to the edge of the container using a thermoplastic adhesive (Beeway, Middlesex, UK), applied at 160°C, above the Mylar barrier, to maintain humidity within the headspace of the microcosm. Two plastic cuvettes were also attached to the wall of the container, into one of which 13.5 µl ^{14}C-$NaCO_3$ (2.146 GBq mmol^{-1} specific activity; PerkinElmer,

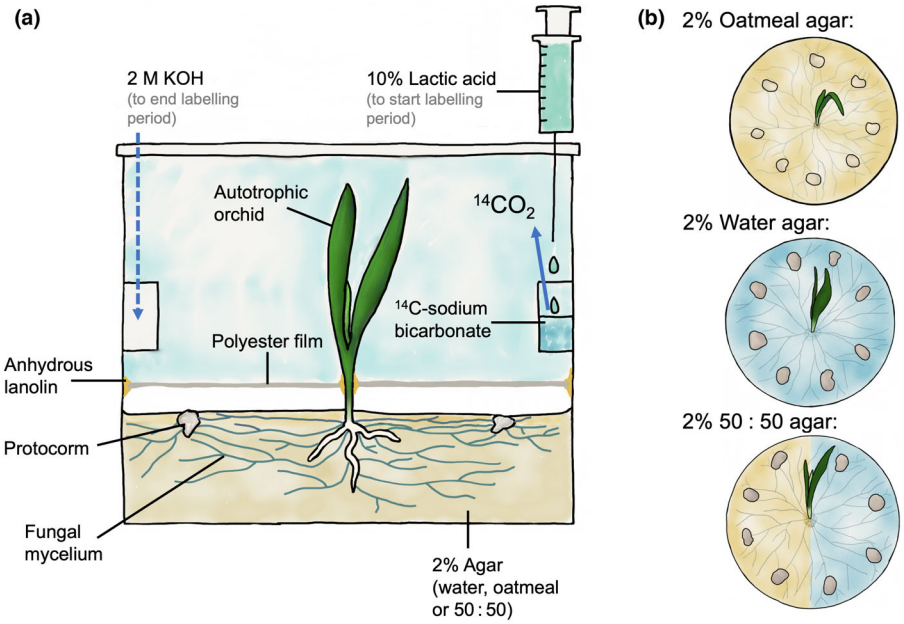

Fig. 1 Design of microcosms showing central autotrophic orchid and mycoheterotrophic protocorms connected by fungal mycelium on 2% agar media as (a) side view, and (b) plan view showing growth media treatments (oatmeal, water, and 50 : 50 oatmeal : water agar) in a 2% agar base.

Waltham, MA, USA) was introduced before the lids of the containers were closed and sealed with electrical tape. Using a flamed needle, 0.5 MBq $^{14}CO_2$ was liberated into the chamber headspace containing the green shoot of the orchid, above the Mylar barrier, by introducing 2 ml 10% lactic acid to the cuvette containing the ^{14}C-$NaCO_3$. The needle hole was sealed with electrical tape and microcosms were maintained at room temperature and in natural day light for 3 d. Drawdown of $^{14}CO_2$ by orchids was monitored regularly throughout the labelling period by gas sampling from the microcosm headspace into gas-evacuated scintillation vials containing 10 ml of the CO_2 trapping chemical CarbonTrap (Meridian Biotechnology, UK), mixed with 10 ml of the liquid scintillation cocktail CarbonCount (Meridian Biotechnology, Epsom, UK) and the activity determined by liquid scintillation counting (Tri-Carb 4910TR; PerkinElmer, Beaconsfield, UK). After 72 h, 2 ml 2 M KOH (Sigma) was injected into the second empty cuvette within each microcosm to absorb any unfixed $^{14}CO_2$ in the microcosm headspace and end the labelling period (Fig. 1b). After 1 h, KOH was removed from the microcosms which were then resealed and incubated for a further 48 h to allow sufficient time for movement of recent photosynthates into the extraradical OM mycelium.

Microcosm harvest and sample analysis

The microcosms were carefully dismantled 48 h after the $^{14}CO_2$ had been removed from the headspace, by removing the agar intact from the containers with the plants and fungal mycelium undisturbed. The agar was placed intact and upright onto a sheet of absorbent paper on a Perspex sheet and covered with Mylar polyester film and photographed. The spatial distribution of ^{14}C in the plant shoots, fungal mycelium and protocorms was then visualised by digital autoradiography (Packard Instant Imager, Isotech, Chesterfield, UK), counting for 420 min per sample. The data were exported at pixel scale (0.5 × 0.5 mm) and imported into a spectral plotter in STANFORD GRAPHICS (v.3.0; Visual Numeric Inc., Houston, TX, USA), using a 12-colour scale with the blend option to give a smooth colour transition in converting counts per pixel to provide false colour images. Photographs taken of the agar were imported into POWERPOINT (v.16.81; Microsoft, Redmond, WA, USA) and standard sized circles overlaid to define the agar margin and smaller circles placed symmetrically over each protocorm to mark their positions relative to each other and the edge of the agar disk. These templates were then grouped and copied and overlaid on the imported false colour digital autoradiographs.

After imaging, autotrophic orchids were removed intact from the agar and separated into roots and shoots. Protocorms were removed from the agar and, together with autotrophic orchid shoots, roots, and agar containing fungal mycelium were freeze dried (ScanVac; Labogene, Lillrød, Denmark) and weighed. Between 0.5 and 50 mg of each component was then weighed into paper cups and oxidised (Model 307 Sample Oxidiser; Perkin Elmer, UK) and the ^{14}C quantified in each sample by liquid scintillation counting (Tri-Carb 4910TR; Perkin Elmer, UK) for 5 min per sample. Total carbon ($^{12}C + {}^{14}C$) content of protocorms and fungal mycelium on agar was calculated as a function of the total volume and CO_2 content of the microcosm headspace and the proportion of $^{14}CO_2$ supplied that was fixed by the plants using equations adapted from Cameron *et al.* (2007):

$$M_C = \left((A/\mathrm{SAct})M^{14}C\right) + (P_r \times M_{wtC})$$

where M_C, mass of carbon; A, radioactivity of the sample (Bq); SAct, specific activity of the source (Bq mol^{-1}); $M^{14}C$, atomic mass of ^{14}C; P_r, proportion of ^{14}C label supplied detected in the sample; M_{wtC}, mass of C in the CO_2 in the labelling chamber (g) – from the ideal gas law:

$$M_{cd}(PV_{cd}/RT) \therefore m_c = m_{cd} \times (0.27292)$$

where m_{cd}, mass of CO_2 (g); M_{cd}, molecular mass of CO_2 (44.01 g mol^{-1}); P, total pressure (kPa); V_{cd}, volume of CO_2 in the chamber (0.0005 m^3); R, universal gas constant (J K^{-1} mol^{-1}); T, absolute temperature (K); m_c, mass of carbon in the CO_2 present in the labelling chamber (g), where 0.27292 is the proportion of carbon in CO_2 on a mass fraction basis.

Statistical analysis

All statistical analyses were performed in RSTUDIO (RStudio Team, 2020) using the R programming language (R Core Team, 2021). Figures were constructed in GRAPHPAD (v.10.12; GraphPad Software, Boston, MA, USA). Mixed effects linear models with the 'type of agar medium' set as a fixed effect and the 'experimental microcosm' included as a random intercept to account for the nonindependence among protocorms growing on the same microcosm were performed for the amount of plant C allocated to the protocorms and OM fungal mycelium in agar. Assumptions for using general linear models were validated by plotting residuals vs fitted values, square root residuals vs fitted values, normal qq plot and constant leverage using the 'autoplot' function of the GGPLOT2 package (Wickham, 2016). The protocorm total carbon and concentration of carbon data did not satisfy the assumptions of the model, so the data were transformed using the natural logarithm. The significance of the model parameters was assessed using the 'anova' function in R, with the LMERTEST package (Kuznetsova *et al.*, 2017) being used for all mixed effects models and the 'Kenward-Roger's method' specified for the calculation of the degrees of freedom. Where relevant, a *post hoc* Tukey test was performed to assess individual differences between treatments, with the EMMEANS package (Lenth *et al.*, 2018) used for the mixed effects models.

Results

Transfer of carbon between autotrophic green orchids and mycoheterotrophic protocorms via mycorrhizal hyphae connecting generations

Using autoradiography, we visualised the allocation of ^{14}C photoassimilated by the green-leaved plants into the external

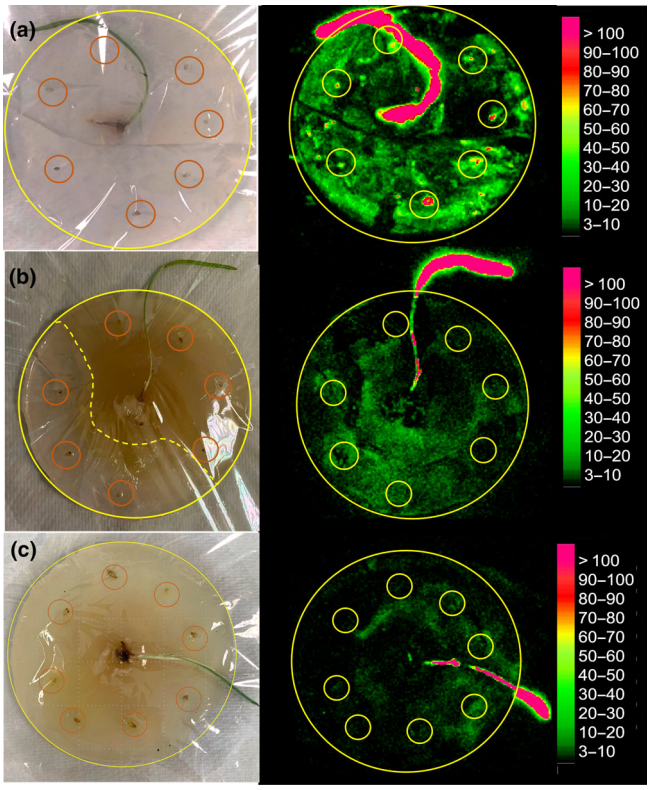

Fig. 2 Photographic (left) and digital autoradiographic images (right) of autotrophic orchids with mycorrhizal mycelium connecting to achlorophyllous protocorms showing relative intensity and spatial distribution of ^{14}C fixed by shoots when cultured on (a) water agar medium, (b) 50 : 50 oatmeal : water agar medium, where water agar is on the left of the dotted line, and (c) oatmeal agar medium. Intensity of colouration is indicative of number of radioactive counts per minute detected in pixel areas of 0.25 mm^2 over a period of 420 min. Large yellow circles indicate peripheral edge of agar, smaller circles highlight location of protocorms in each microcosm.

mycelium of the mycorrhizal fungi at the surface of the growth substrate, and its subsequent accumulation from this mycelium into nonphotosynthetic protocorms developing on all three media treatments. The images (Fig. 2) revealed that ^{14}C accumulated to the greatest extent in the fungal mycelium at the surface of the growth medium and protocorms of microcosms containing water agar (Fig. 2a) relative to that seen on oatmeal medium (Fig. 2c). In microcosms containing both media types, greater ^{14}C is visualised in the fungal mycelium at the surface of the water agar compared to the oatmeal medium, with limited accumulation in protocorms (Fig. 2b).

The total ^{14}C accumulation in plant shoots, roots, protocorms and fungal mycelia, was quantified by liquid scintillation counting and a full carbon budget was constructed for each microcosm, taking into account the $^{14}CO_2$ relative to nonradioactive CO_2 in the microcosms during the pulse-labelling (Fig. 3). We found green orchid plants fixed equivalent amounts of CO_2 across the three media treatments and moved similar amounts to roots in all microcosms (Fig. 3a). From this, we calculate that between 1% and 11% of the total carbon fixed by the plant was moved into the extraradical OM fungal mycelium (Fig. 3b), representing between *c.* 200 and 800 ng C in total (Fig. 4a). Overall, the composition of the agar influenced the amount of carbon that was allocated to OM mycelia (Fig. 4a; F-value = 23.2, $P < 0.001$). Despite the visual observation suggesting greater accumulation of photosynthate in water agar (Fig. 2), the quantitative analysis of fungal mycelium showed the mycelium growing on 50% oatmeal in fact received more carbon than that grown on water agar, whether in separate microcosms (Fig. 4a; t-ratio = 3.1, $P = 0.058$) or combined within the same microcosm (Fig. 4a; t-ratio = 8.73, $P < 0.01$). This pattern did not change when the data were normalised according to the dry mass of the media and fungus, although in this case, there was not an overall statistically significant difference between treatments (Fig. 4b; F-value = 3.98, $P = 0.06$).

We detected significant amounts of C in protocorms, representing up to *c.* 0.5% of the total carbon fixed by the green orchids during the labelling period (*c.* 0.75–2.5 ng, 8–10 ng g^{-1}; Fig. 3c,d) in all the microcosms tested (Fig. 4c,d). Of these, more carbon was allocated to protocorms in microcosms with water agar compared to those growing in all the other media, both in terms of total carbon (Fig. 4c; t-ratio = 4.87, $P < 0.01$; t-ratio = 5.65, $P < 0.01$; t-ratio = 5.43, $P < 0.01$ for treatments 50% water, 50% oatmeal and 100% oatmeal, respectively) and carbon concentration (Fig. 4d; t-ratio = 3.56, $P < 0.05$; t-ratio = 3.34, $P < 0.05$; t-ratio = 3.57, $P < 0.05$ for treatments 50% water, 50% oatmeal and 100% oatmeal, respectively).

Discussion

These results provide experimental evidence of a physiological mechanism underpinning observations that, in nature, the protocorms and seedlings of many terrestrial orchids develop preferentially near parent plants (Batty *et al.*, 2001; Diez, 2007; Jacquemyn *et al.*, 2007, 2009; McCormick & Jacquemyn, 2014; McCormick *et al.*, 2018; Těšitelová *et al.*, 2022). Detailed spatial studies show that a decline in frequency of seedling establishment is paralleled by a decrease in OM fungal abundance away from adults (Waud *et al.*, 2016a,b). Our experiments are the first to include both a potential source (the autotrophic host) and sink (protocorms) for the investigation of carbon transfer between the two trophic phases of orchids. Furthermore, the data confirm experimentally that not only can photosynthetic orchid plants provide a source of mycorrhizal inoculum for seedlings, but they also supply significant quantities of carbon in the form of photosynthate, essential for protocorm development. Given the relatively small size of the green-leaved photosynthetic 'adult' plants and early stage of protocorm development investigated in our experiments, we suggest that the magnitude of carbon transfer in nature has the potential to be substantially larger. It has the potential to sustain increases of protocorm size up to the point of development of green leaves, which can take several months to emerge in some species (e.g. Leeson *et al.*, 1991). In our experiment, there was a tendency for absolute amounts of ^{14}C recovered in protocorms to correspond positively to absolute amounts of ^{14}C captured by green plants (see Fig. 4d).

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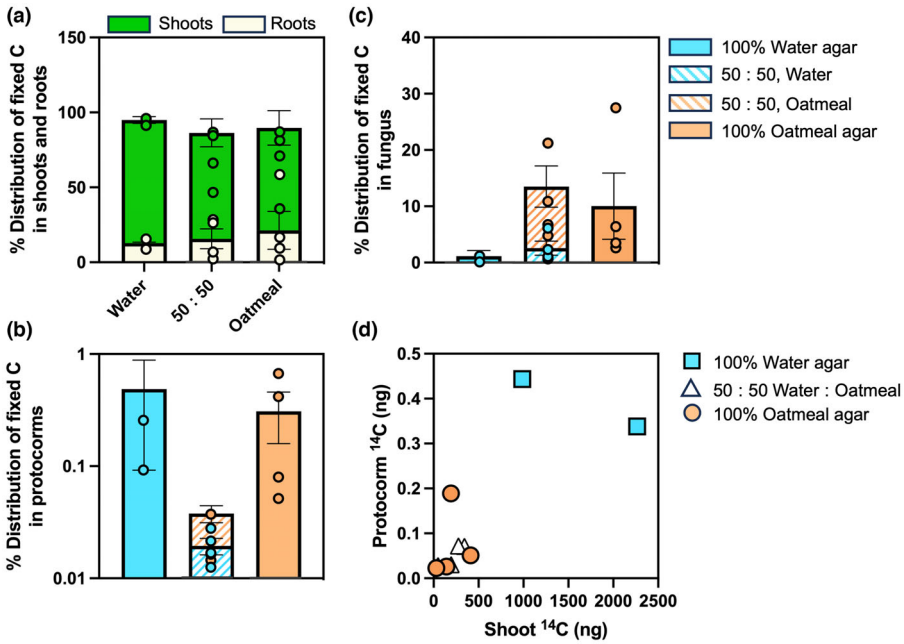

Fig. 3 Mean (±SE) % distribution of carbon (C) fixed by autotrophic orchids during labelling period in (a) green orchid shoots (green bars) and roots (cream bars), (b) achlorophyllous orchid protocorms, (c) extraradical fungal mycelium, and (d) mean total ^{14}C detected in protocorms per microcosm vs total ^{14}C detected in autotrophic orchid shoots in each microcosm.

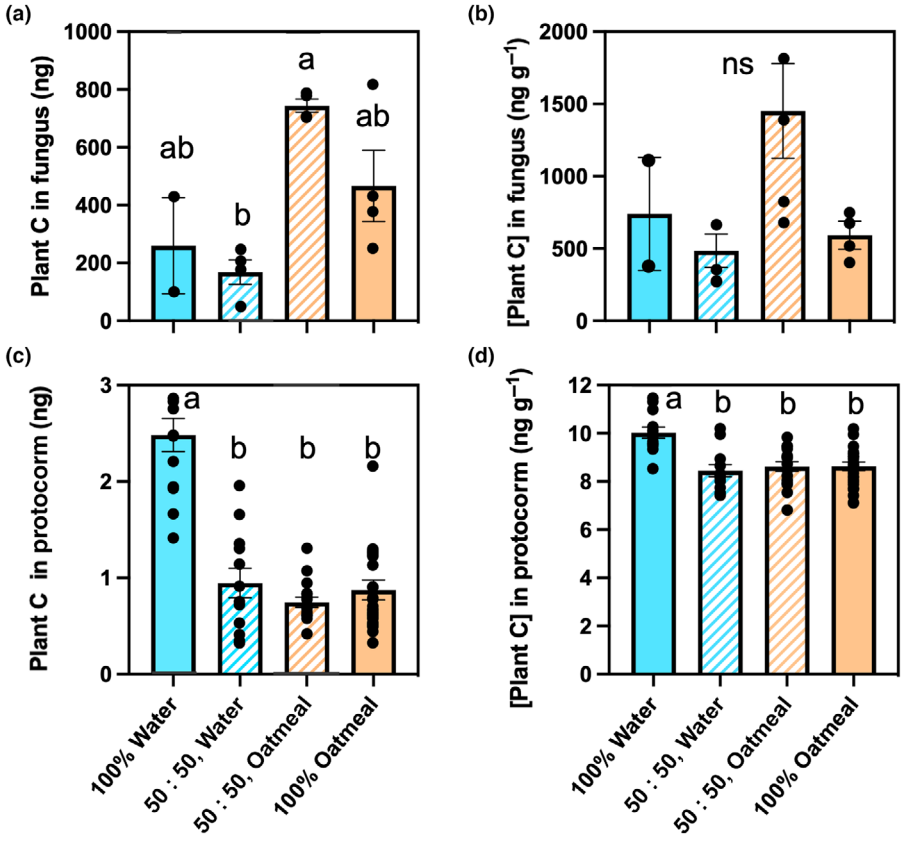

Fig. 4 Mean (±SE) total (a) and concentration (b) of carbon (C) transferred from autotrophic orchid to fungal mycelium and mean (±SE) total (c) and concentration (d) of carbon transferred from autotrophic orchid to protocorms ($\log_e + 1$ transformed) in each media treatment. Bars sharing a letter are not significantly different ($P < 0.05$, Tukey test on mixed effects general model).

These findings provide the physiological underpinning necessary to explain the occurrence of 'parental nurture' via intergenerational orchid mycorrhizal connections in nature. Clearly, they also reveal the potential for repayment of the carbon invested by fungi in protocorms once these develop into autotrophic adults (i.e. 'take now, pay later'; Cameron *et al.*, 2006; Field *et al.*, 2015). Providing they continue to support the fungal partner with photosynthate, the potential for 'parental nurture' may be maintained, with likely mutual benefits to the fitness of both the plants and to the fungi involved. Selection pressure on OM fungi forming common mycorrhizal networks may also provide an additional explanation of carbon transfer from

photosynthetic orchids to achlorophyllous protocorms. In view of the fact that most soils are starved of labile carbon (Soong *et al.*, 2020), the fungus may be under selective pressure to secure sources of autotrophically derived carbon.

In orchids, carbon supply via common mycorrhizal networks is not the only, or even necessarily the main source of support for developing protocorms. If the adult orchids maintain a higher abundance of critical symbionts involved in nourishing protocorms in soil, these fungi will not obtain all their carbon from the conspecific autotrophic adults. Whilst Těšitelová *et al.* (2022) found almost no germination of *O. mascula* seeds in packets sown away from adults, the addition of straw-based inoculum of a *Tulasnella* species boosted germination over the following year to rates higher than under adults not provided with inoculum. Similar findings have been reported for *G. conopsea* reported in Jiang *et al.* (2022). These observations suggest that preinoculation enables competition with saprotrophs in the substrate, supporting the hypothesis that OM fungi may use host plants as 'refuge' for persistence in competitive soil environments (Calevo *et al.*, 2021). Since most temperate terrestrial orchids are deciduous, in many species new tubers and roots being produced each year, the retention of fungal partners seasonally and inter-annually would be required for consistent nurture of seedlings via shared mycorrhiza. A recent systematic review of temporal turnover of mycorrhizal partners found that adult orchids engaged in interacting with more fungal partners than juveniles. From a total of 73 records 19% showed strict fidelity of partners from protocorms to adults, and 11% retained their original partners but gained more (Ventre Lespiaucq *et al.*, 2021), so that 30% of the records showed potential intergenerational fidelity. In the majority of cases (53%) partial replacement of fungal partners occurred, with complete change of species occurring in only 12% of cases. The ability of individual orchids, including *D. fuchsii*, to establish in isolated positions at considerable distances from parental seed sources and so to enhance both the geographical range and population size of a species is recorded (Arditti & Ghani, 2000; Denholm & Mashanova, 2020). Such distribution patterns must be dependent upon an ability of the seed to recruit fungal symbionts from local sources that are growing saprotrophically or in association with other plant species.

For those orchid species that do show aggregated distributions clumped around established adults, our study suggests that carbon transfer via common mycorrhizal networks may be of fundamental importance and thus have a role in orchid conservation and management. Species such as *Cypripedium calceolus*, which are rare, have very specific *Tulasnella* mycorrhizal symbionts (Shefferson *et al.*, 2005, 2007) and show strongly clumped distributions of green-leaved young plants (Kirillova & Kirillov, 2021; Rusconi *et al.*, 2023) might benefit from the use of transplanted 'nurse plants' to help facilitate regeneration from seed – but this needs to be tested. Loss of adults as a resource, if important for facilitating mycoheterotrophic seedling germination and development, might explain why over-collecting of some wild orchids like *C. calceolus* has proved difficult to reverse by reintroductions using plants raised asymbiotically under laboratory conditions (Gargiulo *et al.*, 2021). Certainly in species shown to have strong concentrations of new recruits around adults (e.g. Waud *et al.*, 2016a,b), the use of adult transplants hosting the native fungal partners to help founding new populations germinating from seeds dispersed from the adults would be worth trialling.

We assert that our tracing and quantification of photosynthate transport from green-leaved orchids to conspecific mycoheterotrophic protocorms via a shared mycorrhizal fungal mycelium provides a physiological basis for the widely observed tendency of many terrestrial orchids to reproduce preferentially close to established adults. It also challenges the widely held assumption that the association is not mutualistic but is based simply on orchid digestion of fungal biomass (Rasmussen & Rasmussen, 2007). The dynamic transfers of organic carbon between generations and trophic stages of *D. fuchsii* shown here provide the physiological basis of a fully mutualistic mycorrhizal symbiosis, as first demonstrated by Cameron *et al.* (2008) for *G. repens*. Specificity and fidelity through mycoheterotroph-to-autotroph lifecycles in orchids, and some other plants, will underpin selection for net fitness benefits for partners across their lifetimes and may be the primary stabilising factor in such symbioses (Field *et al.*, 2015).

Clearly, the axenic conditions employed in our experiments diverge significantly from those encountered in natural environments. This leaves uncertainties regarding the relative accessibility to OM fungi of alternative sources of soil carbon. These uncertainties are compounded by the fact that OM fungi scavenging for soil-borne carbon sources do so in competition with other classes of mycorrhizal fungi (arbuscular and/or ectomycorrhiza) as well as with saprotrophic bacteria and fungi. Our results indicate that the OM fungal mycelium receives significant photosynthate carbon from the green orchid irrespective of the availability of complex carbon and nutrients in the substrate. Despite the visual observation suggesting greater accumulation of photosynthate in mycelia on water agar (Fig. 2a), quantitative analysis of agar and OM fungal mycelium (Fig. 4a,b) showed that even when a 'choice' of substrate is available (in 50 : 50 treatments), there was no greater plant-derived carbon detected in the OM mycelium growing on the water vs oatmeal agar. This leads us to challenge the assertion of Rasmussen & Rasmussen (2007) that the results reported by Cameron *et al.* (2006) were due to 'starvation'. According to Rasmussen & Rasmussen (2007), the OM fungal mycelium in these experiments was artificially dependent on the plant for carbon resources, whereas in their view, in nature 'complex carbon sources abound'. In fact, there is now clear evidence that mineral soils of the kinds supporting orchids such as *D. fuchsii* are generally depleted in labile carbon, a feature that leads to intense microbial competition for this limited resource (Soong *et al.*, 2020). It is possible that the greater quantities of plant-derived carbon detected in OM fungal mycelia on oatmeal relative to water agar plates, quantified via complete sample oxidation and liquid scintillation (Fig. 4), reflected increased mycelial growth, biomass and metabolic activity. Such enhanced growth and activity is likely to increase the carbon sink strength of the OM mycelium, potentially driving increased movement of plant photosynthates to the fungi. Interestingly, despite greater quantities of photosynthate to OM mycelium growing on oatmeal agar vs water agar in 50 : 50 treatments, the amount of

carbon reaching protocorms appeared similar across the two types of media. This finding suggests of translocation of carbon from OM mycelium in the oatmeal part of the microcosm to protocorms in the water agar part to support protocorm development.

It will be important in subsequent studies to determine whether the clearly demonstrable impact of sink strength upon the patterns of carbon movement in our agar-based systems are replicated in experiments employing soils encompassing the natural complexity of microbial communities. Since the carbon stocks of soils are both low and transient, it can be hypothesised that gradients of carbon concentration from photosynthetic orchid to protocorms in soil-based systems will be at least as strong as in those using agar, and potentially stronger with larger plants having greater leaf-areas. Ongoing field and soil-based studies of the parental nurture hypothesis will be necessary to improve our understanding of the broader significance of our observations.

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Competing interests

None declared.

Author contributions

DJR, DJ and KJF conceptualised and designed the research. JH generated and supplied the plant and fungal materials. KJF, EM and ED conducted the isotope tracing experiment and collected the data. JRL, EM and KJF analysed and visualised the data. DJR, JRL, DJ and KJF interpreted the data. DJR and KJF led the writing of the manuscript with all authors contributing to and revising the text. All authors approved the final draft.

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Data availability

The data that support the findings of this study are openly available in Dryad at doi: 10.5061/dryad.g1jwstqzt.

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