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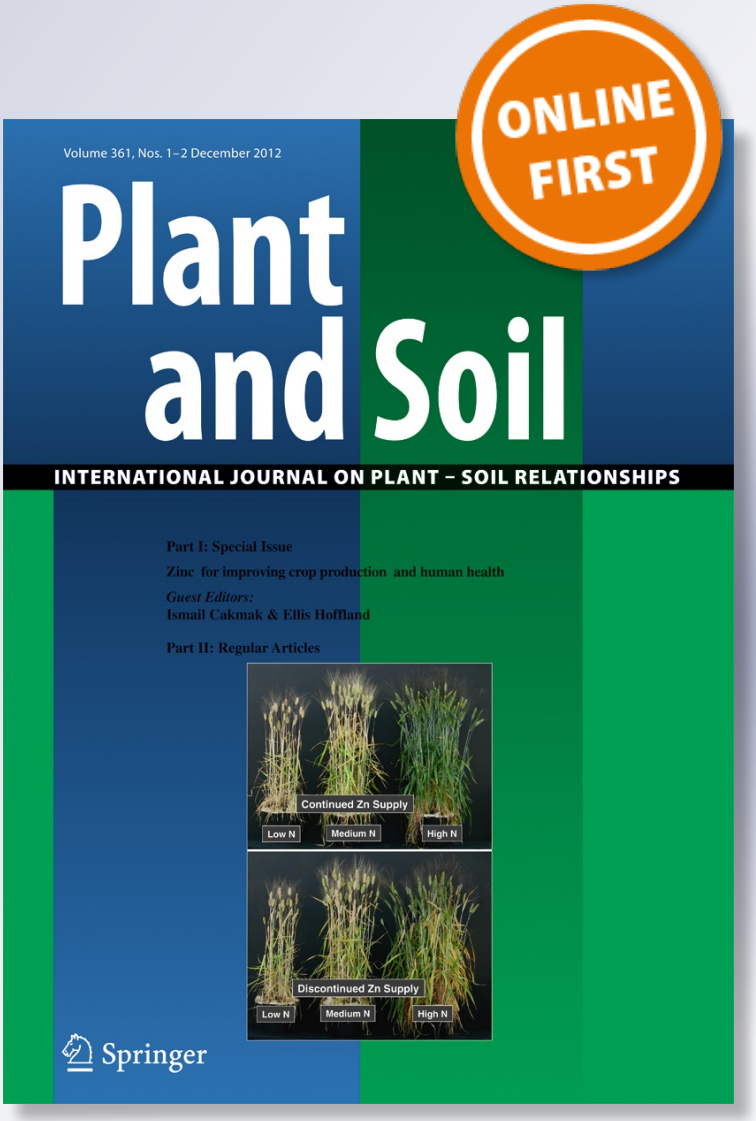

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REGULAR ARTICLE

Seasonal carbon allocation to arbuscular mycorrhizal fungi assessed by microscopic examination, stable isotope probing and fatty acid analysis

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Abstract
Background and Aim Climate change models are limited by lack of baseline data, in particular carbon (C) allocation to – and dynamics within – soil microbial communities. We quantified seasonal C-assimilation and allocation by plants, and assessed how well this corresponds with intraradical arbuscular mycorrhizal fungal (AMF) storage and structural lipids (16:1ω5 NLFA and PLFA, respectively), as well as microscopic assessments of AMF root colonization.
Methods Coastal *Hypochoeris radicata* plants were labeled with $^{13}CO_2$ in February, July and October, and ^{13}C-allocation to fine roots and NLFA 16:1ω5, as well as overall lipid contents and AM colonization were quantified.
Results C-allocation to fine roots and AMF storage lipids differed seasonally and mirrored plant C-assimilation, whereas AMF structural lipids and AM colonization showed no seasonal variation, and root colonization exceeded 80 % throughout the year. Molecular analyzes of the large subunit rDNA gene indicated no seasonal AMF community shifts.
Conclusions Plants allocated C to AMF even at temperatures close to freezing, and fungal structures persisted in roots during times of low C-allocation. The lack of seasonal differences in PLFA and AM colonization indicates that NLFA analyses should be used to estimate fungal C-status. The implication of our findings for AM function is discussed.

Keywords Arbuscular mycorrhiza · Carbon allocation · Grassland · Lipid analysis · Season · Stable isotope probing

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Introduction

Global climate change is expected to cause milder winters and extended growing seasons in many

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European countries, and phenological databases show that spring has arrived on average 2.5 days earlier for every decade since 1971 (Menzel and Fabian 1999; Menzel et al. 2006). This milder climate may allow plants and soil microbial communities to be active year-round even in northern latitudes (Keeling et al. 1996; Myneni et al. 1997). Indeed, coastal heath vegetation in Denmark takes up significant amounts of nitrogen (N) during the winter months (Andresen and Michelsen 2005), and experimental warming of Alaskan soils by 1.5 °C during the winter months has resulted in a doubling of respiratory CO_2 losses to the atmosphere (Natali et al. 2011). In order to construct good, predictive models of how ecosystems may respond to climate change, greater baseline data – including processes operating during the winter months – is sorely needed.

Grasslands cover a large area of the global land surface, and most plants within these communities form a root symbiosis with arbuscular mycorrhizal fungi (AMF) in which the fungi deliver phosphorus (P), nitrogen (N) and possibly other services (Smith and Read 2008) in exchange for carbon (C). Up to 30 % of C fixed during photosynthesis can be allocated to the AMF (Drigo et al. 2010), and some of this C is accessed by other soil microbes, which could make AMF the largest contributor to soil microbial biomass (Miller et al. 1995; Olsson et al. 1999). However, C-allocation to AMF is not constant, but depends on soil nutrient availability and atmospheric CO_2 concentrations (Treseder 2004), which in turn will influence AM function (Johnson 2010). Based on this, it is quite remarkable that AMF – as well as other soil microbes – are not explicitly considered in any current climate change models but are simply represented by kinetic constants that assume that microbial processes do not change across environmental conditions, and are never limited by microbial abundances or dependent on community compositions (Allison and Martiny 2008).

Previous studies have shown that AMF may respond to shifts in both temperature and moisture. For example, experimental manipulations with warmer winters and summer droughts have shown shifts in allocation patterns between storage structures in roots (vesicles), and growth in soil by extraradical hyphae (ERH), as well as changes in fungal abundance and richness (Staddon et al. 2003; Hawkes et al. 2008, 2011). These shifts can significantly impact mycorrhizal function and nutrient cycles, because the ERH can acquire and translocate the majority of plant P (Smith et al. 2003) and access organic N sources (Barrett et al. 2011).

A second issue in need of further study is the appropriateness of current methods to evaluate responses to various perturbations. Traditional microscopic evaluations of AMF abundance based on morphological structures may have low resolution in detecting temporal patterns of fungal abundance and activity. It is questionable if the lack of seasonal shifts in AM colonization observed in some studies (Sanders and Fitter 1992; Merryweather and Fitter 1998, but see Bentivenga and Hetrick 1992) truly reflects a steady C supply from the plant.

The objectives of this study were to track seasonal C-assimilation by plants and allocation to AMF intraradical structures, and to assess how well traditional microscopy reflects fungal C-status. We used stable $^{13}CO_2$ isotope labeling of the perennial forb *Hypochoeris radicata* growing in a Danish coastal grassland, and subsequent compound specific isotope ratio mass spectrometry to estimate seasonal C-flow from the atmosphere to the AMF signature fatty acid 16:1ω5 (Olsson and Johnson 2005). Finally, AMF community composition was analysed in order to determine if seasonal C-allocation may be explained by changes in AMF communities.

Materials and methods

Field site and $^{13}CO_2$ labeling

The study was conducted in a species-rich coastal grassland of North Zeeland, Denmark (56°01′N-11° 59′E; Rosendahl and Stukenbrock 2004). *Hypochoeris radicata* plants were exposed to a $^{13}CO_2$-enriched atmosphere on three occasions over the year; 13–15 October, 2007, 4–6 February and 21–23 July, 2008. On each occasion, 21 individual plants were randomly selected from a 50 × 100 m area and labeled for three consecutive days by enclosing the entire shoot in a 1 L plastic bag and injecting 5 mL 99.9 % $^{13}CO_2$ gas (Cambridge Isotope Laboratories; Andover MA, USA) with a syringe. Each of the three pulse-labeling periods lasted 2 h at mid-day. The average temperature during labeling was 12 °C in October, 5 °C in February, and 20 °C in July, and the cumulative precipitation over the period from 2 weeks prior to labeling was 49 mm for October, 34 mm for February, and 42 mm for July (http://

www.dmi.dk/dmi/index/danmark/ugeberetning.htm). While we did not measure soil temperature, data loggers placed at 5 cm depth at a nearby research site (Mikkelsen et al. 2008) recorded maximum soil temperatures (corresponding to our time of labeling) of 13.8 °C (± 0.15 SD, n = 4) in October, 6.1 °C (± 0.11) in February, and 21.2 °C (± 0.80) in July, which corresponds closely to our measured air temperatures. Two small shoot samples (1 cm^2) per plant were cut off following the last labeling day to estimate the ^{13}C assimilated during labeling. Seven plants were harvested 1, 3, and 7 d after the last labeling using a trowel, and care was taken to retrieve the whole root system, which never exceeded 20 cm. Non-labeled control plants (one per season) were also harvested for measurements of background ^{13}C-values. All plant samples were stored at 4 °C and processed within 24 h.

Shoots and coarse roots (>1 mm ∅; including tap roots) were washed free of soil and dried at 65 °C for 48 h for dry weight (DW) measurements. Fine roots (<1 mm ∅) were washed, blotted dry with paper towels and the fresh weight (FW) was recorded. Subsamples of known weight were removed for measurements of AM colonization, frozen in Tris-EDTA buffer at -20 °C until molecular analyses, and freeze-dried and stored at -20 °C until lipid extractions. Roots from 12 randomly selected plants in February, July and October were cleared and stained in trypan blue (Brundrett et al. 1996) and overall AM colonization and vesicle abundance were determined using the gridline intersect method based on approximately 50 intercepts per sample (McGonigle et al. 1990).

To estimate ^{13}C-allocation within the plant, between 1 and 4 mg of dried and ground shoot (both from immediately after labeling, and at harvest), tap root and fine root tissue were used for analysis of excess tissue ^{13}C-concentration in an Eurovector CN analyzer (EuroVector SpA., Milan, Italy) coupled to an Isoprime (Isoprime Ltd., Cheadle, UK) isotope ratio mass spectrometer. Total plant ^{13}C was calculated by multiplying the ^{13}C concentration (standardized by the appropriate control plant for background ^{13}C) by the total tissue DW.

Fatty acid extraction, quantification and ^{13}C-enrichment

Lipids were extracted from freeze dried fine roots (15–65 mg, depending on the quantity of root material that was available) in a one-phase mixture of citrate-buffer, methanol and chloroform (0.8:2:1, v:v:v, pH 4) following van Aarle and Olsson (2003). The lipids were fractionated into neutral lipids, glycolipids and phospholipids on silica columns by eluting with chloroform, acetone and methanol, respectively. The fatty acid residues in neutral lipids and phospholipids were converted into free fatty acids methyl esters and analyzed by gas chromatography using 50 m HP5 capillary fused silica column (Hewlett Packard, Wilmington, DE, USA) with H_2 as carrier gas (Frostegård et al. 1993). In accordance with previous work (e.g. Olsson and Johnson 2005), we used 16:1ω5 as a signature lipid for AMF, where its neutral lipid (NLFA) and phospholipid (PLFA) fractions were identified from their retention times in relation to that of the internal standard (fatty acid methyl ester 19:0). While the specificity of PLFA 16:1ω5 to AMF has been questioned in field studies due to its presence in soil-dwelling bacteria (Frostegård et al. 2010), lack of AMF specificity is less of a concern for this study because we extracted lipids from roots and not soil. Furthermore, we have observed negligible amounts of 16:1ω5 in roots of non-mycorrhizal plants and plants with low AM colonization at the same field site (e.g. *Dianthus deltoides* and *Carex arenaria*), which indicates that this lipid fraction is suitable for quantifying AMF abundance in the field (Lekberg, unpublished data). Whereas the neutral lipid fatty acid (NLFA) 16:1ω5 was analyzed as an indicator of AMF storage lipids, the phospholipids fatty acid (PLFA) 16:1ω5 was chosen as an important part of AMF membranes and a measure of structural lipids (van Aarle and Olsson 2003). In order to determine the C-allocation to AMF storage lipids, we measured the ^{13}C atom-% in the NLFA 16:1ω5 by isotope ratio mass spectrometry (Olsson et al. 2005) by an ANCA-NT-20-20 Stable Isotope Analyser (PDZ Europa Scientific Instruments, Crewe, UK) interfaced to a gas chromatograph (6890, Hewlett Packard, Palo Alto, CA, USA). We only measured ^{13}C atom-% in NLFA 16:1ω5 (not PLFA 16:1ω5) as it is the dominant lipid fraction in AMF. The gas chromatograph was equipped with a 50-m column (HP-5, Agilent, Palo Alto, CA, USA) with He as a carrier gas. The ^{13}C-enrichment was calculated by subtracting the natural ^{13}C-abundance of 16:1ω5 measured in the control samples from that in samples. AMF excess ^{13}C in the 16:1ω5 was calculated by multiplying the ^{13}C-enrichment in the

NLFA fraction 16:1ω5 with the total amount of NLFA 16:1ω5.

AMF community composition

AMF identities were determined in eight 0.5 cm root pieces per plant according to Rosendahl and Stukenbrock (2004). Briefly, root pieces were crushed separately in 40 μL Tris EDTA pH 8.0 buffer and 10 μL Chelex, heated to 95 °C for 2 min and centrifuged. Two μL of diluted template were used in PCR 1 in which the 3' end of the nuclear large ribosomal subunit (LSU) was amplified with the eukaryotic primers 0061 and NDL22 (van Tuinen et al. 1998) at the following thermocycling parameters: 1 min 95 °C, 30 cycles of 1 min at 95 °C, 1 min of 54 °C and 1 min at 72 °C (+ 4 s per cycle) with a final extension phase of 5 min at 72 °C. PCR1 products were diluted 50× and amplified with the primer pair FLR3-FLR4 (van Tuinen et al. 1998) for 25 cycles as before, except for an annealing temperature of 60 °C and no cycle time extension. All positive PCR products were sequenced (Macrogen, Seoul, Korea) and aligned with sequences obtained from the same grassland and elsewhere (Rosendahl and Stukenbrock 2004). We used a neighbor joining tree clustering algorithm to identify operational taxonomic units (OTUs) as clades with greater than 97 % bootstrap support. Representative sequences have been deposited in the EMBL public sequence database under the accession numbers HE855879 to HE855887.

Statistical analyses

The impact of season and harvest date (1,3,7 days after last labeling) on ^{13}C-allocation within the plant was analyzed using a three-way ANOVA with harvest day, season and tissue type as the three factors. Seasonal differences in plant DW, NLFA and PLFA contents, AM colonization, and ^{13}C-concentration and content in 16:1ω5 were analyzed by one-way ANOVAs in Minitab (Minitab Inc., State College, USA). Residuals were plotted and data transformed when necessary to improve normality and homoscedacity. Mean comparisons were based on the least significant difference method at alpha = 0.05. Tap root and fine root DW values were ln-transformed, as were vesicle colonization, whereas NLFA concentration and content, and ^{13}C-concentration and content to 16:1ω5 was square root transformed. Seasonal changes in OTU abundances were evaluated with a Chi-square test (http://statpages.org/) in a 3 (month) × 9 (OTU) contingency table. Sampling effort curves of OTUs at the three sampling times were constructed in EstimateS (Version 8.2, R. K. Colwell, http://purl.oclc.org/estimates).

Results

Plant biomass, AM colonization and AMF indicator 16:1ω5 PLFA and NLFA

Shoot and tap root DW differed over the season ($F_{2.60}$ = 21.03, p<0.001 and $F_{2.60}$ = 7.57, p=0.001, respectively) with smallest values in February and largest in July (Table 1). Fine root DW, on the other hand, did not show seasonal differences ($F_{2.60}$ = 2.18, p=0.12; Table 1). Total AM colonization did not differ seasonally ($F_{2.35}$ = 1.18, p=0.32) and exceeded 80 % in all months, whereas the colonization by vesicles differed seasonally ($F_{2.35}$ = 3.81, p=0.032) and was highest in July (Table 1).

The concentration and content of PLFA 16:1ω5 (a measure of AMF structural lipids contained in membranes) mirrored the AM colonization and did not differ seasonally ($F_{2.58}$ = 1.99, p=0.15 and $F_{2.58}$ = 2.85, p = 0.07, respectively; Table 2), whereas the NLFA 16:1ω5 concentration and content (a measure of AMF storage lipids) differed among seasons ($F_{2.58}$ = 18.72, p<0.001 and $F_{2.58}$ = 17.46, p<0.001, respectively) and was highest in October and lowest in February (Table 2).

^{13}C-assimilation and allocation

As expected, plants assimilated less ($F_{2.59}$ = 23.7, p<0.001) ^{13}C in February (0.65 ± 0.05 μg ^{13}C mg tissue^{-1}, mean ± se) than in July (1.27 ± 0.12) and October (1.32 ± 0.09). This was reflected in the ^{13}C-concentration and contents of shoots, tap roots and fine roots, which all showed seasonal differences (Tables 3 and 4, Fig. 1). There was also a difference in allocation patterns, with the highest ^{13}C-allocation to shoots and tap roots in July and fine roots in October (Tables 3 and 4, Fig. 1). Surprisingly, even though ^{13}C tended to decline in shoots the first week of post labeling in July and October (data not shown), we detected no effect of harvest date (1, 3 and 7 days

Table 1 Shoot, tap root, and fine root DW, and percentage total AM and vesicular colonization of *Hypochoeris radicata* in a Danish coastal grassland in February, July and October

Harvest	Shoot DW (mg)	Tap root (mg)	Fine root (mg)	AM colonization (%)	Vesicles (%)
Feb	135 (15.1)^{b}	267 (36.9)^{b}	67.6 (8.66)	89.5 (2.16)	16.3 (6.63)^{ab}
July	310 (22.9)^{a}	486 (59.1)^{a}	65.4 (6.05)	82.2 (3.67)	23.3 (5.76)^{a}
Oct	269 (21.2)^{ab}	380 (49.1)^{ab}	82.0 (6.66)	87.4 (4.17)	6.28 (1.51)^{b}

Values with different superscripts differ from each other at $p \leq 0.05$. Means (SE) with $n = 21$ for biomass and n=12 for AM colonization

after harvest) and there was no harvest date × season interaction (Table 3). This suggests that ^{13}C was allocated rapidly to the various tissue types and that only small portions were lost through respiration during the first week after labeling. Because there was no effect of harvest day and its interactions with other factors (Table 3), the ^{13}C values for the three harvest days are hereafter pooled. Carbon allocation to NLFA 16:1ω5 correlated with the total amount of ^{13}C allocated to fine roots ($p = 0.001$, $r = 0.43$). Both ^{13}C concentration and content of NLFA 16:1ω5 differed seasonally ($F_{2.53} = 22.9$, $p<0.001$ and $F_{2.53} = 20.4$, $p<0.001$, respectively) and were highest in October and lowest in February (Table 4, Fig. 1).

AMF community composition

Overall, we obtained 246 sequences that could be aligned with sequences obtained previously from this grassland (Rosendahl and Stukenbrock 2004) and elsewhere (Fig. 2). This represents a sequencing success of approximately 50 % of all root segments distributed equally across all three months, with the other half of root segments either not amplifying, resulting in mixed sequences (due to multiple AMF OTUs within the 0.5 cm root segment) or failed sequencing. To avoid pseudo-replication, we only recorded the presence, not the abundance, of the various OTUs in each root system (Table S1). We detected no seasonal shift in the frequency of occurrence of OTUs (χ^2=22.2, p=0.14), and the sampling effort curves suggest that – given our methodological approach – sampling was sufficient to detect dominant OTUs, although sampling in October could have been more intense (Fig. S1). All in all, we identified nine OTUs (Fig. 2), including two *Acaulospora* OTUs that clustered closely with *A. lacunosa* and *A. paulinae* respectively. Six OTUs belonged to Glomus group A, three of which clustered with known taxa (*Rhizophagus intraradices, Funneliformis mosseae* and *Glomus sinuosum*), and three that are only known from environmental samples (Glomus group D, F, G). We used the same names for the *Glomus* OTUs as in Rosendahl and Stukenbrock (2004) for easy comparisons. The BLAST hit of the last OTU was within Glomeromycota but it did not align within any known family and may therefore constitute a member of a yet undescribed family.

Table 2 Concentration and content of neutral lipid (NLFA) and phospholipid (PLFA) fatty acid 16:1ω5 in fine roots of *Hypochoeris radicata* in February, July and October. NLFA 16:1ω5 is an indicator of AMF storage lipids and PLFA 16:1ω5 is an indicator of AMF structural lipids (van Aarle and Olsson 2003)

Lipid fraction	Treatment	Concentration (nmol g fine roots^{-1})	Content (nmol root system^{-1})
PLFA	Feb	42.7 (6.93)^{a}	2.70 (0.38)^{a}
	July	31.2 (3.56)^{a}	2.09 (0.34)^{a}
	Oct	45.6 (6.25)^{a}	3.43 (0.45)^{a}
NLFA	Feb	156 (36.4)^{b}	10.1 (2.17)^{b}
	July	713 (141)^{a}	48.5 (9.74)^{a}
	Oct	894 (137)^{a}	70.9 (12.2)^{a}

Different letters within each lipid fraction differ significantly from each other at $p \leq 0.05$. Means (SE) with n=21

Discussion

We found substantial seasonal differences in plant C-assimilation. It was lowest in February, but in accordance with previous research (Larsen et al. 2007), we found that plants were active and allocated C to AMF even at air temperatures close to 0 °C. Whether or not AMF are active during times of low plant C assimilation is uncertain but could be important, because a substantial amount of plant litter is consumed and

Table 3 Three-way ANOVA on ^{13}C-concentrations and ^{13}C-contents in the tissue types (shoot, tap root and fine root) at the harvest days 1, 3, 7 days after last labeling, and seasons (February, July, October) as factors

Source	df	^{13}C-concentration F (conc.)	^{13}C-content P (conc.)	F (cont.)	P (cont.)
Tissue type (Tt)	2	313	<0.001	343	<0.001
Harvest day (Hd)	2	1.78	0.17	1.25	0.29
Season (S)	2	219	<0.001	264	<0.001
Tt x Hd	4	0.80	0.53	0.20	0.94
Tt x S	4	13.4	<0.001	15.5	<0.001
Hd x S	4	1.62	0.17	0.81	0.52
Tt x Hd x S	8	0.59	0.78	1.46	0.18

mineralized by soil microbes during winter, leading to high mineral nutrient availability at this time for uptake by AMF (Bardgett et al. 2005). There is some indications that AMF can acquire and transfer N to host plants at soil temperatures equivalent to our October temperatures (Barrett et al. 2011) and store P during the winter months to be used by host plants in the spring when photosynthetic activity increases (Merryweather and Fitter 1998). This requires further study, because seasonal dynamics in AMF activity and abundance could impact nutrient cycling on an ecosystem scale.

Carbon allocation to AMF intraradical storage lipids corresponded with overall allocation to fine roots, but was approximately three orders of magnitudes smaller (Fig. 1). Total photosynthate allocation to AMF has been estimated to be as high as 20–30 % (Jakobsen and Rosendahl 1990; Drigo et al. 2010), but we only measured the amount of ^{13}C in the NLFA 16:1ω5, and earlier studies have indicated this portion to be at best 20 % of the ^{13}C that is allocated to AMF (Olsson et al. 2002). Also, a large proportion of neutral lipids in AMF are found in extraradical spores (Olsson and Johansen 2000), which were not considered here since all soil-dwelling AMF structures were excluded in our measurements. Finally, the transfer from intraradical mycelium to extraradical may be rapid (Drigo et al. 2010) and respiration and turnover in the soil may be high (Staddon et al. 2003).

We did not observe significant changes in ^{13}C-content 1, 3 and 7 days after labeling (Table 3), which contradicts with some previous findings that show rapid losses of ^{13}C-label with time (Johnson et al. 2002). It is possible that we missed a peak before the first, or between harvests, but differences could also be due to the tissue fractions analyzed. For example, a pulse in respired CO_2 may peak more rapidly than C incorporated into storage compounds, and Olsson and Johnson (2005) showed that ^{13}C in certain lipid fractions are stable for up to 30 days. An alternative explanation would be that labeling over three consecutive days blurred the peak of ^{13}C.

Table 4 ^{13}C-content in shoots, tap roots, fine roots of *Hypochoeris radicata* and AMF (based on ^{13}C and NLFA 16:1ω5 content) in February, July and October. Values from the three harvests (1, 3, and 7 days after pulse-labeling) were pooled for each season, because the values did not differ significantly and there was no significant season × harvest day interaction (Table 3)

Month	Shoot (μg ^{13}C)	Tap root (μg ^{13}C)	Fine root (μg ^{13}C)	AMF (ng ^{13}C)
Feb	140 (17.5)^{c}	17.8 (2.44)^{c}	14.0 (2.40)^{c}	2.13 (0.53)^{b}
July	951 (86.3)^{a}	336 (42.9)^{a}	44.7 (5.31)^{b}	38.8 (9.45)^{a}
Oct	698 (55.2)^{b}	144 (23.1)^{b}	80.2 (7.98)^{a}	53.7 (16.6)^{a}

Different letters within each tissue type indicate significant differences at $p \leq 0.05$. Means (SE), n=21. Note that ^{13}C–contents of AMF are given in ng and not μg

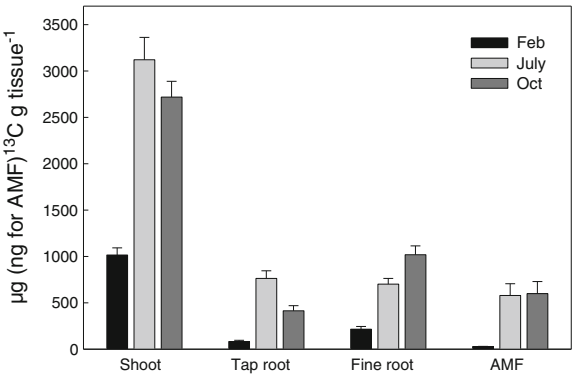

Fig. 1 ^{13}C-concentration in shoots, tap roots (TR), fine roots (FR) and AMF (based on ^{13}C-concentration in NLFA 16:1ω5 in roots) after pulse-labeling of *Hypochoeris radicata* in a Danish coastal grassland in February, July and October. The three harvests (1, 3 and 7 days after last labeling) were combined as harvest day was not significant. Mean (se), *n*=21. Please note that the ^{13}C-concentration in AMF is in ng, not μg

In accordance with some previous studies in the UK and Denmark (Sanders and Fitter 1992; Merryweather and Fitter 1998; Andresen and Michelsen 2005; but see Staddon et al. 2003), no seasonal shift in AM colonization was observed. This was supported by a lack of differences in AMF structural lipids (Table 2), but contrasts drastically with the large fluctuations in AMF storage lipids, which were significantly lower in February (Table 2). Overall, the discrepancy between AM colonization measures, structural lipids and storage lipids in our study has two important implications. First, intraradical fungal structures remain in the roots throughout the year in spite of large fluctuations in belowground C allocation. Second, NLFA may be superior to AM colonization and PLFA to assess the energy status of AMF, because NLFA content tracks C-availability and can be an order of magnitude greater than PLFA 16:1ω5 contents within roots. It is curious that no correspondence between vesicle abundance (highest in July) and AMF storage lipids (highest in October) was detected, although this agrees with previous findings (van Aarle and Olsson 2003) and may suggest that vesicles may not be the most important mode of internal C storage. It is also interesting to note that seasonal differences in AM colonization *have* been documented in some sites (e.g. Bentivenga and Hetrick 1992; Staddon et al. 2003), and identifying what may cause these differences among sites could be informative.

Seasonal shifts in AMF communities have been observed in some (Merryweather and Fitter 1998) but not all studies (Rosendahl and Stukenbrock 2004). AMF families differ in their C-allocation strategies, and some allocate relatively more C to extraradical relative to intraradical structures (Hart and Reader 2002). These differences could complicate seasonal studies such as the one conducted here, but since no significant shift in AMF community composition was observed, the recorded seasonal difference in C-allocation appear not to have been driven – or caused – by shifts in the relative abundance of AMF OTUs.

Given that stressors, such as drought, can result in resilient communities (Hawkes et al. 2011), it is

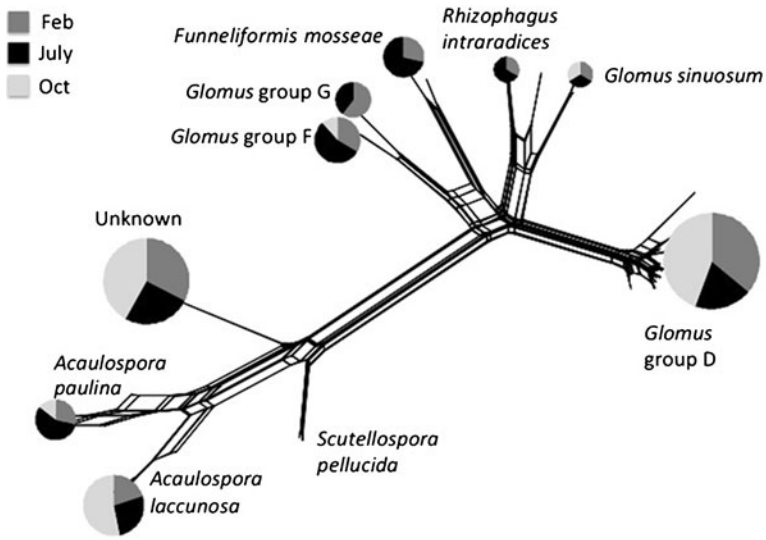

Fig. 2 NeighborNet split network of arbuscular mycorrhizal fungal (AMF) operational taxonomic units (OTUs) in *Hypochoeris radicata* individuals in February, July, and October (*n*=21) based on 97 % sequence identity in the nuclear large subunit rRNA gene. The "unknown" falls within the Glomeromycota but lacks a close match to any known family. The relative size of the circles reflects their log10-transformed abundances across all three months

plausible that seasonal shifts in C-allocation make AMF more tolerant to some perturbations associated with climate change. Hawkes et al. (2008) recorded a shift from storage (vesicles) to growth (extraradical hyphae) with warmer temperatures, and a faster translocation and respiration by hyphae and roots. Contrary, Staddon et al. (2003) found that both winter warming and summer drought increased AMF root colonization, but decreased the amount of extraradical hyphae in soil. How these responses influence AMF resilience and mycorrhizal function is largely unknown and additional studies are sorely needed. Future studies should collect data that can be used to improve climate change models as it is clear that the static, kinetic constants currently used are insufficient. Based on results obtained here, we argue that fatty acid stable isotope probing is a valuable tool that should be utilized more as it allows for in situ physiological studies.

In summary, we showed that plant C-allocation to AMF intraradical structures differed seasonally and corresponded with that to fine roots. Seasonal differences were reflected in AMF storage, but not structural lipids, which suggest that AMF structures can persist under times of low C-supply, but their abundance does not reflect fungal energy status.

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References

Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci USA 105:11512–11519

Andresen LC, Michelsen A (2005) Off-season uptake of nitrogen in temperate heath vegetation. Oecologia 144:585–597

Bardgett RD, Bowman WD, Kaufmann R, Schmidt SK (2005) A temporal approach to linking aboveground and belowground ecology. Trends Ecol Evol 20:634–641

Barrett G, Campbell CD, Fitter AH, Hodge A (2011) The arbuscular mycorrhizal fungus *Glomus hoi* can capture and transfer nitrogen from organic patches to its associated host plant at low temperature. Appl Soil Ecol 48:102–105

Bentivenga SP, Hetrick BAD (1992) Seasonal and temperature effects on mycorrhizal activity and dependence of cool- and warm-season tallgrass prairie grasses. Can J Bot 70:1596–1602

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. ACIAR Monograph 32. ACIAR, Canberra

Drigo B, Pijl AS, Duyts H, Kielak AM, Gamper HA, Houtekamer MJ, Boschker HTS, Bodelier PLE, Whiteley AS, van Veen JA, Kowalchuk GA (2010) Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO_2. Proc Natl Acad Sci USA 107:10938–10942

Frostegård A, Tunlid A, Bååth E (1993) Phospholipid fatty-acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy-metals. Appl Environ Microb 59:3605–3617

Frostegård Å, Tunlid A, Bååth E 2010. Use and misuse of PLFA measurements in soils. Soil Biol Biochem 42:1621–1625

Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Hawkes CV, Hartley IP, Inseson P, Fitter AH (2008) Soil temperature affects carbon allocation within arbuscular mycorrhizal networks and carbon transport from plant to fungus. Glob Chang Biol 14:1181–1190

Hawkes CV, Kivin SN, Rocca JD, Huguet V, Thomsen MA, Suttle KB (2011) Fungal community responses to precipitation. Glob Chang Biol 17:1637–1645

Jakobsen I, Rosendahl L (1990) Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. New Phytol 115:77–83

Johnson NC (2010) Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. New Phytol 185:631–647

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

Keeling CD, Chin JFS, Whorf TP (1996) Increased activity of northern vegetation inferred from atmospheric CO2 measurements. Nature 382:146–149

Larsen KS, Ibrom A, Beier C, Jonasson S, Michelsen A (2007) Ecosystem respiration depends strongly on photosynthesis in a temperate heath. Biogeochemistry 85:201–213

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. New Phytol 115:495–501

Menzel A, Fabian P (1999) Growing season extended in Europe. Nature 397:659–659

Menzel A, Sparks TH, Estrella N, Koch E, Aasa A, Ahas R, Alm-Kubler K, Bisolli P, Braslavksa O, Briede A et al (2006) European phenological response to climate change matches the warming pattern. Glob Chang Biol 12:1969–1976

Merryweather J, Fitter A (1998) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* - II. Seasonal and spatial patterns of fungal populations. New Phytol 138:131–142

Mikkelsen TN, Bier C, Jonasson S, Holmstrup M, Schmidt IK, Ambus P, Pilegaard K, Michelsen A, Albert K, Andresen LC et al (2008) Experimental design of multifactor climate change experiments with elevated CO_2, warming and drought: the CLIMAITE project. Funct Ecol 22:185–195

Miller RM, Reinhardt DR, Jastrow JD (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. Oecologia 103:17–23

Myneni RB, Keeling CD, Tucker CJ, Asrar G, Nemani RR (1997) Increased plant growth in the northern high latitudes from 1981 to 1991. Nature 386:698–702

Natali SM, Schuur EAG, Trucco C, Pries CEH, Crummer KG, Lopez AFB (2011) Effects of experimental warming of air, soil and permafrost on carbon balance in Alaskan tundra. Glob Chang Biol 17:1394–1407

Olsson PA, Johansen A (2000) Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycol Res 104:429–434

Olsson PA, Johnson NC (2005) Tracking carbon from the atmosphere to the rhizosphere. Ecol Lett 8:1264–1270

Olsson PA, Thingstrup I, Jakobsen I, Bååth E (1999) Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. Soil Biol Biochem 31:1879–1887

Olsson PA, van Aarle IM, Allaway WG, Ashford AE, Rouhier H (2002) Phosphorus effects on metabolic processes in monoxenic arbuscular mycorrhiza cultures. Plant Physiol 130:1162–1171

Olsson PA, van Aarle IM, Gavito ME, Bengtson P, Bengtsson G (2005) ^{13}C incorporation into signature fatty acids as an assay for carbon allocation in arbuscular mycorrhiza. Appl Environ Microbiol 71:2592–2599

Rosendahl S, Stukenbrock EH (2004) Community structure of arbuscular mycorrhizal fungi in undisturbed vegetation revealed by analyses of LSU rDNA sequences. Mol Ecol 13:3179–3186

Sanders IR, Fitter AH (1992) The ecology and functioning of vesicular arbuscular mycorrhizas in coexisting grassland species. 1. Seasonal patterns of mycorrhizal occurrences and morphology. New Phytol 120:517–524

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Academic, Cambridge

Smith SE, Smith FA, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiol 133:16–20

Staddon PL, Thompon K, Jakobsen I, Grime JP, Askew AP, Fitter AH (2003) Mycorrhizal fungal abundance is affected by long-term climatic manipulations in the field. Glob Chang Biol 9:186–194

Treseder KK (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus and atmospheric CO_2 in field studies. New Phytol 164:347–355

van Aarle IM, Olsson PA (2003) Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Appl Environ Microb 69:6762–6767

Van Tuinen DE, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887