Letters

The fungal perspective of arbuscular mycorrhizal colonization in 'nonmycorrhizal' plants

Arbuscular mycorrhiza (AM) is arguably the most abundant symbiosis on Earth. It involves fungi in the Glomeromycota and *c.* 70% of vascular plants (Brundrett, 2009), in which the fungal partner aids in nutrient uptake, pathogen protection and possibly other services in exchange for plant carbon (C) (Smith & Read, 2008). A smaller, but not insignificant, number of plant species are considered to be nonmycorrhizal, especially those within the families Amaranthaceae, Chenopodiaceae, Carophyllaceae and Brassicaceae (Brundrett, 2009). However, fungal colonization that resembles AM is often observed in these 'nonmycorrhizal' plants when they co-occur with mycorrhizal plants (Hirrel *et al.*, 1978; Miller *et al.*, 1983), although arbuscules are rarely if ever present (Meney *et al.*, 1993; Muthukumar *et al.*, 1996; Sengupta & Chaudhuri, 2002). Because arbuscules are the structures where AM fungi deliver phosphorus (P) to plants (Smith & Read, 2008), and since the hyphal and vesicular colonization is often observed in old or dying roots (Brundrett, 2004), the function of this symbiosis has been questioned (Hirrel *et al.*, 1978; Koide & Schreiner, 1992; Brundrett, 2004). Nonetheless, responses to AM fungal inoculations by these plants range from negative (Allen *et al.*, 1989) to positive (Williams *et al.*, 1974), which suggests that mycorrhizal associations are not always asymptomatic, and that the distinction between mycorrhizal and nonmycorrhizal plants is more subtle than sometimes believed. Acknowledging its limitation, we will use the term 'nonhost' here to refer to plants that are thought to be nonmycorrhizal but often appear to be colonized by AM fungi.

AM fungal colonization of nonhost plants has been quantified for decades (Hirrel *et al.*, 1978), but we know surprisingly little about what the benefits might be for the fungus. For example, if colonization mainly occurs in old or dying nonhost roots (Brundrett, 2004), does this represent an important P source for the fungus to be allocated to other, better host plants, similar to what has been observed when killing or defoliating highly mycorrhizal plants (Eason *et al.*, 1991; Mikkelsen *et al.*, 2008)? Also, does the C stored in vesicles come from the nonhost, or does the fungus simply use the roots as shelter and C storage (Lekberg *et al.*, 2010)? And finally, because these plants may differ from mycorrhizal plants in root anatomy and chemistry (Koide & Schreiner, 1992), are nonhost plants colonized by a broad array of AM fungi or restricted to a narrow lineage, similar to what has been shown for mycoheterotrophic plants (Bidartondo *et al.*, 2002)?

Here we set out to explore the latter two questions using a putative nonhost plant, *Dianthus deltoides* in the Caryophyllaceae (Wang & Qui, 2006), which co-occurs with mycorrhizal plants in a Danish coastal grassland. In June, 2007, we sampled 16 *Dianthus* plants in a 50 m × 100 m area for assessments of AM colonization and fungal community composition. We also sampled 16 *Hypochoeris radicata* plants (a highly mycorrhizal species) to allow for comparisons between *Dianthus* and a typical host plant. Plants were sampled in a pairwise manner (*Dianthus* and *Hypochoeris* plants were no more than 30 cm apart) to reduce confounding effects of AM fungal spatial patterns, which have been documented at this site previously (Rosendahl & Stukenbrock, 2004). We assessed AM colonization on trypan blue stained roots using the gridline intersect method on at least 50 intersects (McGonigle *et al.*, 1990), and extracted and amplified DNA from roots using the same method as in Lekberg *et al.* (2012). Due to the visually lower AM fungal abundance in *Dianthus* roots, we extracted DNA from eight 2-cm root pieces per plant compared with eight 0.5-cm pieces per *Hypochoeris* plant. Briefly, we targeted the large ribosomal subunit (LSU) and amplified DNA using a nested PCR with the eukaryotic primers 0061 and NDL22 followed by FLR3-FLR4 (Van Tuinen *et al.*, 1998). Because we extracted DNA from short root fragments, all positive PCR products were sequenced directly without cloning (Macrogen, Seoul, Korea). High quality sequences were aligned with published sequences obtained from the same grassland and elsewhere (Rosendahl & Stukenbrock, 2004; Stukenbrock & Rosendahl, 2005; Lekberg *et al.*, 2012). We considered sequences a match with previous operational taxonomic units (OTUs) if they clustered within their monophyletic clades (see Supporting Information Table S1 for original OTU names, updated nomenclature and published accession numbers). We used a chi-square test (with Yates correction; http://statpages.org/) and a 2 (plant species) × 10 (OTU) contingency table to test for differences in AM fungal community composition between *Dianthus* and *Hypochoeris* based on presence/absence of OTUs in each plant.

To verify that AM fungi were responsible for the observed root colonization, and to assess if *Dianthus* allocates C to AM fungi, we used neutral lipid fatty acid (NLFA) stable isotope probing (Olsson *et al.*, 2005) and conducted a $^{13}CO_2$ pulse-chase labeling on seven *Dianthus* individuals in July 2008. Given that this sampling addressed a separate question from the sampling in 2007, the different sampling times should present no problem. The labeling and post-labeling processing was identical to that described in Lekberg *et al.* (2012), and results from the *Dianthus* plants were compared with seven *Hypochoeris* individuals from the study by Lekberg *et al.* (2012). These *Hypochoeris* plants were from the same 50 m × 100 m area, and had an identical labeling time (three consecutive days starting 21 July for 2 h d^{-1}) and chase period (3 d after the last labeling). All plants were at least 5 m away from each other to eliminate any risk of ^{13}C cross-contamination. Carbon-13

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(^{13}C) concentration (expressed as δPDB values) was quantified at harvest in shoots and roots to assess C-assimilation and allocation belowground, and one nonlabelled plant per species was harvested for background ^{13}C concentration. Lipids were extracted from freeze-dried fine roots ($n = 8$ per species) and analyzed as described earlier (van Aarle & Olsson, 2003; Lekberg *et al.*, 2012). In order to assess the C-allocation specifically to AM fungi, we measured the ^{13}C concentration in the AM fungal storage lipid NLFA 16:1ω5 in all labeled plant roots by compound specific isotope ratio mass spectrometry (Olsson *et al.*, 2005). To control for differences in overall allocation belowground between the two plant species, we also compared ^{13}C concentration in NLFA 16:0 (occurs both in plants and AM fungi) and in NLFA 18:2ω6,9 (in plant roots but not in AM fungi).

Similar to previous studies involving nonhost plants (Hildebrandt *et al.*, 2001), *Dianthus* roots were colonized by hyphae and vesicles but rarely by arbuscules (two *Dianthus* plants had < 5% arbuscular colonization), and overall AM colonization was substantially lower ($P < 0.001$, *t*-test) than in *Hypochoeris* plants (Fig. 1). This was supported by large differences ($P < 0.001$, *t*-test on $\log_e$-transformed values) in 16:1ω5 concentrations between *Dianthus* (11.1 ± 5.7 nmol g^{-1} root, mean ± standard error (SE)) and *Hypochoeris* roots (536 ± 180 nmol g^{-1} root). We do not know if *Dianthus* can form AM in the absence of mycorrhizal plants (*sensu Atriplex confertifolia* in Miller *et al.*, 1983), but the colonization of field plants suggests that the nearly nonhost status of this plant is not due to cell wall structures or antifungal compounds that deter hyphal growth and penetration. Lack of signaling may explain the low AM colonization (discussed in Koide & Schreiner, 1992) and this could be tested in controlled inoculation experiments with *Dianthus* grown with and without good host plants.

Reflecting the lower AM colonization, only three *Dianthus* plants had roots with detectable NLFA 16:1ω5, which clearly limits our ability to test if *Dianthus* allocates C to AM fungi. Due to this, and because of the large variation among *Dianthus* replicates, we chose not to analyze the data statistically but instead to plot individual values for visual comparisons against mean values for *Hypochoeris* (Fig. 2). The ^{13}C concentrations in NLFA 16:1ω5 in the three *Dianthus* roots are visually lower than the mean values for *Hypochoeris*, and because ^{13}C concentration measures recently allocated C per unit fungal storage lipid (as indicated by NLFA 16:1ω5), these lower values are not due to a lower AM fungal abundance in *Dianthus*. Nor are potential differences driven by plant size and ^{13}C assimilation because they were comparable between the two species (Table S2), as was the C-allocation to roots (indicated by similar ^{13}C concentration in the plant specific NLFA 18:2ω6,9, Fig. 2). It is interesting to note, however, that NLFA 16:1ω5 was enriched in *all Dianthus* samples compared to the unlabeled plants (Fig. 2), suggesting that at least *some* C in the AM fungi came from *Dianthus*. This enrichment cannot be explained by C-fractionation during the transfer of C from plant to AM fungi, because the difference in natural abundance of ^{13}C between the two symbionts is < 2‰ (Staddon *et al.*, 1999), and the least enriched sample showed an enrichment of > 14‰. The ^{13}C concentration in NLFA 16:1ω5 varied substantially within *Dianthus* for unknown reasons, and the low enrichment in two of the samples may simply have resulted from an assimilation of apoplastic C without any active allocation from – or real cost to – the plant (see discussions in Lekberg & Koide, 2014). The sample with the higher enrichment may suggest a more active symbiosis, but careful pairing of costs and benefits is needed to assess that. We want to stress that more samples are required for robust conclusions, and we urge readers to view our results as preliminary. However, our findings agree with Allen & Allen (1990) who showed that nonhost plants contribute at least some C for spore production, although spore numbers were much lower than under a good host. As in Allen & Allen (1990) we observed a higher C investment to AM fungi by the good host, because *Hypochoeris* appeared to allocate more C per unit fungal biomass and also supported a much greater fungal biomass than *Dianthus*. Combined with the higher arbuscular colonization in

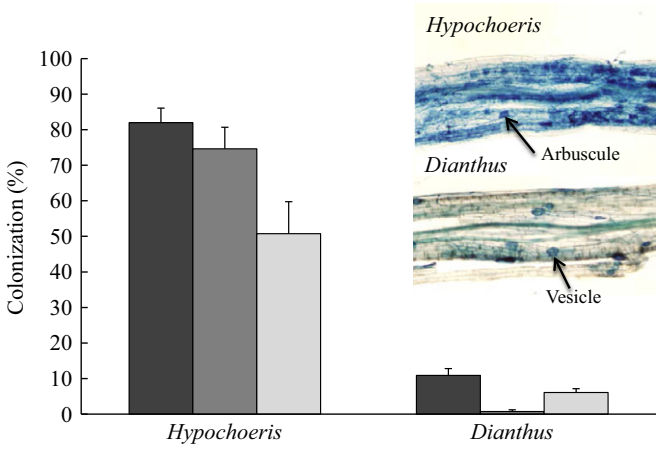

Fig. 1 Overall arbuscular mycorrhiza (AM) colonization (dark gray), arbuscular (medium gray) and vesicular (light gray) colonization of *Hypchoeris radicata* and *Dianthus deltoides* in a Danish coastal grassland and micrographs of stained roots from the same site (inserted pictures).

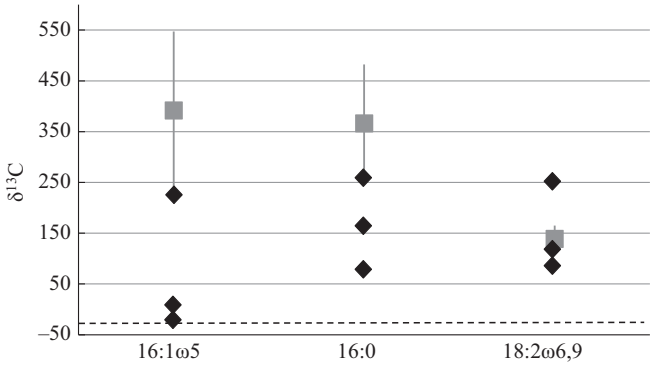

Fig. 2 Carbon-13 (^{13}C)-concentration as indicated by δ^{13}C values for three neutral lipid fatty acids: 16:1ω5 (specific to arbuscular mycorrhiza (AM) fungi), 16:0 (common in all types of organisms) and 18:2ω6,9 (common in plants, but not in AM fungi) in the seven *Hypochoeris* (gray squares, average ± standard error (SE)) and the three *Dianthus* samples that contained sufficient neutral lipid fatty acid (NLFA) 16:1ω5 concentrations (black diamonds). The δ^{13}C values in roots of unlabeled *Hypochoeris* and *Dianthus* were –28.4 and –25.3, respectively (dashed line show the average of the two values).

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Hypochoeris, our field survey support previous findings of a balanced resource trade in the AM symbiosis, in which symbionts that invest more C receive more resources in return (Lekberg *et al.*, 2010; Hammer *et al.*, 2011). Our measure of AM colonization and its different components also show that these measurements can be informative.

Nine of the 16 *Dianthus* plants and all 16 *Hypochoeris* plants yielded at least one PCR product that was sequenced successfully (average PCR success was 12% and 59% of root segments, respectively). We found a total of four OTUs in *Dianthus* roots and eight OTUs in *Hypochoeris* roots. The two plant species harbored different AM fungal communities ($P = 0.04$); *Dianthus* was dominated by an OTU that clustered with *Funneliformis mosseae*, whereas Cluster D fungi dominated in *Hypochoeris* roots (Fig. 3; Table S1). The high abundance of Cluster D in *Hypochoeris* was not surprising given that it is an ubiquitous fungus at this site (Rosendahl & Stukenbrock, 2004; Stukenbrock & Rosendahl, 2005), but the dominance of *F. mosseae* in *Dianthus* was unexpected as it is relatively rare and was only detected in *c.* 10% of *Hypochoeris* plants in a large survey (Lekberg *et al.*, 2012). This nonrandom host association is particularly interesting given that no difference in AM fungal communities has been documented among highly colonized plants in this grassland previously (Stukenbrock & Rosendahl, 2005). We deliberately choose the term 'nonrandom host association' in favor of 'host preference', because the higher abundance of *F. mosseae* in *Dianthus* may have little to do with a preference for *Dianthus*. On the contrary, *F. mosseae* may very well prefer other, better hosts, but could be limited in those root systems by abundant OTUs that may be superior competitors. There are also clear differences in life history strategies between the fungi that dominate in the two plant species that could contribute to the compositional differences observed. For example, Cluster D form large mycelia (Rosendahl & Stukenbrock, 2004) and appear never to sporulate (Lekberg & Rosendahl, personal observations), whereas *F. mosseae* is considered a ruderal species that sporulates frequently (Sykorova *et al.*, 2007). Because AM fungi may differ in their ability to acquire C from hosts (Pearson & Jakobsen, 1993), it is also possible that host quality could be an additional, less recognized, driver of community assembly. Indeed, the distribution of *Dianthus* and another putative nonhost plant, *Carex arenaria* (Cyperaceae) correlated significantly with shifts in OTU compositions in a previous large-scale survey in this grassland (Lekberg *et al.*, 2012), suggesting that our more limited sampling reflects plant–AM fungal community interactions within this grassland.

Work on mycoheterotrophic plants has lead to suggestions that the degree of host specificity in AM is greater in plants where only the plant receives benefits from the association (Brundrett, 2004). For example, nonphotosynthetic plants are predominately colonized by Glomus group A fungi (Merckx *et al.*, 2012) and Bidartondo *et al.* (2002) suggested that these fungi may mediate inter-plant C transfer. Perhaps co-incidental, but *Dianthus* was also colonized by Glomus group A fungi, and it is tempting to speculate about other similarities between nonhost plants and mycoheterotrophs; neither plant group likely delivers much C to the fungus, and arbuscules are seldom observed. Whether or not these similarities mean anything is uncertain, and we do not mean to imply that *Dianthus* receives C from the fungus.

By combining microscopic examinations, fatty acid analysis and molecular identification, our results indicate that the putative nonmycorrhizal plant *Dianthus deltiodes* is colonized by a particular subset of AM fungi in this Danish grassland. This suggests that the traditional distinction between host and nonhost is not very useful and that mycorrhizal associations are more nuanced than this dichotomy indicates. Brundrett (2009) argued that colonization by AM fungi in typical nonhost plants should be referred to as Glomeromycotan fungus colonization (GFC) as opposed to AM given that the ecological significance of these interactions are likely low. Indeed, the absence of arbuscules in most *Dianthus* plants here indicates that resource delivery from AM fungi to the plants is probably minimal. However, if the intraradical colonization is high, AM fungi may start competing with putative pathogens for space and resources and result in some pathogen protection (Sikes *et al.*, 2009). From the fungal perspective, these associations may be more important, and our results suggest that *some* C can be delivered from *Dianthus* to AM fungi. Also, because AM fungi can store a substantial amount of the C it acquires from one host in other neighboring roots (Robinson & Fitter, 1999), it is possible that *F. mosseae* largely uses *Dianthus* roots as a refuge to store C that it gains from spotty colonization of better hosts. Colonization for the sole purpose of acquiring nutrients from dying and decomposing roots (Brundrett, 2004) seems unlikely here, because *Dianthus* roots all appeared healthy. In summary, while most

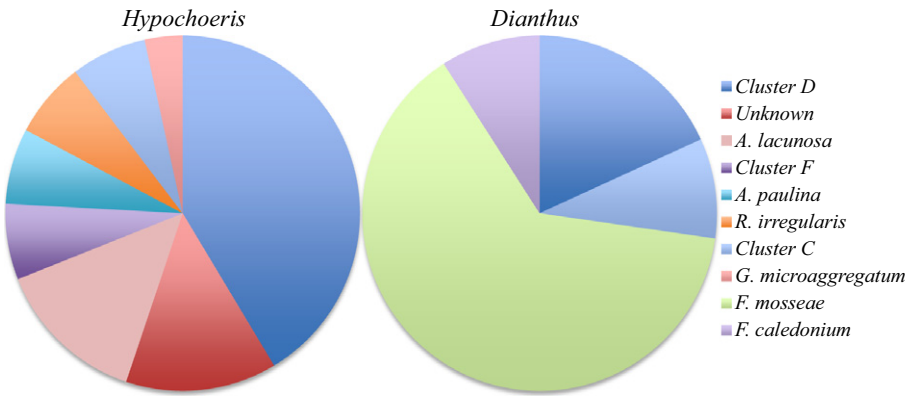

Fig. 3 The distribution of sequence types differed ($P = 0.04$) between *Hypochoeris radicata* and *Dianthus deltoides*. The dominant sequence type in *Dianthus* (*Funneliformis mosseae*) was not found in *Hypochoeris*. Sequence names are adopted from Rosendahl & Stukenbrock (2004), Stukenbrock & Rosendahl (2005) and Lekberg *et al.* (2012) with some updated nomenclature based on Redecker *et al.* (2013).

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research on the mycorrhizal status of nonhost plants was published decades ago, modern DNA- and isotope-based tools allow us to probe the physiological and ecological significance of this specific symbiosis, which can increase our understanding of plant–AM fungal interactions in general. It is our hope that this small study will inspire others to address these questions elsewhere to substantiate (or refute) findings presented here.

Acknowledgements

The authors thank Roger Koide, Philip Ramsey, Lauren Waller and three anonymous reviewers for their constructive comments on the manuscript. Y.L. is grateful to MPG Operations and P.A.O. to the Swedish Research Council for funding.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Operational taxonomic units (OTUs), accession numbers and their presence in 16 *Hypochoeris radicata* and nine *Dianthus deltoides* individuals

Table S2 Shoot and fine root dry weight (DW) as well as ^{13}C-concentration as indicated by δ^{13}C values of shoots and fine roots at harvest, 3 d after labeling in *Hypochoeris radicata* and *Dianthus deltoides*

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Key words: arbuscular mycorrhiza (AM), carbon allocation, *Dianthus deltoides*, grassland, *Hypochoeris radicata*, lipid analysis, nonmycorrhizal plants, stable isotope probing.

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