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Fungi Dominated the Incorporation of ^{13}C-CO$_2$ into Microbial Biomass in Tomato Rhizosphere Soil under Different CO$_2$ Concentrations

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Abstract: An elevated CO$_2$ (eCO$_2$) fumigation experiment was carried out to study the influence of various CO$_2$ concentrations on microorganisms involved in the incorporation of root-derived C in greenhouse soil systems. In this study, 400 and 800 µmol·mol^{-1} CO$_2$ fumigation treatments were conducted during tomato planting. Phospholipid fatty acid (PLFA) profiling based on the stable isotope probing (SIP) technique was applied to trace active microorganisms. The absolute total abundance of ^{13}C-PLFAs was much higher under eCO$_2$ treatment. Most of the ^{13}C-CO$_2$ was incorporated into the ^{13}C-PLFAs 18:2ω6,9 (fungi), 16:0 (general PLFA), 18:1ω9c (Gram-negative bacteria, G$^-$) and i17:0 (Gram-positive bacteria, G$^+$) via rhizodeposition from tomato under ambient CO$_2$ (aCO$_2$) and eCO$_2$ treatments, suggesting similar responses of active microorganisms to different CO$_2$ treatments. However, the fungi (characterized by the ^{13}C-PLFA 18:2ω6,9) played a much more dominant role in the incorporation of root-derived C under eCO$_2$. Actinomycetes, marked by the ^{13}C-PLFA 10-Me-18:0, occurred only on labeling day 15 under the eCO$_2$ treatment, indicating that the actinomycetes fed on both soil organic carbon and fresh rhizodeposition. It was indicated that eCO$_2$ significantly affected microbial biomass and microbial community structures involved in the incorporation of ^{13}C-CO$_2$ via tomato root secretions, as supported by Adonis analysis and the Mantel test.

Keywords: eCO$_2$; PLFA-SIP; fungi; actinomycete; microbial community structures

1. Introduction

Atmospheric CO$_2$ concentrations have been increasing annually since the Industrial Revolution, reaching 400 µmol·mol^{-1} for the first time in 2013 [1]. eCO$_2$ could improve the net primary production and quality by accelerating photosynthetic rates and increasing water use efficiency [2–5]. At the same time, plant rhizodeposition, which accounted for 17% of photosynthates [6], was altered quantitatively and qualitatively under eCO$_2$ treatment [4,7–9]. As a consequence, microbial biomass was generally significantly increased under eCO$_2$ because of the increased carbon flow in plant root secretions [8,10], and the microbial community structure was affected by eCO$_2$ to different degrees depending on plant species [11], soil characteristics [11] and experimental designs [12,13]. However, whether the changes in microbial biomass and community structure were derived from increased rhizodeposition or soil organic matter remains largely unknown in most cases [11,14,15], which could ultimately affect the soil's nutrient balance and the sustainable development of ecosystems.

Under this condition, studies on the microorganisms responsible for the incorporation of root-derived C have received more and more attention and can provide insights into this phenomenon. Fungi (PLFAs 18:1ω9 and 18:2ω6,9) and G$^-$ (PLFAs 16:1ω7, 18:1ω7 and cy19:0) incorporated most of the ^{13}C-CO_2 in a 5 h stable isotope labeling experiment in grassland soils [16]. Similarly, the ^{13}C-PLFA 18:2ω6,9 was shown to significantly incorporate root-derived C, while bacterial PLFAs showed no significant changes in another experiment conducted on young beech trees [17]. For anaerobic incubation with ^{13}C-labeled urea in wheat-rice rotation soils, the ^{13}C-PLFA 18:1ω9c was much more abundant when the wheat season changed into the rice season [18]. Furthermore, microbes involved in the incorporation of root-derived C were found to be significantly different under various CO_2 concentrations with the support of Deoxyribonucleic acid-stable isotope probing (DNA-SIP) [19]. Bacilli, Gammaproteobacteria and Clostridia were dominant under aCO$_2$ treatment, while Bacilli and Betaproteobacteria were dominant under the eCO$_2$ treatment. Based on the above discoveries, it was found that fungi (18:2ω6,9) played a dominant role in incorporating root-derived C, and the changes in active microbial community structures were mainly dependent on ecosystems. eCO$_2$ stimulates the C flow from plant roots to soils and promotes microbial activity. Greater utilization of additional C resources and root secretions [20,21], a significantly higher abundance of genes associated with C and N cycling [22] and increased enzyme activity [23,24] indicate rapid nutrient cycling and the stimulation of microbial activity under eCO$_2$. Unfortunately, the isotope pulse-labeling technique used in previous studies was less stable than the steady isotope labeling technique, and little research has focused on the effects of various CO_2 concentrations, especially in greenhouse soil ecosystems.

Soils managed under plastic tunnel greenhouses, which are widely used in fruit and vegetable plantations and characterized by lower pH levels and nutrient-poor conditions under longer planting durations, have rarely been studied under various CO_2 treatments [22,25,26]. The soil microbial community composition in the rhizosphere soils of tomato changed insignificantly under various CO_2 treatments in our previous study [27]. However, the changes in active microbial community structures incorporating root-derived C were largely unknown. The Phospholipid fatty acid-stable isotope probing (PLFA-SIP) technique based on steady $^{13}CO_2$ labeling can be used to reflect the changes in active PLFAs that are characteristic of diverse microbes [28].

A study demonstrated that the ideal concentration of CO_2 for the production of vegetables in greenhouses is 800–1000 μmol·mol^{-1} [29]. It has been shown that eCO$_2$ can significantly improve crop photosynthesis, including that in tomatoes [30,31]. According to reports by the Intergovernmental Panel on Climate Change (IPCC, 2014) [1], atmospheric CO_2 has been reached 400 μmol·mol^{-1} in 2013, and it will increase at the rate of 1.9 μmol·mol^{-1}·y^{-1}, reaching 550 μmol·mol^{-1} in 2050. In previous studies about eCO$_2$ fumigation, higher CO_2 concentration was usually set as 550 [24], 700 [32] or 800 μmol·mol^{-1} [26,33,34] and so on, and the ambient atmospheric CO_2 concentration was usually regarded as 400 μmol·mol^{-1}. In order to clearly reveal the stimulation of plant biomass under eCO$_2$ condition, 800 μmol·mol^{-1} CO_2 was also chosen in our study. As higher plant dry biomass usually means more root secretions, so only one higher eCO$_2$ concentration was chosen to induce significant changes of microbes.

In this study, a 2% ^{13}C-CO_2 steady-state labeling experiment with the support of the PLFA-SIP technique was conducted in both tomato planted and unplanted soils, in which 400 or 800 μmol·mol^{-1} CO_2 fumigation was performed for 15 days because of the rapid metabolism of PLFAs. Based on our previous study that fungal biomass was significantly increased under eCO$_2$, we hypothesized that fungi would play a dominant role in the incorporation of root-derived C under eCO$_2$ treatment. The aim was to reveal the differences in the compositions of active microbial communities that assimilate plant-derived carbon under various CO_2 concentrations.

2. Materials and Methods

2.1. Study Site

In August 2020, the plow layer of the soil was sampled from a 10-year-old tomato greenhouse located in Wuhan city (30°17'44" N, 114°16'34" E). The field holding capacity of the soil was 24.36%, and the moisture content of the air-dried soil was 2.86%. The soil pH was 5.31 (soil:water = 1:2.5). The total carbon and total nitrogen contents were 10.3 and 1.6 g·kg^{-1} dry soil, respectively, and the C:N ratio was 6.49. The dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) contents were 395.75 and 122.88 mg·kg^{-1} dry soil, respectively. The soil NH_4^+-N and NO_3^--N contents were 2.11 and 37.16 mg·kg^{-1} dry soil, respectively. The soil was defined as loamy because sand, silt and clay accounted for 47.7%, 42.6% and 8.2% of its contents, respectively.

2.2. Experimental Set-Up

In this study, each pot was filled with the equivalent of 200 g of soil (dry weight). The soils in each pot were passed through a 5.0 mm sieve, the water content was adjusted to 65% of the field holding capacity, and 0.2 g NPK fertilizer was mixed before the transplantation of the tomato plants.

The seeds of the tomato plants (*Solanum lycopersicum* L., Jinguan 28) were soaked in tap water (37 °C, 10 h) and then germinated at room temperature (25 °C) according to the planting instructions. Tomato seedlings were placed into larger pots (15 × 12 × 14 cm) for seedling cultivation. After 13 days, three plants of similar sizes (5 cm length with 4 leaves) were transplanted into each small pot (9 × 6 × 9.5 cm). All the pots were transferred to a controlled environment room (day 22–26 °C/night 15–19 °C; photoperiod, 12 h light; watered every two days with deionized water; CO_2 concentration, 400 μmol·mol^{-1}, controlled with a volumetric flow meter and a control panel).

All pots were divided into two parts: pots for plant growth and pots for soil incubation. In each part, two variants (CO_2 concentration and labeling days) were set. In total, 28 pots for plant growth (2 CO_2 concentrations (400 and 800 μmol·mol^{-1} CO_2) × 3 labeling days (labeling days 5, 10 and 15) × 4 replicates + 4 pots sampled on the day before labeling); 12 pots for soil incubation (2 CO_2 concentrations (400 and 800 μmol·mol^{-1} CO_2) × 1 labeling days (labeling days 15) × 4 replicates + 4 pots sampled on the day before labeling).

One week after transplantation (day 21), the pots were separated equally into two different CO_2 concentration treatment groups (400 or 800 μmol·mol^{-1} CO_2), and 2% ^{13}C-CO_2 steady-state labeling was performed. The two growth chambers were placed in the above-mentioned controlled-environment room, so the growth conditions were the same as those in the plant growth period. The steady-state labeling of $^{13}CO_2$ was accomplished by mixing compressed air without CO_2, natural $^{12}CO_2$ and $^{13}CO_2$ at fixed rates controlled by mass-flow controllers, which were described previously [30]. Briefly, the flow rates of $^{13}CO_2$, $^{12}CO_2$ and air without CO_2 were set as 0.072 mL·min^{-1}, 7.128 mL·min^{-1} and 18 L·min^{-1}, respectively, in the 400 μmol·mol^{-1} CO_2 chamber, while they were 0.144 mL·min^{-1}, 14.256 mL·min^{-1} and 18 L·min^{-1}, respectively, in the 800 μmol·mol^{-1} CO_2 chamber.

In the pots used for plant growth, destructive sampling of tomato plants and rhizosphere soils was performed on days 21 (the day before labeling), 26, 31 and 36 (every 5 days after labeling). From the pots used for soil cultivation, soils were collected only on day 21 (the day before labeling) and day 36 (the end of the labeling experiment). Rhizosphere soils were harvested by collecting the soils adhering to the root after shaking and then freeze-dried for PLFA analysis. Plant shoots and roots were separated and dried (105 °C for 45 min and 60 °C for 48 h) for further analysis.

2.3. Analysis of Biochemical Properties

To reflect the changes in the biochemical properties of the soils, the soil pH, moisture, DOC, DON, NO_3^--N and NH_4^+-N were measured. The measurement details are available in a previous study [27]. In brief, the total C and total N levels in the soils were measured by

an elemental analyzer (Elementar Vario MACRO cube, Langenselbold, Germany) according to the operating instructions. The NO_3^--N and NH_4^+-N in the soil were extracted with 2 M KCl at a 1:5 fresh soil to KCl ratio, and the extract was analyzed with a continuous flow analyzer (SKALAR, Delft, The Netherlands). The DOC and DON in the soil were extracted with 0.5 M K_2SO_4 at a 1:5 fresh soil to K_2SO_4 ratio and analyzed with a Multi N/C 2100S TOC/TN b analyzer (Analytik Jena, Jena, Germany). The % C and 13C/12C ratio of the soils and tomato roots and shoots were analyzed with a Flash EA 2000 Series Elemental Analyzer connected via a Conflo IV to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Scientific, Germany) [35]. The isotope ratios were calculated according to a previous study [36].

2.4. PLFA Analysis

PLFA extraction and analyses were performed according to the modified Bligh and Dyer method [37], the details of which were provided in a previous study [27]. The concentration and ^{13}C labeling of fatty acid methyl esters (FAMEs) were analyzed by using a Trace GC 1310 system with a combustion column attached via a GC IsoLink II system to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Scientific, Germany) [36]. Other details were the same as those described in previous studies [36,38].

In total, 38 PLFAs were detected across all the treatments. Taking into account the concentration of ^{13}C among these 38 PLFAs, 19 PLFAs accounting for 90% of the total amount of PLFAs were considered valid and were finally retained for further analyses. These PLFAs were divided into the following categories according to previous reports [39]: general PLFAs (14:0, 15:0, 16:0, 17:0, 18:0 and 20:0), PLFAs from G$^-$ (16:1ω7c, cy17:0, 18:1ω9c and cy19:0), PLFAs from G$^+$ (i15:0, a15:0, i16:0, i17:0 and a17:0), actinomycetes (10-Me-16:0 and 10-Me-18:0), fungi (18:2ω6,9) and arbuscular mycorrhizal fungi (AMF) (16:1ω5c) [40].

The proportion (Pi) of ^{13}C in each PLFA was determined according to the mass balance equation below [36].

$$P_i = (AT\% \ ^{13}C_t - AT\% \ ^{13}C_0)/(AT\% \ ^{13}C_g - AT\% \ ^{13}C_0) \tag{1}$$

where AT% means the ratio of an isotope atom to the total atomic number of an element, AT% $^{13}C_t$ and AT% $^{13}C_0$ are the AT% ^{13}C/^{12}C enrichment (%) of each PLFA in the rhizosphere soil at the end and beginning of labeling, respectively, and AT% $^{13}C_g$ is the AT% ^{13}C/^{12}C enrichment (%) of labeled CO_2 (%). The absolute amount of labeled ^{13}C in each PLFA was defined as the product of Pi and the absolute concentration of carbon in each PLFA (the number of carbons multiplied by the absolute abundance of each PLFA).

2.5. Statistical Analysis

Basic data processing, including the calculation of means and standard deviations (SDs), was performed with Microsoft Excel 2010. Tests of significant variation in the effects of CO_2 concentrations and labeling days on biochemical properties and plant biomass were conducted with the Scheirer–Ray–Hare test in R 3.6.3 with the rcompanion package [41]. Analyses of the effects of CO_2 concentrations and labeling days on microbial community structure (PLFAs) were conducted via Adonis analysis with 999 permutations in R 3.6.3. The assessment of whether the mol% of PLFAs or ^{13}C was correlated with environmental factors was carried out with Mantel tests. The principal component analysis (PCA) of 19 PLFAs was conducted with the help of IBM SPSS Statistics 22. Other figures were plotted by using Origin 2018.

3. Results

3.1. Effect of CO_2 Concentration on the Tomato Biomass and AT% ^{13}C/^{12}C

The eCO_2 concentration significantly ($p < 0.001$) increased the shoot and root dry weights of tomato at the end of the labeling experiment (Figure 1a). However, both the labeling days and CO_2 concentration:labeling days had no effect on plant biomass during the whole labeling period ($p > 0.05$).
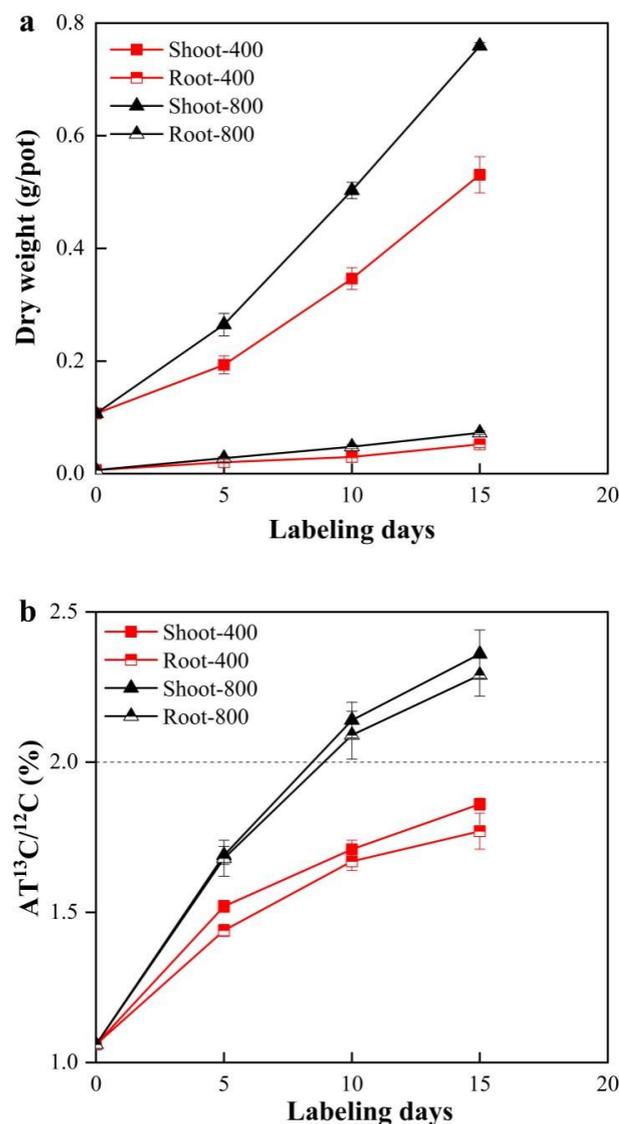
Figure 1. Changes in plant dry weights (**a**) and AT% $^{13}C/^{12}C$ values of shoots and roots (**b**) during the 15 labeling days. 400:400 $\mu mol \cdot mol^{-1}$ CO_2 treatment; 800:800 $\mu mol \cdot mol^{-1}$ CO_2 treatment. Bars represent the standard errors of the means (n = 4).

The natural AT% $^{13}C/^{12}C$ values of the tomato shoots and roots were 1.06 before labeling. The AT% $^{13}C/^{12}C$ values of the shoots and roots were significantly ($p < 0.001$) higher under the eCO_2 treatment, while the CO_2 concentration:labeling days produced no significant ($p > 0.05$) effects on these induces during the labeling period (Figure 1b). Specifically, the AT% $^{13}C/^{12}C$ values of both tomato shoots and roots increased significantly ($p < 0.05$) with the labeling days under both the 400 and 800 $\mu mol \cdot mol^{-1}$ CO_2 treatments. Moreover, the AT% $^{13}C/^{12}C$ values of the shoots and roots were greater than 2 (the AT% $^{13}C/^{12}C$ value of CO_2 used in this experiment) on labeling days 10 and 15 under the 800 $\mu mol \cdot mol^{-1}$ CO_2 treatment (Figure 1b), indicating that most of the carbon in the tomato shoots and roots was derived from photosynthesate after labeling day 10 under eCO_2.

3.2. Effect of CO_2 Concentration on the Biochemical Properties of the Soils

The changes in the biochemical properties of the soils during the labeling period were analyzed. In the planted treatment groups, the soil pH was nonsignificantly influenced ($p > 0.05$) by the CO_2 concentration but decreased significantly ($p < 0.001$) with labeling days (Figure 2a). The soil moisture decreased significantly ($p < 0.05$) under the eCO_2 treatment

(Figure 2b). The CO_2 concentration significantly ($p < 0.05$) increased the soil DOC, while labeling days significantly ($p < 0.05$) decreased it, and CO_2 concentration:labeling days had no effect ($p > 0.05$) during the whole labeling period (Figure 2c). In particular, the soil DOC increased significantly ($p < 0.05$) with the CO_2 concentration but decreased significantly ($p < 0.001$) with labeling days. The soil DON was not influenced by the CO_2 concentration ($p > 0.05$) but decreased significantly ($p < 0.001$) with labeling days over the entire incubation period (Figure 2d). Similar to the soil DON, both the soil NO_3^--N and NH_4^+-N decreased significantly ($p < 0.001$) with labeling days and were much lower ($p < 0.05$) under the eCO_2 treatments on labeling days 10 and 15 (Figure 2e,f). In the soil cultivation treatments, the soil DOC increased significantly ($p < 0.05$), while the soil NO_3^--N and soil NH_4^+-N decreased significantly ($p < 0.05$), under the eCO_2 treatment (Table S1). The AT% $^{13}C/^{12}C$ values of the soils remained at 1.082 before labeling day 5 in both the aCO_2 and eCO_2 treatments, while they increased significantly ($p < 0.05$) on labeling days 10 and 15 under the eCO_2 treatment.

3.3. Effect of CO_2 Concentration on the Soil Microbial Community

In the tomato-planted treatment groups, the absolute abundance of total PLFAs was significantly ($p < 0.05$) higher under the eCO_2 treatments (30.8 ± 0.6 vs. 38.5 ± 3.6 nmol·g^{-1} soil) at the end of labeling. However, the total PLFA contents of soils from the unplanted treatment groups were not significantly ($p > 0.05$) affected by the CO_2 concentration, as the values in the 400 and 800 μmol·mol^{-1} CO_2 treatment groups were 19.3 ± 0.4 and 20.1 ± 0.2 nmol·g^{-1} soil, respectively.

Based on the distributions of 19 PLFAs on each sampling day, general bacteria (PLFA 16:0), G$^-$ (PLFAs cy19:0 and 18:1ω9c) and fungi (PLFA 18:2ω6,9c) were considered to be most abundant in both the planted and unplanted treatment groups (Figure S1). However, minor differences in the relative abundance of the abovementioned PLFAs existed between the planted and unplanted treatment groups. For example, the abundance of the fungal PLFA marker 18:2ω6,9 was much higher ($p < 0.05$) in the planted treatment groups than in the unplanted treatment group. For the significantly ($p < 0.05$) changed microbial PLFAs under various CO_2 concentrations, both the PLFA species and numbers (6, 3 and 5) changed with labeling days in the planted treatment groups. Specifically, the relative abundance of the PLFA 18:2ω6,9 (fungi) was not significantly affected by eCO_2 in the earlier labeling period (day 5) but was affected by eCO_2 on labeling days 10 and 15, finally accounting for more than 20% of the total PLFAs at the end of labeling (Figure S1).

Principal component analysis (PCA) based on the relative abundance of 19 PLFAs was conducted to reveal the effects of tomato plants and CO_2 concentrations on the microbial community composition (Figure 3). The microbial community compositions in the planted and soil cultivation treatment groups were separated on the first principal component axis (PC1), explaining 63.41% of the total variation, indicating the important role of tomato plants in shaping the microbial community structure. Analyses of the loading scores of PLFAs on the PC1 axis suggested that significant ($p < 0.05$) increases in i16:0 (score 0.081) and 10-Me-16:0 (score 0.081) and decreases in 18:2ω6,9 (score -0.082) and i17:0 (score -0.068) occurred in the soils without tomato (Figure S2), which was the same as the distribution of the mol% of PLFAs (Figure S1). Though the microbial community structures of soils from the 400-plant and 800-plant treatment groups were nicely clustered by direct visual observation (Figure 3), the *p* value of the Adonis analysis was above 0.05, which indicated that the CO_2 concentration had no significant effects on microbial community structure in soils from the planted treatments during the 15 days of CO_2 fumigation.
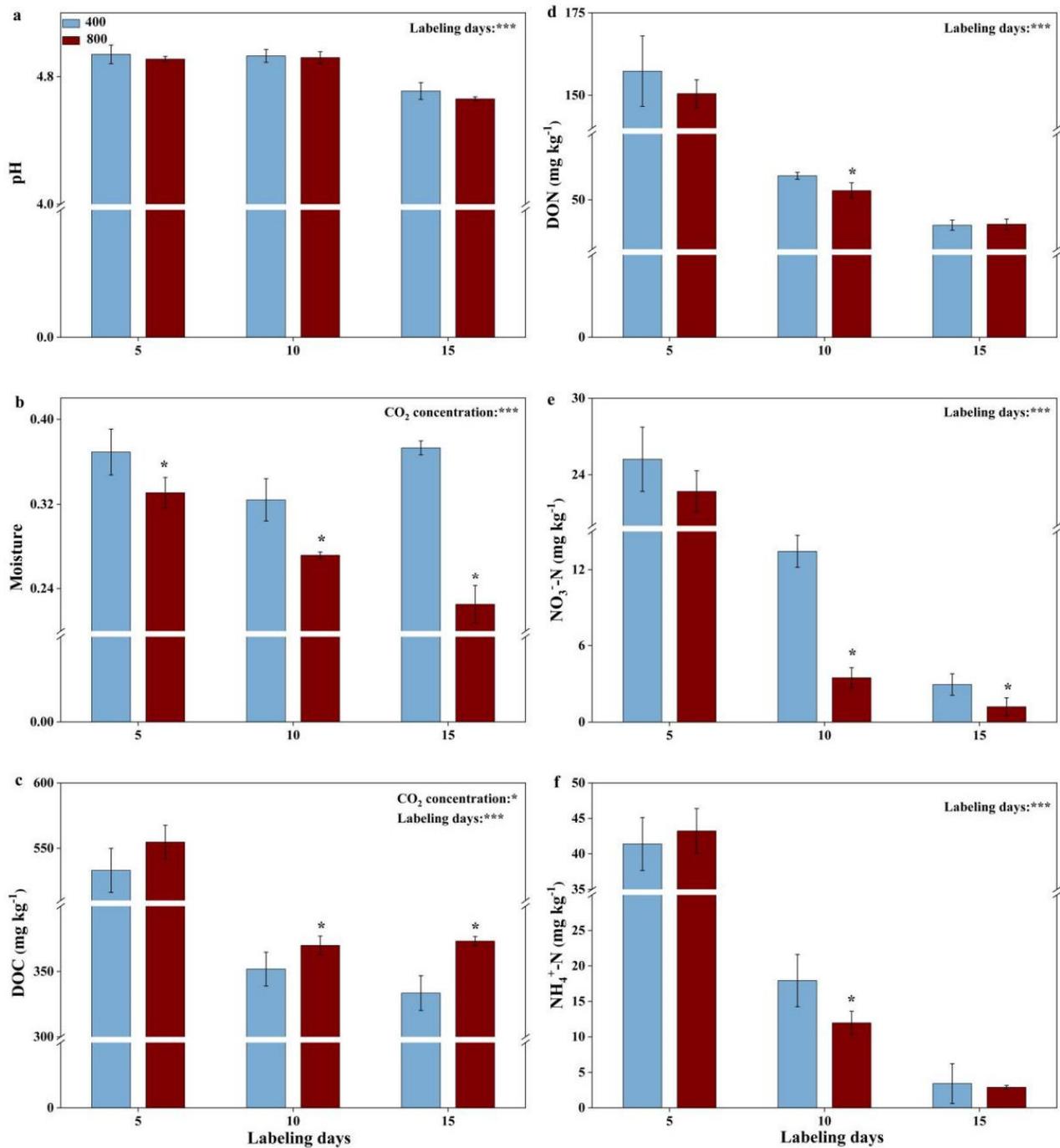
Figure 2. Changes in pH (**a**), moisture (**b**), DOC (**c**), DON (**d**), $NO_3^- - N$ (**e**) and $NH_4^+ - N$ (**f**) during the 15 labeling days under both the 400 and 800 treatments. 400:400 $\mu mol \cdot mol^{-1}$ CO_2 treatment; 800:800 $\mu mol \cdot mol^{-1}$ CO_2 treatment. Bars represent the standard errors of the means (*n* = 4). *: *p* < 0.05; ***: *p* < 0.001.
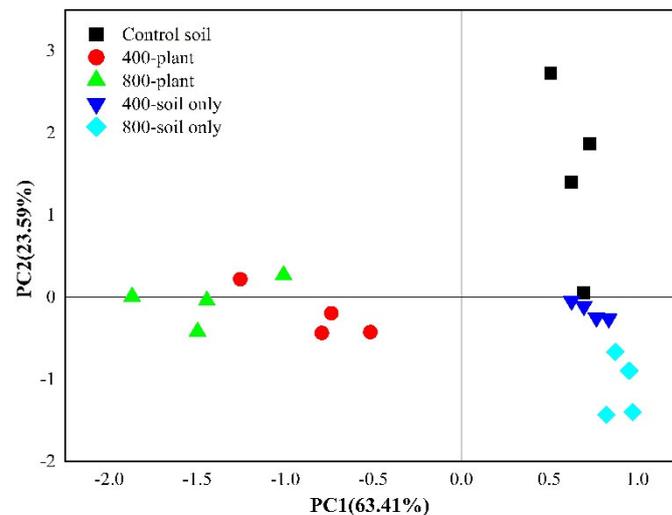
Figure 3. Principal component analysis of the PLFA composition of soil samples at the end of the 15−day labeling experiment. Values in parentheses on the axis labels indicate the percentage variation accounted for by each axis. 400:400 $\mu mol \cdot mol^{-1}$ CO_2 treatment; 800:800 $\mu mol \cdot mol^{-1}$ CO_2 treatment; the control soil is the original tomato soil.

3.4. Variation in Soil Microbial Composition Incorporating ^{13}C-Rhizodeposition under Different CO_2 Concentrations

In the tomato planted treatment groups, the total ^{13}C incorporation into PLFAs was significantly stimulated by the CO_2 concentration ($p < 0.05$) and labeling days ($p < 0.001$), reaching 188.71 ± 16.14 and 456.76 ± 67.91 $nmol \cdot g^{-1}$ soil in the 400-day 15 and 800-day 15 treatment groups, respectively (Table 1). The average enrichment rate of ^{13}C under the eCO_2 treatment was almost twice as high as that under the aCO_2 treatment (69% vs. 36%) (Table 1). However, in the soil incubation treatment groups, the average enrichment rate of ^{13}C was less than 2%, and ^{13}C labeling was not affected by the CO_2 concentration ($p > 0.05$) (Table 1). Based on the distribution of ^{13}C among fungal, general, G^-, G^+ and actinomycete PLFAs, fungi and general bacteria were dominant in the planted treatments, while general bacteria and G^- were dominant in the unplanted treatments (Figure S3).

Table 1. The total enrichment of ^{13}C into the PLFA of individual soil samples under varied CO_2 concentration treatments.

Labeled Days	CO_2 Concentration ($\mu mol \cdot mol^{-1}$)	Labeled Amount ($nmol \cdot g^{-1}$) Average $\pm$ STD	Enrichment Rate (%) Average $\pm$ STD
5	400 [1]	24.43 $\pm$ 4.39 ab [2]	7.43 $\pm$ 1.35 b
	800	60.05 $\pm$ 11.37 b	15.13 $\pm$ 1.45 c
10	400	49.66 $\pm$ 9.6 ab	12.65 $\pm$ 1.95 c
	800	150.46 $\pm$ 45.25 c	34.27 $\pm$ 6.29 d
15	400	188.71 $\pm$ 16.14 c	35.82 $\pm$ 2.33 d
	800	456.76 $\pm$ 67.91 d	68.89 $\pm$ 4.25 e
CO_2 concentrations	*		
Labeling days	***		
CO_2 concentration:Labeling days	ns		
Unplanted soil	400	3.52 $\pm$ 0.45 a	1.08 $\pm$ 0.14 a
	800	5.61 $\pm$ 0.79 a	1.65 $\pm$ 0.22 a

[1] 400:400 $\mu mol \cdot mol^{-1}$ CO_2 treatments; 800:800 $\mu mol \cdot mol^{-1}$ CO_2 treatments. [2] Values are means $\pm$ standard deviation (n = 4). Values with different lowercase letters within a column and same stage are statistically significantly different at $p < 0.05$. * $p < 0.05$; *** $p < 0.001$.

The percentage distribution of ^{13}C in the PLFAs revealed the composition of microorganisms that incorporated root-derived ^{13}C (Figure 4). In the unplanted soils, the ^{13}C in a general PLFA (16:0) and G^- (16:1ω7c and 18:1ω9c) accounted for 60% of the total ^{13}C (Figure 4a), which was in accordance with previous results (Figure S3). In contrast, 18:2ω6,9 (fungi), 16:0 (general PLFA), 18:1ω9c (G^-) and i17:0 (G^+) were the most abundant ^{13}C-PLFAs under both 400 and 800 μmol·mol^{-1} CO_2 in the planted treatment groups, accounting for more than 85% of the total ^{13}C-PLFAs (Figure 4b–d).

The differences in the soil microbial community structure in terms of ^{13}C incorporation between the 400 and 800 μmol·mol^{-1} CO_2 treatment groups was dependent on the labeling days, as indicated by both PCA and Adonis analyses (Figure 5, Table S2). Adonis analyses demonstrated that significant ($p < 0.05$) differences in the microbial community structure between the 400 and 800 μmol·mol^{-1} CO_2 treatment groups occurred on labeling days 10 and 15 (Table S2). PCA further revealed that the 400-day 10 and 800-day 10 treatment groups were separated by PC1, while the 400-day 15 and 800-day 15 treatment groups were separated by PC2. In detail, the plant-derived ^{13}C contents of the PLFAs 16:0 (a general PLFA), 18:1ω9c (G^-) and 16:1ω7c (G^-) were much higher ($p < 0.05$) under the 400 μmol·mol^{-1} CO_2 treatment, while the plant-derived ^{13}C contents of the PLFAs 18:2ω6,9 (fungi), 20:0 (general PLFA) and 10-Me-18:0 (actinomycetes) were much higher ($p < 0.05$) under the 800 μmol mol^{-1} CO_2 treatment at the end of labeling period (Figure 4d). In terms of the AT% $^{13}C/^{12}C$ values of PLFAs, 18:2ω6,9 and 18:1ω9c were the top two of the 19 discussed ^{13}C-PLFAs (Figure S4). Moreover, the AT% $^{13}C/^{12}C$ values of 18:2ω6,9 exceeded 2 (the AT% $^{13}C/^{12}C$ value of CO_2 used in this experiment) on labeling days 10 and 15 under in 800 μmol·mol^{-1} CO_2 treatment group (Figure S4). In addition, the incorporation of ^{13}C from CO_2 into the PLFAs 10-Me-18:0 and 20:0 occurred only in the 800-day 15 treatment group (Figure 4b–d), although the difference in the mol% of the PLFAs 10-Me-18:0 and 20:0 between the 400-day 15 and 800-day 15 treatment groups was not significant ($p > 0.05$), suggesting that microorganisms containing the PLFAs 10-Me-18:0 and 20:0 were dependent mainly on the original soil carbon source (such as organic matter) in the 400 μmol·mol^{-1} CO_2 treatment group and in the earlier period of eCO_2 treatment (up to labeling day 10).

3.5. Relationship between PLFAs and Environmental Factors

To reveal the associations between microbial compositions and soil and plant characteristics, Mantel test analyses were conducted based on the mol% of PLFAs or ^{13}C and three key factors (CO_2 concentration, DOC and total dry weight (TDW)). The whole PLFA profile was significantly ($p < 0.05$) related to DOC and TDW, while the whole ^{13}C-PLFA profile was significantly ($p < 0.05$) correlated with CO_2, DOC and TDW (Table 2). However, when TDW or DOC was set as a covariate, the influence of DOC or TDW on the whole PLFA profile was not significant ($p > 0.05$), indicating that the soil DOC and plant TDW interacted with each other. When TDW or DOC was set as a covariate, the effect of CO_2 on the whole ^{13}C-PLFA profile was not significant ($p > 0.05$), indicating that CO_2 made an influence on ^{13}C-PLFA profile by stimulating the TDW and DOC. Furthermore, when DOC was set as a covariate, the effect of TDW on the whole ^{13}C-PLFA profile was significant ($p < 0.05$), suggesting that TDW could directly influence microbial community structures.

When specific PLFAs were considered alone, the results showed that CO_2 produced a significant ($p > 0.05$) influence on ^{13}C-18:1ω9c by increasing the tomato biomass, while it had a direct influence on 18:1ω9c ($p < 0.05$) (Table S3). In addition, both 18:2ω6,9 and ^{13}C-18:2ω6,9 were significantly ($p < 0.05$) related to TDW. However, CO_2 and TDW were significantly ($p < 0.05$) correlated with only the labeled ^{13}C-10-Me-18:0 and ^{13}C-20:0 (Table S3). The above results indicated that specific ^{13}C- PLFAs were correlated with environmental factors in various manners.
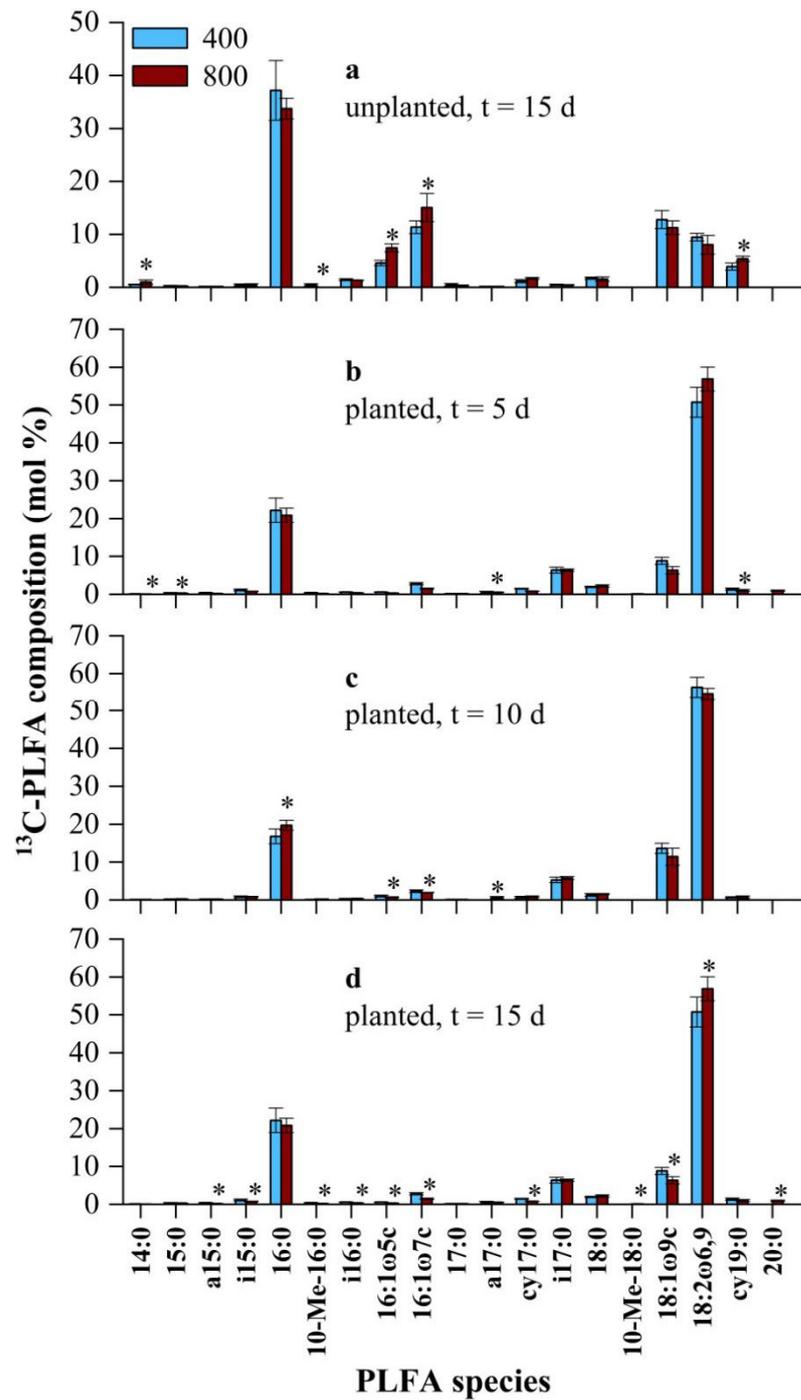
Figure 4. Differences in ^{13}C-PLFA composition (mol%) between the 400 and 800 μmol·mol^{-1} CO$_2$ treatment groups in unplanted (**a**) and planted (**b**–**d**) soils on each sampling day. Labels shown in the figure represent significant ^{13}C-PLFA biomarkers (*p* < 0.05). 400:400 μmol mol^{-1} CO$_2$ treatment; 800:800 μmol·mol^{-1} CO$_2$ treatment. Bars represent the standard errors of the means (*n* = 4). *: *p* < 0.05.
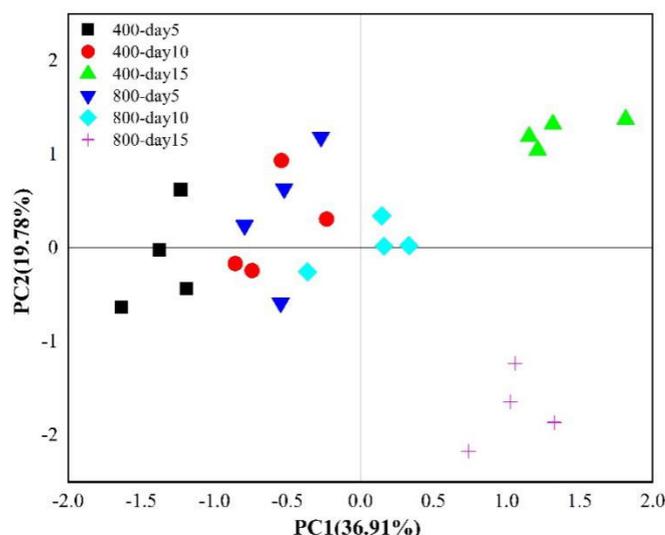
Figure 5. Principal component analysis of the ^{13}C−PLFA composition (mol%) of soil samples under various CO_2 treatments during the 15-day labeling experiment. Values in parentheses on the axis labels indicate the percentage variation accounted for by each axis. 400:400 µmol·mol^{-1} CO_2 treatment; 800:800 µmol·mol^{-1} CO_2 treatment.

Table 2. Mantel tests between PLFA or ^{13}C-PLFA and environmental factors under 400 and 800 µmol·mol^{-1} CO_2 treatments.

Factors	PLFA Composition		^{13}C-PLFA Composition	
	Mantel Statistic r	Significance	Mantel Statistic r	Significance
CO_2	0.3887	0.0562	0.4556	0.0289 *
DOC [1]	0.3636	0.0489 *	0.4281	0.0256 *
TDW	0.4905	0.0204 *	0.7088	0.0008 ***
CO_2_DOC	0.1648	0.1881	0.1958	0.1594
CO_2_TDW	−0.0714	0.6301	−0.4199	0.9494
DOC_TDW	−0.02641	0.5555	−0.2638	0.846
TDW_DOC	0.3543	0.0685	0.6581	0.0035 **

[1] Abbreviations: DOC, dissolved organic carbon; TDW, total dry weight; CO_2_DOC, the DOC was set as a covariate; CO_2_TDW, the TDW was set as a covariate; DOC_TDW, the TDW was set as a covariate; TDW_DOC, the DOC was set as a covariate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4. Discussion

Similar responses of soil microorganisms to root-derived C under aCO_2 have been shown in previous studies [16,17]. The ^{13}C-PLFA 18:2ω6,9 was shown to significantly ($p < 0.05$) incorporate root-derived C, while bacterial PLFAs showed no significant changes in an experiment conducted on young beech trees [17]. In our results, the significantly higher abundance of ^{13}C among fungal and actinomycete PLFAs under eCO_2 treatment partially supports our hypothesis and is consistent with previous findings.

4.1. Effect of CO_2 Concentration on ^{13}C-Plant Biomass

The AT% ^{13}C/^{12}C values of tomato shoots and roots were significantly ($p < 0.05$) increased in the 800 µmol·mol^{-1} CO_2 treatment group (Figure 1b), in accordance with the general theory that eCO_2 is beneficial for plant photosynthesis [30,31]. In addition, the greater tomato root biomass and AT% ^{13}C/^{12}C values of tomato roots (exceeding 2, the AT% ^{13}C/^{12}C of CO_2 value used in this experiment) (Figure 1) might indicate that the quantity of ^{13}C-root secretions increased in the 800 µmol·mol^{-1} CO_2 treatment group, as rhizodeposition accounted for approximately 17% of the photosynthates [6] and an increase in fine roots usually indicates increased root secretion. The significant ($p < 0.05$) increases

in soil DOC (Figure 2c) and ^{13}C among the PLFAs (Table 1) observed under eCO_2 further supported the above hypothesis.

4.2. Effect of CO_2 Concentration on Microbial Community Structure

During the 15 days of CO_2 fumigation, the absolute abundance of the total PLFAs was significantly ($p < 0.05$) stimulated by eCO_2 treatment, while the overall microbial community structure (the PLFA profile) was not influenced by CO_2 concentration (Figure 3), as confirmed by Adonis analysis and Mantel tests ($p > 0.05$). The above results were similar to those of previous studies in which short-term eCO_2 fumigation usually led to increases in root rhizodeposition and microbial biomass [42] but had little influence on microbial community structure [27,43,44]. In fact, because of the differences in plant species, soil characteristics and experimental designs, the observed response of the microbial community structure to eCO_2 is not always the same [27]. A study conducted in a grassland revealed that even a longer period of eCO_2 fumigation produced no effect on the microbial community structure [45], which might be attributed to the specific ecosystem involved and the large pool of soil carbon underground. Therefore, a focus on the changes in microbes involved in the incorporation of root-derived C is essential for understanding the effect of eCO_2.

4.3. Effect of CO_2 Concentration on ^{13}C-Microbial Community Structure

In this study, the ^{13}C-PLFAs 18:2ω6,9, 16:0, 18:1ω9c and i17:0 showed the highest activity in terms of the incorporation of rhizodeposited ^{13}C compared with other PLFAs under both 400 and 800 μmol·mol^{-1} CO_2, accounting for 85% of the total ^{13}C (Figure 4d), which was in accordance with previous studies conducted in forests or other nonflooded ecosystems [16,17]. Moreover, both the absolute abundance of total ^{13}C (Table 1) and the distribution of ^{13}C-PLFAs (Figure 5) changed significantly ($p < 0.05$) under eCO_2 at the end of the labeling period.

On the one hand, ^{13}C-18:2ω6,9 (fungi) showed a significantly ($p < 0.05$) higher relative abundance under eCO_2 (56.9% vs. 50.8%) (Figure 4d). The dominant role of fungi (18:2ω6,9) in incorporating root-derived carbon has also been observed in previous studies [16,17,46]. The significantly increased relative abundance of the ^{13}C-PLFA 18:2ω6,9 under eCO_2, in accordance with experiments conducted in young beech trees [17], could be attributed to the characteristics of fungi. First, the suitable pH range for fungi is acidic; in the present study, it was 4.8 on average and decreased under eCO_2 (Figure 2a) [47]. Second, fungi can efficiently absorb nutrients through their long hyphal network to overcome the significant decrease in the soil N status, especially in the later stages of eCO_2 fumigation (Figure 2d–f) [48]. Third, mutualism occurs between AMF and plant roots, and eCO_2 further stimulates the growth of AMF and plants by increasing plant rhizodeposition secretion into rhizosphere soils [49,50]. Finally, fungi contain phenol oxidase, and this enzyme can mediate the absorption of sufficient N resources from recalcitrant C forms (such as lignin) with increased levels under eCO_2 [51,52]. Therefore, the increase in fungi might be a general trend for eCO_2 treatments in most studies. In addition, Mantel tests suggested that ^{13}C-18:2ω6,9 was significantly ($p < 0.05$) related to TDW (Table S3), which means that fungi were affected mainly by the plant biomass or root secretions in this study. Therefore, the above results were consistent with the hypothesis that fungi played a much more dominant role in the incorporation of root-derived carbon under the eCO_2 treatment.

On the other hand, 20:0 (general PLFA) and 10-Me-18:0 (actinomycete) were involved in the incorporation of ^{13}C-CO_2 later in the eCO_2 treatment (such as 15 days) (Figure 4d). However, no significant ($p > 0.05$) variations in the mol% of 20:0 or 10-Me-18:0 existed between the 400 and 800 μmol·mol^{-1} CO_2 treatment groups (Figure S1d). This result suggested that C among these two PLFAs was mainly derived from original soil carbon sources under aCO_2 treatment, but root-derived carbon (rhizodeposition) could also be utilized under the eCO_2 treatment, which was in accordance with the wide range of substrates utilized by actinomycetes [53]. Mantel tests demonstrated that ^{13}C-20:0 and

^{13}C-10-Me-18:0 were significantly ($p < 0.05$) correlated with the CO_2 concentration, TDW and CO_2 _TDW (Table S3). Therefore, both general bacteria and actinomycetes also play an important role in the incorporation of root-derived carbon under long-term eCO_2 fumigation [19].

The PLFA 18:1ω9c is usually regarded as a biomarker of G^- and fungi in agroecosystems and forest ecosystems, respectively [40]. In this study, ^{13}C-18:1ω9c (G^-) was much more abundant ($p < 0.05$) under aCO_2 than under eCO_2 (8.8% vs. 6.3%) on labeling day 15 (Figure 4d). The Mantel test results further indicated that the CO_2 concentration affected ^{13}C-18:1ω9c by increasing the TDW (Table S3). A recent study of the anammox reaction of urea revealed that the relative abundance of ^{13}C-PLFA 18:1ω9c increased significantly ($p < 0.05$) during crop rotation from wheat to rice [18]. This might explain why ^{13}C-18:1ω9c was much more abundant under aCO_2, and it could be attributed to the higher water status under aCO_2 (Figure 2b).

In addition, the whole ^{13}C-PLFA profile was also identified to be significantly related to CO_2 concentration (Mantel test, $p < 0.05$) (Table 2). The increase in plant biomass was considered to be the main mechanism underlying the above responses.

5. Conclusions

In this study, we focused on the changes in the overall microbial community structure and the subset of microbes involved in ^{13}C-CO_2 incorporation during 15 days of CO_2 fumigation at different levels. We demonstrated that the overall microbial community structure was not changed, while the microbes involved in ^{13}C-CO_2 incorporation significantly ($p < 0.05$) differed between the 400 and 800 μmol·mol^{-1} CO_2 treatment groups from labeling day 10 onward. The Mantel test further revealed that the ^{13}C-PLFA profile was significantly related to the CO_2 concentration. In addition, the mol% of ^{13}C in the PLFAs 18:2ω6,9 (fungi), 20:0 (general PLFA) and 10-Me-18:0 (actinomycetes) were higher ($p < 0.05$) in the 800 μmol mol^{-1} CO_2 treatment group, while those of 18:1ω9c (G^-) and 16:1ω7c (G^-) were much higher ($p < 0.05$) in the 400 μmol·mol^{-1} CO_2 treatment group. In summary, this study successfully revealed the important role of 18:2ω6,9, 10-Me-18:0 and 18:1ω9c in incorporating rhizodeposits under eCO_2. The structures of ^{13}C rhizodeposits need to be analyzed to completely understand the mechanism of the effects of eCO_2 on soil microbes in future work.

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