The impact of increased atmospheric carbon dioxide on microbial community dynamics in the rhizosphere

Drigo, B.

Chapter 5

Tracking microbial responses in the rhizosphere of plants subjected to elevated CO_2

Barbara Drigo, Johannes A. van Veen, Henricus T.S. Boschker and George A. Kowalchuk

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Abstract

To assess the effects of elevated atmospheric CO_2 on microbial communities that respond to plant-derived C substrates in the rhizosphere, a ^{13}C-CO_2 pulse-chase labelling experiment was performed with *Festuca rubra* plants grown in ambient (350 µl/l) versus elevated (700 µl/l) CO_2 environments, and results compared with those observed for a non-mycorrhizal plant species, *Carex arenaria.* Fatty acid biomarker analyses revealed an initial rapid transfer of plant assimilates to arbuscular mycorrhizal fungi (AMF), with a gradual release of C to the bacterial community. The bacterial genera *Burkholderia* and *Pseudomonas*, were strongly influenced by elevated CO_2, whereas the genus *Bacillus* and actinomycetes were not, suggesting that effective accumulation of plant-derived carbon in the short term is restricted to efficient rhizosphere colonizers. Our results indicate that effects of plant-derived carbon are principally mediated by AMF particularly at elevated CO_2, with direct plant/bacterial interactions initially playing a minor role.

Introduction

Given that soil is the largest reservoir of organic carbon (C) in the terrestrial biosphere, significant efforts have focused on understanding the soil processes involved in terrestrial C flow and the impact of rising CO_2 levels on these. Although considerable attention has been paid to assessing aboveground ramifications of increased atmospheric CO_2 levels, relatively little is known about the associated changes in belowground community dynamics. In order to better understand and predict future responses of terrestrial ecosystems to increasing atmospheric CO_2 levels the studies on below-ground dynamics, including roots and associated microorganisms, are also necessary.

Root-associated microorganisms play a major role in the flow of C through the plant-soil system, as they are the primary utilizers of root-derived C. Furthermore, the microbial community within the rhizosphere is known to exert a feedback on plant productivity, as well as the quantity and quality of root-derived substrates (Hu *et al.* 1999; Moore & de Ruiter 2007).

Microbial responses to increase plant C fixation, as a result of elevated atmospheric CO_2, are expected via both direct interactions with the plant and its exudates, as well as via indirect interactions. Rhizosphere bacteria and arbuscular mycorrhizal fungi (AMF) have been postulated to be the most important potential sequesters of plant-derived carbon in plant-soil systems (Phillips 2007; Staddon 2005). Rhizosphere bacteria are known to feed directly on plant-derived exudates, and this nutritional source has been demonstrated to exert a selective pressure on the structure and function of bacterial communities inhabiting the rhizosphere (Phillips *et al.* 2007). AMF, which form symbioses with the majority of land plants and depend on plant-derived C, have also been put forth as the key functional group of soil organisms involved in the sequestration of plant-derived C, and excesses thereof, in response to elevated CO_2 in the atmosphere (Staddon 2005). Although some recent progress has been made in our understanding of C fluxes from the plant, through AMF, rhizosphere communities and the soil food-web, knowledge is still rather scarce with respect to the relative flow of C to different biological groups of the plant-soil ecosystem (Olsson & Johnson 2005; Carney *et al.* 2007; Kreuzer-Martin 2007). Such knowledge is critical to not only our understanding of soil foodweb, but also to predicting the future impacts of increasing CO_2 levels.

The recent development of ^{13}C Stable Isotope Probing (SIP) (reviewed in Neufeld *et al.* 2007), and its applications for tracking plant-derived C fluxes into microbial nucleic acids (Lu & Conrad 2005) or biomarkers (Treonis *et al.* 2004; Carney *et al.* 2007), provides a means of understanding the flux of C through plant-associated microbial communities and the impact of elevated CO_2 on these relationships. In order to track the fate of plant-assimilated C to the belowground microbial community, and to examine the impact of elevated atmospheric CO_2 levels on these processes, we conducted a $^{13}CO_2$ pulse-chase labelling experiment. The experiment involved a mycorrhizal plant species, *Festuca rubra* ssp. *arenaria*, as well as a non-mycorrhizal plant, *Carex arenaria*, for comparison, and examined microbial community responses for plants grown under ambient (350 µl/l) versus elevated (700 µl/l) CO_2 conditions. To gain insight into the flow of carbon to different soil-borne microbial groups, specific fatty-acid biomarkers for AMF, total bacteria, *Pseudomonas* spp., *Burkholderi*a spp., *Bacillus*, actinomycetes and protozoa were used to track the ^{13}C allocation from the atmosphere into rhizosphere communities.

Methods

Soil and plant pretreatment

Soil was collected in the spring of 2005 from *F. rubra* and *C. arenaria* clonal growth site classified as river dune, at Bergharen (51°51'31.37"N; 5°40'9.86"E; the Netherlands). The soil had a sandy texture with a pH of 4.32, low calcium carbonate contents, 1.97 % organic C and 1.7 mg/kg fungal biomass (based on ergosterol data). The sampling site was divided into 4×4 m subplots. For each subplot, ten cores were taken within tussocks of *F. rubra* and *C. arenaria* and the 5-15 cm layer was collected. Soil samples were put in plastic bags and transported in a cooling box to the lab. The material was sieved (4 mm mesh), homogenized and stored at 4 °C until use (within one week after sampling).

Plastic containers (1100 cm^3) were filled with 1 kg of soil and wetted to 10% volumetric water content (based on dry weight). Prior to planting, soil-filled containers were kept in a greenhouse for 4 weeks, in order to allow weeds germination, and germinated weeds were subsequently removed prior to the experiment.

Seeds of *F. rubra* and *C. arenaria* were sterilized and germinated on sterilized glass beads (3 mm diameter) in a growth chamber at 25 ºC light (16 h) and 15 ºC dark (8 h). Four-week-old seedlings (plumule length 3-5 cm) were selected and transferred to the containers (three seedlings per container), which were divided over four controlled CO_2 flow cabinets. A detailed description of the CO_2 flow cabinets is provided in Drigo *et al*. (2007). Briefly, four identical flow cabinets (1.9 m × 2.4 m × 0.9 m, l × h × d; Vötsch, Industrietechnik GmbH, Germany) provided an airtight system, which facilitated the maintenance of a constant atmospheric CO_2 level of 350 µl/l or 700 µl/l (two cabinets each). Onboard infrared gas analysers (IRGA), calibrated for $^{12}CO_2$ and $^{13}CO_2$ (prior labelling) were fitted in each of the flow cabinets and linked to a controller to regulate the CO_2 concentration either by automated injection of $^{12}CO_2/^{13}CO_2$ from a pressurized cylinder or by removal of $^{12}CO_2/^{13}CO_2$ by a solid carbon soda filter (Sofnoline, SIGMA). Maximum daily temperatures ranged from 21 – 22 ºC and minimum temperatures ranged from 16 – 18 ºC. Light intensity averaged 250 µE, with a 16 h photoperiod and a relative humidity of 70%.

For 180 days, 200 *F. rubra* and 200 *C. arenaria* plants were grown, half at an ambient atmospheric CO_2 concentration (350 µl/l) and half at double this concentration (700 µl/l). For each CO_2-treatment 10 pots with unplanted soil were also incubated. Soil moisture

content was maintained at 10% using demineralized water. Within each chamber, all pots were shuffled after each watering (twice a week) to reduce potential position effects.

$^{13}CO_2$ pulse-labeling

$^{13}CO_2$ pulse-labeling was carried out 181 days after germination, when *F. rubra* plants were previously found to be heavily colonized by AMF (Drigo *et al.* 2007). A total of 96 *F. rubra* and 96 *C. arenaria* plants, plus 16 unplanted pots, were subjected to $^{13}CO_2$ pulse-labeling, half from the 350 µl/l CO_2 treatment and the other half at from the 700 µl/l CO_2. The remaining pots, used for natural abundance and background $^{13}C/^{12}C$ measurements, were incubated in two separate CO_2 flow cabinets (350 and 700 µl/l), to ensure that there was no contamination with respired ^{13}C enriched CO_2. Pulse labeling used 99 at. % ^{13}C enriched CO_2 (Cambridge Isotope Laboratories, Andover, MA, USA). Prior to labeling, plants were allowed to assimilate CO_2 until the concentration fell to 150 µl/l in the ambient CO_2 cabinets and to 272 µl/l in the elevated ones. During this period, the overall photosynthetic rate was determined and subsequently used to predict the rate of $^{13}CO_2$ assimilation. Once these CO_2 levels were reached, $^{13}CO_2$ was injected using a gas tight pumping system at a rate of 1.04 l/h for 24 h. The CO_2 concentrations increased to 500 µl/l and 906 µl/l for the ambient and elevated $^{13}CO_2$ cabinets, respectively, resulting in ^{13}C-labeling levels of 77% CO_2 in both cases. To enhance photosynthetic $^{13}CO_2$ assimilation during the labeling, the photoperiod was increased to 24 hours during labeling. Care was taken to minimize any shading effects or labeling biases by spacing out the plants in the $^{13}CO_2$ flow cabinets and by circulating the labeled air with an internal ventilation system. At the end of the labeling episode (24h), all plants and flow cabinets were set back to the conditions present prior to labeling. The amount of $^{13}CO_2$ added during labeling was sufficient to label plants to 2545 ‰ $\delta^{13}C$ at ambient CO_2 levels and 2892 ‰ $\delta^{13}C$ at elevated CO_2.

Harvesting procedure

At each sampling (24h, 48h, 72h, 96h and 5, 6, 14, 21 days after labeling), six replicates of labeled *F. rubra* and *C. arenaria* plants per CO_2 concentration were selected randomly. The frequency of the harvesting was chosen in accordance with an expected C turnover rate of ≤ 1 week (Staddon *et al.* 2003). In addition, four unlabelled *F. rubra* and *C. arenaria* plants were harvested per CO_2 concentration prior to labeling, and two at each sample period to serve as controls for background of $\delta^{13}C$ values. Ten unplanted containers of soil per CO_2 concentration were also harvested: two served as unlabelled controls and eight underwent the labeling process at days 1, 6, 14 and 21. Four *C. arenaria* plants per CO_2 treatment were used for non-mycorrhizal assessments at days 1, 6, 14 and 21. Four extra *F. rubra* and *C. arenaria* plants per CO_2 level were used for plant biomass assessments prior to labeling, as well as on days 6, 14 and 21. All plant parts and soil were analyzed for total C and ^{13}C abundance by elemental analyzer-isotope ratio mass spectrometry (EA-IRMS, Boschker *et al*. 1999).

Upon harvest, shoots, roots, rhizosphere soil and bulk soil were separated. Half of the shoot samples were oven dried and weighed and the other half frozen immediately and freeze dried for $\delta^{13}C$ measurements. Roots were shaken gently to remove loosely adhering soil, and the remaining attached soil was considered rhizosphere soil. Subsequently, the rhizosphere soil was carefully removed from the roots with a probe and forceps. Root

fragments remaining in the bulk or rhizosphere soil samples were removed by passing through a 1mm sieve. All soil samples (rhizosphere, bulk and unplanted), were frozen immediately following harvest in liquid nitrogen, freeze-dried and stored at -80 °C until lipid analysis. A sub-sample of roots was stained with trypan blue (0.1% in lactic acid, glycerol and water 1:2:2, v/v/v) for determinations of percent AMF root colonization, via a gridline intersect method (Giovanetti & Mosse 1980). Sub-samples of bulk soil were used for ergosterol (fungal biomarker) extractions as per Baath (2001).

Lipid biomarker and stable isotope analysis

Neutral (NLFA) and phospholipid (PLFA) lipid fatty acids were extracted and analyzed according to the protocol described by Boschker (2004). Briefly, the soil was extracted and lipids were fractionated into neutral lipids, glycolipids and phospholipids fractions using silicic-acid columns. The neutral and phospholipids fractions were esterified to yield NLFA and PLFA, respectively. NLFA and PLFA short-hand nomenclature is according to Guckert *et al.* (1985). The isotopic composition of the lipid fractions was determined on a gas chromatograph (Hewlett Packard HP G1530) coupled to a Thermo Finningan Delta-plus IRMS via a type III combustion interface (GC-C-IRMS). An a-polar analytical column HP5-ms (Hewlett-Packard, 60 m × 0.32 mm × 0.25 μm) was used with helium as carrier gas, providing adequate separation of most LFAs.
To calculate $\delta^{13}C$ ratios in NLFA and PLFA, the measured carbon isotope ratios of fatty acid methyl esters (FAME) were corrected for the additional carbon atom in the methyl group added during derivation:

$$\delta^{13}C_{NLFA/PLFA} = ((n+1) \times \delta^{13}C_{FAME} - 1 \times \delta^{13}C_{methanol})/n \qquad (1)$$

where n is the number of carbon atoms in the individual NLFA and PLFA (Boschker 2004). Actual ^{13}C-content (excess ^{13}C) in individual pools (shoots, roots, soil), NLFA and PLFAs was also calculated as described in Boschker (2004). Excess ^{13}C in the bacterial biomass was calculated by dividing the sum of the ^{13}C enrichment in all bacteria-specific PLFA by a conversion factor of 350 μmol bacterial PLFA per g of C biomass, assuming similar enrichment in the total bacterial biomass as in PLFA (Boschker 2004).
The NLFA 16:1ω5 was used as a signature for arbuscular mycorrhizal fungal biomass and ^{13}C incorporation (Olsson 1999; Van Aarle & Olsson 2003; Olsson & Johnson 2005). The PLFA 16:1ω5 was also used as a marker for AMF. However, as PLFA 16:1ω5 also occurs in bacteria, the ratio of this biomarker to other bacterial markers was examined to determine the relative importance of AMF for this signature biomarker (Olsson & Johnson 2005). The AMF ^{13}C excess was calculated by subtracting the background levels of 16:1ω5 fatty acids determined from non-mycorrhizal *C. arenaria* harvested at each sampling period and by multiplying with the conversion factor 2.7 (Olsson & Johnson 2005). The following fatty acids were used as biomarkers for bacterial biomass: i14:0, i15:0, a15:0, i16:0, 16:1ω7t, i17:1ω7, 10Me16:0, a17:1ω7, i17:0, a17:0, cy17:0, 10Me17:0, 18:1ω7c, 10Me18:0 and cy19:0 (Frostegard *et al.* 1993). Within the bacterial community, we chose *Pseudomonas* spp. (Lugtenberg *et al.* 2001) and *Burkholderia* spp. (Berg *et al.* 2005) as representative groups known to be efficient colonizers of the rhizosphere. Actinomycetes and the genus *Bacillus* (Smalla *et al.* 2001) were chosen as representative groups for the bulk soil bacterial community. The phospholipid fatty acids (PLFA) cy17:0 and cy19:0 were used as biomarkers for *Pseudomonas* spp. and *Burkholderia* spp. respectively (Vancanneyt *et al.*

1996). 10Me16:0, 10Me17:0 and 10Me18:0 were used for actinomycetes (Frostegard *et al.* 1993) and i17:0 for *Bacillus* (Kaneda 1991). The signature 20:4ω6 was used to assess the ^{13}C incorporation and biomass of the protozoan community (Findlay and Dobbs 1993; Mauclaire *et al*. 2003).
Although considerable effort went into removing all visible root material from soil during harvest, it was possible that some of the highest labeled PLFAs (i.e., 16:0, 18:1ω9 and 18:2ω6c, which are abundant in plants) were coming from root material remaining in the soil. We therefore excluded these fatty acids signatures from our analyses, which prevented us from addressing total fungal labeling, which typically relies on 18:2ω6c. Ergosterol was therefore used as and alternative estimator of non-AMF fungal biomass. Although ergosterol levels were higher in the rhizosphere of *F. rubra* compared to *C. arenaria*, no effect of elevated CO_2 treatments were observed (3.80 and 3.39 mg/kg at ambient and elevated CO_2 for *F. rubra*, respectively, and 1.73 and 1.80 mg/kg for *C. arenaria*).

Statistical analysis

Soil parameters, NLFA/PLFA and $\delta^{13}C$ abundance in soil and plant material were analyzed using analysis of variance (ANOVA). Analyses were carried out according to a split-plot design as described by Filion *et al*. (2000). We considered as whole plot the two different CO_2 treatments, while *F. rubra* and *C. arenaria* un/labeled, un/labeled unplanted soil, and the time courses were considered as sub-treatments within each whole plot. The F statistic, used for testing the significance of main effects of the CO_2 treatment applied to whole plots (CO_2 flow cabinets), was obtained by dividing the treatment mean-square by the mean-square for CO_2 flow cabinets nested within CO_2 treatments. The error term to test for interactions between CO_2 and soil origin or plant species was based on the mean square of the interaction between those treatments and cabinets nested in CO_2 (Filion *et al.* 2000). Analyses were carried out using Statistica 7.0 (StatSoft Inc., Tulsa, OK). Normality was tested with a Shapiro-Wilks test and by inspection of residuals, and variance homogeneity by Levene's test. When data failed to satisfy one of these tests, an appropriate transformation was applied (log or square-root transformation). Tukey's honestly significant difference (HSD) method and the modified version for unequal sample size (Unequal N HSD in Statistica) were used for post-hoc comparisons with a 0.05 grouping baseline.

Results

Mycorrhizal infection and ^{13}C incorporation in AMF biomass

Root colonization by AMF was observed in all root fragments of *F. rubra* examined. As expected, no AMF colonization was found in the roots of the non-mycorrhizal plant species, *C. arenaria*. Levels of AMF colonization were not different across the eight harvests as determined by percent root length colonization (results not shown). In line with these results, the content of NLFA and PLFA 16:1ω5, used as surrogates for intraradical (NLFA and PLFA 16:1ω5) and extraradical (NLFA 16:1ω5) AMF biomass (Olsson & Johnson 2005), respectively, were significantly higher ($F_{2,4}$ = 88.44; $P < 0.001$) in the mycorrhizal plant species (*F. rubra*) as compared to the non-mycorrhizal plant species (*C. arenaria,* $F_{2,4}$ = 2.91, P = 0.23). The PLFA 16:1ω5 content in *F. rubra* roots was 9.1 nmol/g (rhizosphere dry soil) at ambient CO_2 and 285.4 nmol/g at elevated CO_2, while the non-

mycorrhizal *C. arenaria* contained 0.2 nmol/g at ambient CO_2 and 5.9 nmol/g at elevated CO_2. The content of NLFA 16:1ω5 in mycorrhizal plant increased significantly ($F_{2,4}$ = 571.19, P < 0.001) at elevated CO_2 as compared to ambient CO_2 by a factor of 22. PLFA 16:1ω5 increased significantly at elevated CO_2 ($F_{2,4}$ = 2171.82, P < 0.001) by a factor of 143. Although PLFA 16:1ω5 occurs in AMF as well as bacteria, the increase due to the CO_2 enrichment followed the same patterns observed for NLFA 16:1ω5, supporting the assumption that this biomarker was mostly of AMF origin in our study.
In our pulse-chase labelling experiment, we observed that *F. rubra* AMF signature biomarkers incorporated ^{13}C label within one day of labelling, after which ^{13}C enrichment in NLFA and PLFA 16:1ω5 in the rhizosphere soil decreased significantly (P < 0.001) reaching a plateau 14 days after labelling (Fig. 1a, b).

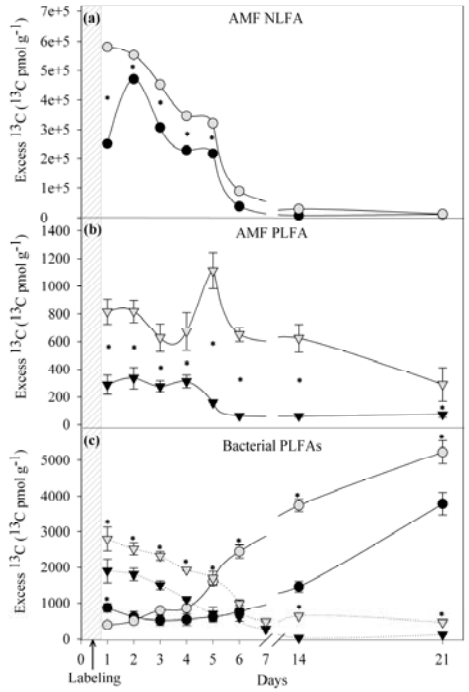

Figure 1: (a) ^{13}C enrichment in the arbuscular mycorrhizal fungal signature 16:1ω5 was determined in *F. rubra* rhizosphere soil for NFLA (circles) at ambient CO_2 (black) and at elevated CO_2 (grey) and (b) for PLFA 16:1ω5 (triangles) fractions at ambient CO_2 (black) and at elevated CO_2 (grey). The ^{13}C enrichment in the bacterial PLFAs (c) was determined as the sum of 15 bacteria-specific PLFAs (see methods) at ambient CO_2 (black) and at elevated CO_2 (grey) in the rhizosphere soil of *F. rubra* (circles) and *C. arenaria* (triangles). ^{13}C enrichment denotes the excess ^{13}C after subtraction of natural background as determined for non-labeled systems. Asterisks designate significant differences (P < 0.001) between CO_2 concentrations. Shaded area indicates period of ^{13}C-CO_2 incubation.

Excess ^{13}C in NLFA 16:1ω5 decreased from a maximum of approximately 4.7×10^5 pmol ^{13}C/g on day 2 to 1.2×10^4 pmol ^{13}C/g on day 21 at ambient CO_2 and from approximately 5.8×10^5 on day 1 to 1.1×10^4 pmol ^{13}C/g on day 21 at elevated CO_2 (Fig. 1a). Enrichment of the NLFA 16:1ω5 ^{13}C was significantly increased by the elevated CO_2 treatment from day 1 to day 5 (days × CO_2: $F_{7,14} = 920.14$; $P < 0.001$). In contrast, PLFA 16:1ω5 ^{13}C enrichment at elevated CO_2 was significantly greater during the entire incubation period (Fig. 1b; days × CO_2: $F_{7,4} = 1682.53$, $P < 0.001$).

^{13}C incorporation into bacterial PLFAs

The ^{13}C enrichment of 15 bacteria-specific fatty acids of the rhizospheres of *F. rubra* and *C. arenaria* revealed that there was a significantly higher transfer of plant-assimilated C to the soil bacterial community under elevated CO_2 as compared to ambient CO_2 levels (Days × CO_2: $F_{7,14} = 85.41$, $P < 0.001$; Fig. 1c). For *F. rubra*, bacterial PLFAs enrichment increased from 886.90 pmol ^{13}C/g on day 1 to 3.8×10^3 pmol ^{13}C/g by day 21 at ambient CO_2, and from 392.55 pmol ^{13}C/g on day 1 to 5.2×10^3 pmol ^{13}C/g by day 21 at elevated CO_2 (Fig. 1c). Initial bacterial PLFAs labelling was low, only showing more significant ^{13}C incorporation after a period of four days. Initially, greater ^{13}C allocation was found in Gram-positive signatures i15:0 and a15:0 and the Gram-negative 18:1ω7c in both CO_2 treatments. ^{13}C incorporation into the bacterial biomass of the signatures i15:0, a15:0 and 18:1ω7c remained constant over the incubation period. *C. arenaria* bacterial PLFAs were less enriched and showed a different ^{13}C enrichment trend, with the highest peaks at day one (1904.06 and 2799.62 pmol ^{13}C/g at ambient and elevated CO_2, respectively), and lowest at day 21 (124.05 and 463.33 pmol ^{13}C/g at ambient and elevated CO_2, respectively). The ^{13}C incorporation in the bacterial-specific fatty acids of the bare plots showed no significant changes across harvests (days × CO_2 $F_{7,14} = 3.51$, $P = 0.63$) in both CO_2 treatments with an average incorporation of 291.11 and 321.23 pmol ^{13}C/g at ambient and elevated CO_2 respectively. Bacterial biomass decreased under elevated CO_2 in *F. rubra*, yet increased in *C. arenaria* and in the bare plots (data not shown).

PLFA signatures for both *Burkholderia* spp. and *Pseudomonas* spp. in the rhizosphere of *F. rubra* showed an increased ^{13}C enrichment after four days ($P < 0.001$), similar to the patterns observed for total bacterial PLFAs (Fig. 2a, b). For these two genera, ^{13}C incorporation was significantly greater at elevated CO_2 (*Burkholderia,* days × CO_2: $F_{7,14} = 225822.1$, $P < 0.001$; *Pseudomonas*, days × CO_2: $F_{7,14} = 29961.7$, $P < 0.001$). In contrast, *Burkholderia* spp. in the *C. arenaria* rhizosphere showed the highest ^{13}C enrichment on day 1 at elevated CO_2 (1151.16 pmol ^{13}C/g). *Pseudomonas* spp. in *C. arenaria* was hardly labeled and showed higher initial ^{13}C incorporation on day one at ambient CO_2 (111. 40 pmol ^{13}C/g). Biomass of these two genera decreased significantly ($P < 0.01$) for *F. rubra* plants grown at elevated CO_2, indicating an increased turnover for both genera (Fig 2a1, 2b1). In *C. arenaria*, ^{13}C enrichment was correlated with the significant increase in *Burkholderia* biomass at elevated CO_2. PLFA signature molecules for *Bacillus* spp. and actinomycetes on the other hand, showed no significant incorporation of labeled CO_2 for either plant (Fig 2c; *Bacillus,* days × CO_2 $F_{7,14} = 2.91$, $P = 0.23$; actinomycetes*,* days × CO_2, $F_{7,14} = 3.91$, $P = 0.34$), and no changes in the biomass of these groups were observed at either ambient or elevated CO_2 concentrations (Fig. 2c1), except for a significant decrease ($P < 0.001$) of *Bacillus* at elevated CO_2 in *C. arenaria*.

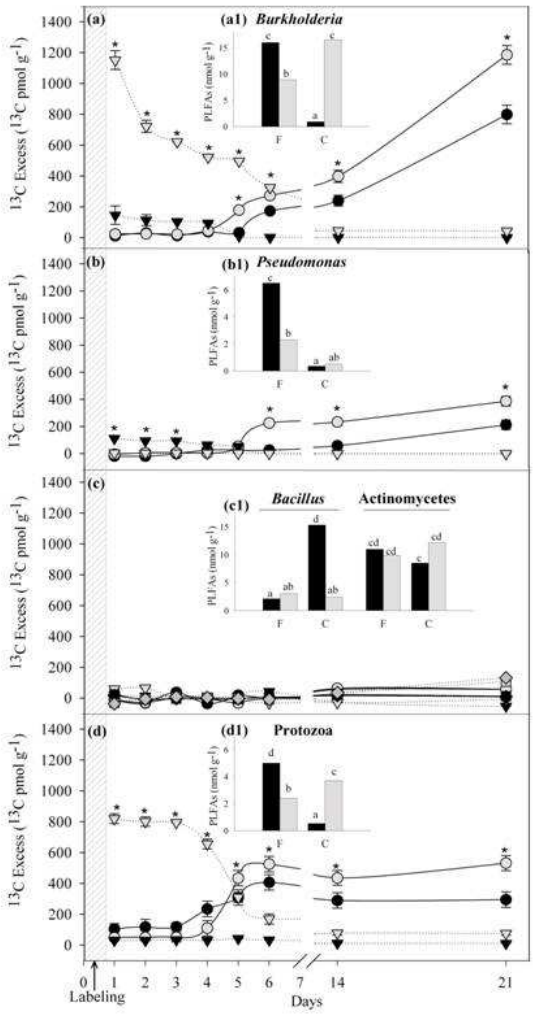

Figure 2: ^{13}C enrichment in the specific bacterial signatures was determined in rhizosphere soil of *F. rubra* (circles) and *C. arenaria* (triangles) at ambient (black) and elevated CO_2 (grey) in the phospholipid fatty acids (PLFA) specific signatures for (a) *Burkholderia* spp., (b) *Pseudomonas* spp., (c) *Bacillus* (circles, *F. rubra*; triangles, *C. arenaria*) and actinomycetes (squares, *F. rubra*; diamonds, *C. arenaria*) and (d) for the protozoa. The abundance of the specific fatty acids was determined in rhizosphere soil of *F. rubra* (F) and *C. arenaria* (C) at ambient CO_2 (black) and at elevated CO_2 (grey) for (a1) *Burkholderia* spp., (b1) *Pseudomonas* spp., (c1) *Bacillus* and actinomycetes, and (d1) protozoa.^{13}C enrichment denotes the excess ^{13}C after natural background subtraction as determined in non-labeled systems. Different letters designates within a graph refer to significantly different averages based upon Tukey HSD test. Asterisks designate significant differences ($P < 0.001$) between CO_2 concentrations. Shaded area indicates period of ^{13}C-CO_2 incubation.

The *F. rubra*-associated protozoan community showed (Fig. 2d) significant (days × CO_2, $F_{7,14} = 38854.28$, $P < 0.001$) enrichment of ^{13}C at elevated CO_2 from 5 days post labelling. The level of labelling remained rather constant after this point for both CO_2 treatments.
In *C. arenaria* at elevated CO_2, ^{13}C incorporation was higher for the first four days post labelling. The protozoan biomass significantly ($P < 0.001$) decreased in *F. rubra* and increased in *C. arenaria* at elevated CO_2 (Fig. 2 d1).

^{13}C pulse allocation budget

The amount of net ^{13}C incorporation was calculated for the shoots, roots and rhizosphere soil of *F. rubra* and *C. arenaria*. The partial budget for ^{13}C pulse allocation at ambient and elevated CO_2 after 6 days from the pulse chase labelling revealed an increased ^{13}C retention in shoots and roots of both plants at elevated CO_2 (Fig. 3; 3.9 and 2.1 ^{13}C g respectively in *F. rubra* and *C. arenaria*). Within the *F. rubra* rhizosphere soil, the majority of pulse-derived ^{13}C was allocated to the mycorrhizal NLFA signature16:1ω5 in both CO_2 treatments (0.4 ^{13}C g at ambient and 1.1 ^{13}C g at elevated CO_2). Increased CO_2 concentration led to a nearly three-fold increase in the ^{13}C incorporation in the *F. rubra* mycorrhizal PLFA (0.08 ^{13}C g). Similar increases were observed in the bacterial PLFAs for both plant species (0.30 ^{13}C g, *F. rubra* and 0.11 ^{13}C g, *C. arenaria*). The total incorporation of ^{13}C in the shoots and rhizosphere soil of *F. rubra* was nearly twice that observed for *C. arenaria.*

Discussion

Our findings suggest that the major pathway of C flux from the roots into the soil microbial community may be via mycorrhizal fungi. We observed a rapid transfer of photosyntates into mycorrhizal biomass and a subsequent slow C release to bacterial genera known to colonize the rhizosphere (Fig. 1 and Fig. 2). This pattern was more pronounced in the elevated CO_2 treatment.
Previous studies highlighted four main aspects of mycorrhizal functioning namely, their impact on the primary production process, the direct and rapid acquisition of recent photosynthates (Johnson *et al.* 2002), the significant contribution to both fast and slow pools of soil organic C through the retention of C in the mycelium (Olsson & Johnson 2005) and the ability of AMF to influence microbial communities in soil via deposition of mycelium products (Toljander *et al.* 2007; Filion *et al*. 1999; Marschner & Baumann, 2003). Our results confirmed the importance of mycorrhizal fungi in terrestrial C fluxes, as previous suggested by Staddon (2005), thereby emphasizing the need to understand the dynamics of these organisms when assessing the current, and predicting the future, effects of rising atmospheric CO_2 concentrations.
AMF neutral lipids are usually stored in intraradical vesicles or in spores and make up a large proportion of AMF biomass (Olsson *et al.* 2002).The dynamics of NLFA in our study indicate that C is primarily assimilated by AMF in the intraradical components and spores, with a postulated residence time of 4-5 days. In accordance with a previous study (Olsson& Johnson 2005), we observed a lower retention of C in the PLFA 16:1ω5 fraction, which might be a reflection of arbuscule formation (Van Aarle & Olsson 2003).

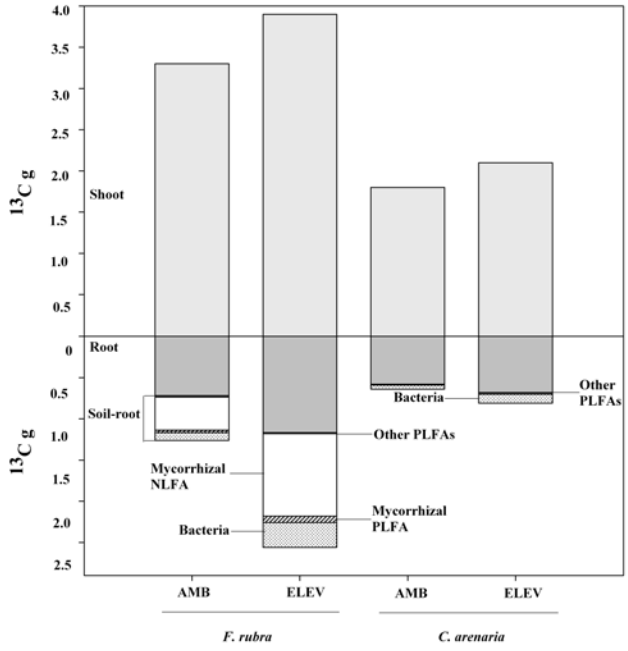

Figure 3: Partial budget for ^{13}C pulse allocation in ambient (AMB) and elevated (ELEV) CO_2 in *F. rubra* and *C. arenaria* showing the mean amounts of pulse-derived ^{13}C in the different C pools (shoot, root and soil-root) 6 days after pulse labeling. Note that the ^{13}C values for roots do not include the ^{13}C derived from mycorrhizal NLFA and PLFA 16:1ω5. This budget does not include respiration from roots and soil micro-organisms.

The initial capacity of AMF to act as a sink for C may be sufficient to cope with the increased C translocation below-ground under elevated CO_2 conditions, without changing the pathways of C turnover in the plant/soil system (Douds *et al*. 2000; Graham 2000; Jones *et al*. 2004; Olsson & Johnson 2005). In particular, the C retention in the PLFA fraction was significantly larger at elevated CO_2, again suggesting either a higher translocation to that fraction due to an increased arbuscular biomass or a slower turnover of the arbuscles compared to the ambient CO_2 treatment.

The increased retention of C in AMF under elevated CO_2 may have altered the carbohydrate metabolism of the roots (Bago 2003), increased root respiration (Douds *et al.*, 2000) and changed root exudation patterns, which may subsequently effect microbial community composition in the rhizosphere (Johansson *et al.* 2004; Rillig 2006; Marschner & Baumann 2003; Drigo *et al*. 2007). Similar effects may also result from an increase in root biomass (Allard *et al.* 2005) and the resulting qualitative and quantitative changes in root exudation and other forms of rhizodeposition (Hodge *et al.* 1998). Although direct root exudation probably represents a relatively minor C transfer pathway compared to other fluxes (e.g. structural root components and litter), its impact on ecosystem functioning may be disproportionably large due to the relatively simple chemical nature of most root exudates (Cardon 1996).

Interestingly, the central role of mycorrhizae in the belowground C-pathway did not change at elevated CO_2 conditions; only the sizes of the pools and the fluxes changed increasing at elevated CO_2. The decrease of the ^{13}C incorporation in AMF-specific biomarkers was accompanied by a significant increase in ^{13}C incorporation for the bacterial community about 4-5 days after labeling in the mycorrhizal plant (*F. rubra*) (Fig. 1c). This may indicate that AMF are an important source of bacterial C. This suggestion is strengthened by our findings for the non-mycorrhizal plant, *C. arenaria*, which showed an opposite trend, with a rapid incorporation of ^{13}C in the bacterial community only at the beginning of labeling experiment.

The cyclopropyl PLFAs, cy17:0 and cy19:0 were used as biomarkers for *Pseudomonas* spp. and *Burkholderia* spp. respectively, due to their high presence in these typically rhizo-competent genera (Berg *et al.* 2005; Lugtenberg *et al.* 2001; Vancanneyt *et al.* 1996; Treonis *et al*. 2004). As judged by these biomarkers, both of these genera, became highly enriched for the mycorrhizal plant during the SIP experiment at elevated CO_2 (Fig. 2a,b). Interestingly, this coincides with a lower biomass, suggesting a more rapid turnover at elevated CO_2. The pattern of ^{13}C incorporation for these genera was similar to the total bacterial labeling in the mycorrhizal and non-mycorrhizal plants, but did not account for the full level of bacterial labeling, indicating that bacterial labeling is not limited to these genera. In both plants, *Burkholderia* spp. were highly labeled at elevated CO_2, whereas *Pseudomonas* spp. received markedly more label in the mycorrhizal plant, supporting the notion of *Pseudomonas* spp. as a particularly active microorganisms in the mycorrhizosphere (Mansfeld-Giese *et al.* 2002; Toljander *et al.* 2007). High resolution techniques such as rRNA-SIP (Manefield et al. 2002) could be used to further strengthen the conclusion that these two genera may be indicative of general rhizosphere bacterial responses to elevated CO_2.

We considered the PLFA signature i17:0 as an indicator for the activity and biomass of *Bacillus* spp. (Kaneda 1991), and 10Me-PLFAs as indicative for actinomycetes (Zelles, 1999; Bardgett *et al.* 1999; Billings & Ziegler 2005). These bacterial groups have previously been recognized as dominant bulk soil inhabitants (Smalla *et al.* 2001). In both plants, the dynamics of these bacterial groups, as judged via these biomarkers, were markedly different from those observed for *Pseudomonas* and *Burkholderia*. In contrast to these presumably rhizo-competent genera, bacilli and actinomycetes showed nearly no ^{13}C incorporation. This showed little fluctuation across the incubation period and was not affected by elevated CO_2 (Fig 2c). Our results are in accordance with Zak *et al.* (1996) and Bardgett *et al.* (1999), who showed that slow-growing soil microorganisms, such as actinomycetes, were unaffected by elevated CO_2.

The biomarker PLFA 20:4ω6 was used as a biomass indicator for the protozoan community. Interestingly, in the mycorrhizal plant, the main ^{13}C incorporation protozoa coincided with the highest ^{13}C incorporation into the bacterial community, 5-6 days of post-labeling. Thereafter, the ^{13}C enrichment of the protozoan fraction did not increase further. Unlike bacteria, the ^{13}C level in the protozoan community remained constant in both CO_2 conditions after 5 days. This suggests that the smaller size and higher activity of the bacterial community under elevated CO_2 was independent of protozoan grazing. In contrast, for the non-mycorrhizal plant, the ^{13}C incorporation (i.e. grazing) by protozoa was strictly correlated with the increased ^{13}C enrichment in the rhizosphere bacterial community under elevated CO_2.
Our results demonstrated that, for the mycorrhizal plant species, the main response to the increased translocation of C at elevated CO_2 conditions proceeded via AMF, which rapidly accumulated plant-assimilated carbon that was subsequently released gradually to rhizo-competent bacterial populations in the soil. These bacterial communities, although highly active, did not decrease in density due to protozoan predation. We therefore provide new evidence of a central role of mycorrhiza in mediating potential impacts of elevated CO_2 in plant-soil systems and the global C cycle (Kuzyakov & Domanski 2000).

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