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REGULAR ARTICLE

# $^{13}C$ isotope fractionation during rhizosphere respiration of $C_3$ and $C_4$ plants

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**Abstract** Stable carbon isotopes are used extensively to partition total soil $CO_2$ efflux into root-derived rhizosphere respiration or autotrophic respiration and soil-derived heterotrophic respiration. However, it remains unclear whether $CO_2$ from rhizosphere respiration has the same $\delta^{13}C$ value as root biomass. Here we investigated the magnitude of $^{13}C$ isotope fractionation during rhizosphere respiration relative to root biomass in six plant species. Plants were grown in a carbon-free sand-perlite medium inoculated with microorganisms from a farm soil for 62 days inside a greenhouse. We measured the $\delta^{13}C$ value of rhizosphere respiration using a closed-circulation 48-hour $CO_2$ trapping method during 40~42 and 60~62 days after sowing. We found a consistent depletion in $^{13}C$ (0.9~1.7‰) of $CO_2$ from rhizosphere respiration relative to root biomass in three $C_3$ species (*Glycine max* L. Merr., *Helianthus annuus* L. and *Triticum aestivum* L.), but a relatively large depletion in $^{13}C$ (3.7~7.0‰) in three $C_4$ species (*Amaranthus tricolor* L., *Sorghum bicolor* (L.) Moench and *Zea mays* L. ssp. *mays*). Overall, our results indicate that $CO_2$ from rhizosphere respiration is more $^{13}C$-depleted than root biomass. Therefore, accounting for this $^{13}C$ fractionation is required for accurately partitioning total soil $CO_2$ efflux into root-derived and soil-derived components using natural abundance stable carbon isotope methods.

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## Introduction

Stable carbon isotopes are commonly used in studying carbon cycles at various temporal and spatial scales (Bowling et al. 2008; Pataki et al. 2007). Studies that partition NEE (Net Ecosystem Exchange) into photosynthetic and respiratory components (e.g. Bowling et al. 2001), partition total ecosystem respiration into aboveground, root and microbial components (e.g. Tu and Dawson 2005), or partition total soil respiration into root-derived and soil-derived components (e.g. Lin et al. 1999) often use the $\delta^{13}C$ values of organic carbon in total or each ecosystem component (leaf, stem, root, and litter) to estimate the $\delta^{13}C$ values of respiration generated by total or each component. If the $\delta^{13}C$ value of plant respiration is different than that of organic carbon substrate or bulk material, the results of these studies will need to be reconsidered and modified accordingly. Therefore, knowing carbon isotopic fractionation during major

carbon cycle processes, such as rhizosphere respiration that includes both respiration of roots and respiration of rhizosphere microorganisms utilizing carbon substrates derived from live roots, is often crucial for the proper use and the reliability of isotope approaches (Werth and Kuzyakov 2010).

Carbon isotopic fractionation during plant respiration was initially assumed to be negligible (Flanagan and Ehleringer 1998; O'Leary 1981). Lin and Ehleringer (1997) cultured leaf mesophyl protoplasts of two plants (*Phaseolus vulgaris* and *Zea mays*) with carbohydrates of known $\delta^{13}C$ values as the carbon source and found no significant differences in $\delta^{13}C$ value between mitochondrial dark respiration and the substrates. Cheng (1996) grew *Triticum aestivum* in sand-vermiculite mixture inoculated with 1 g of soil and found that the $\delta^{13}C$ value of $CO_2$ from rhizosphere respiration measured during 14~16 days after seedling emergence was virtually the same as the $\delta^{13}C$ value of bulk root biomass. However, more recent studies suggested that $CO_2$ from plant respiration was remarkably $^{13}C$-enriched or $^{13}C$-depleted in comparison to bulk plant materials or assumed respiratory substrates (Bowling et al. 2008; Werth and Kuzyakov 2010). For example, Schnyder and Lattanzi (2005) used a special gas exchange system to measure the $\delta^{13}C$ value of shoot or root respiratory $CO_2$ for two herbaceous species (*Lolium perenne* and *Paspalum dilatatum*) grown in quartz sand and found that shoot respiratory $CO_2$ was $^{13}C$-enriched relative to shoot biomass, while root respiratory $CO_2$ was $^{13}C$-depleted compared to root biomass. Moreover, relative to the number of studies on $^{13}C$ fractionation during leaf respiration (e.g. Sun et al. 2009; Xu et al. 2004), there were much fewer studies on $^{13}C$ fractionation during root respiration (e.g. Bathellier et al. 2009; Klumpp et al. 2005) and only one study on $^{13}C$ fractionation during rhizosphere respiration (Cheng 1996), which is likely because the isotopic composition of root respiration and rhizosphere respiration is difficult to measure (Bowling et al. 2008; Werth and Kuzyakov 2010). Because additional $^{13}C$ fractionation may occur during both rhizodeposition (i.e. rhizodeposits may have a different $\delta^{13}C$ value than bulk root tissue, Werth and Kuzyakov 2005) and microbial respiration of rhizodeposits (i.e. microbial respired $CO_2$ may be different in $\delta^{13}C$ value compared to rhizodeposits, Fernandez and Cadisch 2003; Mary et al. 1992), the overall $^{13}C$ fractionation may be different between root respiration and rhizosphere respiration that includes both root respiration and rhizomicrobial respiration.

Most published studies on $^{13}C$ fractionation during root respiration were based on snapshot measurements (minutes to hours) of the $\delta^{13}C$ value of respiratory $CO_2$ from excised roots (Gessler et al. 2007; Wegener et al. 2010) or roots grown in sand or nutrient solution without the presence of soil microorganisms (Bathellier et al. 2008; Klumpp et al. 2005), and thus did not include the rhizomicrobial respiration by rhizosphere microorganisms utilizing materials released from live roots. In order to partition total soil respiration into rhizosphere respiration that includes both root respiration and rhizomicrobial respiration (root-derived, autotrophic respiration) and microbial decomposition of soil organic matter (soil-derived, heterotrophic respiration) using a two end-member isotope mixing model (Cheng 1996), we need to know $\delta^{13}C$ values of rhizosphere respiration integrated over days or seasons. Furthermore, $^{13}C$ fractionation during root respiration varies considerably between plant species (Klumpp et al. 2005; Schnyder and Lattanzi 2005). It remains unclear whether $^{13}C$ fractionation during rhizosphere respiration also differs among species, particularly between $C_3$ and $C_4$ plants. Some previous studies have reported differences in $^{13}C$ fractionation during root respiration between a $C_3$ plant and a $C_4$ plant (Schnyder and Lattanzi 2005), fungal respiration of sucrose derived from $C_3$ and $C_4$ plants (Henn and Chapela 2000), and microbial decompositions of residues of $C_3$ and $C_4$ plants (Fernandez et al. 2003; Schweizer et al. 1999). Undoubtedly, the issue of $^{13}C$ isotopic fractionation associated with rhizosphere respiration requires further investigation.

In this study we grew three $C_3$ plants and three $C_4$ plants in carbon-free sand-perlite mixture inoculated with microorganisms from a farm soil inside a greenhouse for 62 days. We then measured $\delta^{13}C$ values of bulk root biomass and $CO_2$ derived from roots and the associated microorganisms (i.e. rhizosphere respiration) during 40~42 and 60~62 days after sowing, using a closed-circulation 48-hour $CO_2$ trapping method (Cheng et al. 2003). Our primary goal was to answer two questions: (1) Is there a difference in $\delta^{13}C$ value between bulk root biomass and rhizosphere respiration measured during a two-day period? (2) If the answer to (1) is yes, does the

difference vary among species and with growth stages?

## Materials and methods

### Experimental setup

The experiment was conducted in a greenhouse at University of California, Santa Cruz. We made 36 polyvinyl chloride (PVC) pots (diameter 8 cm, height 15 cm). Each pot was closed at the bottom with a rubber stopper and had an air inlet and an air outlet consisting of clear plastic tubing. We filled each pot with 500 g burned and acid-washed carbon-free sand, 90 g carbon-free perlite, and 10 g soil as inoculant. The sand, perlite and soil were well mixed in each pot before planting seeds. The soil was a sandy loam (Mollisol) collected from a farm on the university campus, with 1.5% C, 0.14% N and $\delta^{13}C$ value of −26.65‰. Various crops and vegetables (mostly $C_3$ plants, sunflower, soybean, strawberry, lettuce, etc.) have been grown in the farm since it was converted from a meadow dominated by $C_3$ annual grasses in 1974. Six pots were planted with each of the following six species: soybean (*Glycine max* L. Merr.), sunflower (*Helianthus annuus* L.), wheat (*Triticum aestivum* L.), amaranthus (*Amaranthus tricolor* L.), sorghum (*Sorghum bicolor* (L.) Moench), and maize (*Zea mays* L. ssp. *mays*). We planted four seeds and kept one plant per pot after seedling emergence for all species except wheat (planted 10 seeds, kept three plants). All pots were flushed with full-strength Hoagland solution every day. The volume of Hoagland solution increased from 20 ml per day initially to 100~150 ml per day at the end. The extra solution in excess of the holding capacity of growth medium and plant uptake drained out of the pot through the air outlet tube at the bottom of the pot. During the 62-day plant growth period, air temperature was maintained at 25°C during the day and 15°C during the night, relative humidity was maintained at 40%, and photoperiod was set as 14 h with supplemental lighting when needed.

### Measurements

During 40~42 and 60~62 days after sowing (DAS), we measured rhizosphere respiration of each plant species in three randomly selected pots using a closed-circulation $CO_2$ trapping system (Cheng et al. 2003). Briefly, we sealed the pot at the base of the plant with non-toxic silicone rubber (GI-1000, Silicones Inc., NC) and removed $CO_2$ inside the pot by circulating the isolated air through a soda lime column for 1 h. Then $CO_2$ produced in the sealed pot was trapped in a 400 mL 0.5*M* NaOH solution for 30 min every 6 h during the 48-h period. Three blanks were included to correct for possible contamination from carbonate in the NaOH stock solution and from sample handling. An aliquot of each NaOH solution was analyzed for total inorganic carbon using a Shimadzu TOC-5050A Total Organic Carbon Analyzer and another aliquot was precipitated as $SrCO_3$ and then analyzed for $\delta^{13}C$ (relative to PDB standard) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer at the Stable Isotope Facility at University of California, Davis (Harris et al. 1997). The $\delta^{13}C$ values measured in $SrCO_3$ were corrected for a small amount of contamination from carbonate in the NaOH stock solution and from sample handling (Cheng et al. 2003).

Immediately after $CO_2$ trapping (42 and 62 DAS), the pots were destructively sampled. Shoots and roots were harvested, washed with deionized water, dried in an oven at 60°C for 48 h, and weighed. We then ground the dry shoot and root samples in a ball mill and analyzed them for C%, N%, $\delta^{13}C$ and $\delta^{15}N$ using a Carlo Elba 1108 elemental analyzer interfaced to a ThermoFinningan Delta Plus XP isotope ratio mass spectrometer at the Stable Isotope Laboratory at University of California, Santa Cruz.

### Statistical analyses

Independent-samples *t*-test was used to test whether the variables ($\delta^{13}C$ and rhizosphere respiration rate) were significantly different between the two trapping periods (Table 1). Paired-samples *t*-test was used to test whether the difference in $\delta^{13}C$ values between shoot, root, and $CO_2$ from rhizosphere respiration was significantly different than zero (Fig. 1).

## Results

All plants were at vegetative stage during the first trapping period (40~42 DAS). During the second

**Table 1** $\delta^{13}C$ values (‰) of shoot, root, and rhizospheric $CO_2$ ($C_r$), differences in $\delta^{13}C$ values (‰) between shoot, root, and rhizospheric $CO_2$, and rhizosphere respiration rate ($C_r$, g C/g root N/day). Values represent means of three replicates with standard error in parenthesis. Different letters within each column for each species represent significant differences in mean value between two sampling periods (Independent-samples *t*-test, *n*=3, *P*<0.05); bold values of $\delta^{13}C$ difference (Root—Shoot, $CO_2$—Shoot, and $CO_2$—Root) are statistically different than zero (Paired-samples *t*-test, *n*=3, *P*<0.05); DAS stands for days after sowing

| Species | DAS | $\delta^{13}C$ (‰) | | | Difference in $\delta^{13}C$ (‰) | | | $C_r$ (g C/g root N/day) |
|---|---|---|---|---|---|---|---|---|
| | | Shoot | Root | $CO_2$ | Root—Shoot | $CO_2$—Shoot | $CO_2$—Root | |
| Soybean | 40~42 | −28.43(0.07)a | −27.87(0.16)a | −29.66(0.12)a | 0.56(0.17)a | **−1.23(0.13)a** | **−1.79(0.05)a** | 0.99(0.12)a |
| | 60~62 | −27.93(0.20)a | −27.73(0.28)a | −29.35(0.07)a | 0.20(0.20)a | **−1.42(0.27)a** | **−1.62(0.34)a** | 1.39(0.16)a |
| Sunflower | 40~42 | −28.17(0.26)a | −28.24(0.16)a | −28.97(0.12)a | −0.07(0.23)a | −0.79(0.38)a | −0.72(0.26)a | 0.95(0.10)a |
| | 60~62 | −28.38(0.20)a | −28.46(0.28)a | −29.77(0.17)b | −0.09(0.14)a | −1.39(0.36)b | −1.30(0.43)a | 0.90(0.12)a |
| Wheat | 40~42 | −27.20(0.21)a | −26.48(0.04)a | −27.28(0.32)a | 0.73(0.19)a | −0.07(0.26)a | −0.80(0.33)a | 1.48(0.02)a |
| | 60~62 | −29.28(0.19)b | −27.91(0.08)b | −28.85(0.19)b | **1.37(0.20)b** | 0.43(0.36)a | **−0.94(0.17)a** | 1.14(0.12)a |
| Amaranthus | 40~42 | −15.78(0.20)a | −13.64(0.17)a | −20.71(0.30)a | **2.14(0.32)a** | **−4.93(0.10)a** | **−7.07(0.43)a** | 2.00(0.04)a |
| | 60~62 | −16.65(0.13)b | −13.90(0.31)a | −20.91(0.36)a | **2.74(0.27)a** | **−4.26(0.23)a** | **−7.01(0.34)a** | 2.23(0.06)b |
| Sorghum | 40~42 | −13.46(0.20)a | −13.41(0.09)a | −20.14(0.15)a | 0.05(0.11)a | **−6.68(0.35)a** | **−6.74(0.24)a** | 1.17(0.01)a |
| | 60~62 | −13.50(0.15)a | −13.41(0.12)a | −19.60(0.35)a | 0.09(0.14)a | **−6.09(0.50)a** | **−6.19(0.41)a** | 1.27(0.10)a |
| Maize | 40~42 | −13.98(0.20)a | −13.93(0.16)a | −16.90(0.18)a | 0.04(0.09)a | **−2.92(0.17)a** | **−2.97(0.22)a** | 1.86(0.07)a |
| | 60~62 | −14.04(0.05)a | −13.98(0.03)a | −18.49(0.15)b | 0.06(0.05)a | **−4.44(0.12)b** | **−4.50(0.16)b** | 2.00(0.12)a |

trapping period (60~62 DAS), sunflower and soybean were at flowering stage, wheat was at grain-filling stage, while the three $C_4$ species (amaranthus, sorghum and maize) remained at vegetative stage.

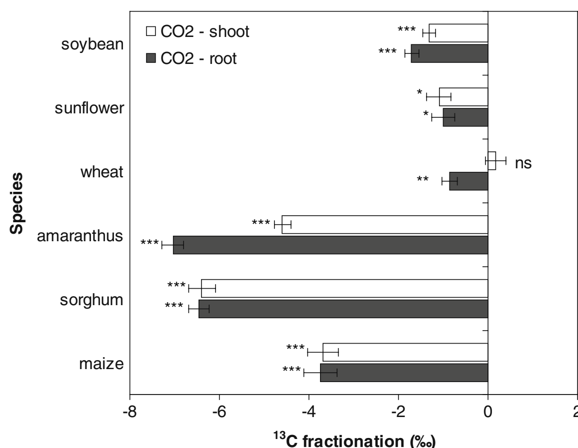


**Fig. 1** The difference in $\delta^{13}C$ values between rhizospheric $CO_2$ ($C_r$) and root or shoot biomass (i.e. carbon isotopic fractionation during rhizosphere respiration relative to root or shoot biomass) in three $C_3$ plants (soybean, sunflower and wheat) and three $C_4$ plants (amaranthus, sorghum and maize) in this study. Error bars represent standard errors of the mean. Statistical comparisons were between the isotopic fractionation and the "0" line (Paired-samples *t*-test, *n*=6), ****P*< 0.001, ***P*<0.01, **P*<0.05, ns-*P*>0.05

Plant biomass (data not shown) was significantly higher during the second trapping period than during the first trapping period, while specific rhizosphere respiration rate (g C/g root N/day, Table 1) and shoot or root N concentration (data not shown) did not change significantly between the two trapping periods. Sunflower and wheat showed higher biomass than other species, while amaranthus and maize had nearly twice specific rhizosphere respiration rate as other species (Table 1).

Shoot and root $\delta^{13}C$ values were approximately −14‰ in the three $C_4$ plants, except that amaranthus shoot $\delta^{13}C$ value was −16‰ (Table 1). However, shoot and root $\delta^{13}C$ values were about −28‰ in the three $C_3$ plants, except that wheat showed relatively large variations in $\delta^{13}C$ value (−26.5‰ to 29.3‰) (Table 1). Most shoot and root $\delta^{13}C$ values did not change significantly between the two trapping periods (*P*>0.05), except that wheat (shoot and root) and amaranthus (shoot) was more $^{13}C$-depleted during the second trapping period than during the first trapping period (*P*<0.05). Overall, root had similar $\delta^{13}C$ values with shoot in soybean, sunflower, sorghum and maize (*P*>0.05), but root was more $^{13}C$-enriched than shoot in wheat (1.0‰, *n*=6, *P*<0.01) and amaranthus (2.4‰, *n*=6, *P*<0.001) (Table 1).

We calculated the difference in $\delta^{13}C$ value between root or shoot bulk organic matter and $CO_2$ from rhizosphere respiration. In general, the $^{13}C$ fractionation during rhizosphere respiration relative to root biomass did not change significantly between the two trapping periods ($P>0.05$), except that it was significantly higher ($P<0.05$) during the second trapping period (4.5‰) than during the first trapping period (3.0‰) in maize (Table 1). Although we noticed the possible effect of plant phenology or growing period on $^{13}C$ fractionation during rhizosphere respiration, particularly for maize, we used the average value during the two trapping periods ($n=6$) to estimate an integrative $^{13}C$ fractionation during rhizosphere respiration for each species (Fig. 1). Overall, $CO_2$ from rhizosphere respiration was slightly but significantly ($n=6$, $P<0.05$) more $^{13}C$-depleted than root biomass by 0.9±0.2‰ in wheat, 1.0±0.3‰ in sunflower, and 1.7±0.2‰ in soybean. However, $^{13}C$ fractionation during rhizosphere respiration was much higher in the three $C_4$ species. Compared to root biomass, rhizospheric $CO_2$ was remarkably ($n=6$, $P<0.001$) more $^{13}C$-depleted by 3.7±0.4‰ in maize, 6.5±0.2‰ in sorghum, and 7.0±0.2‰ in amaranthus. Moreover, because shoot and root had similar $\delta^{13}C$ values, the difference in $\delta^{13}C$ value between shoot biomass and $CO_2$ from rhizosphere respiration was similar to or slightly lower than that between root biomass and $CO_2$ from rhizosphere respiration (Fig. 1)

## Discussion

### Comparison with previous studies

We summarized all available data in the literature on carbon isotopic fractionation during root respiration or rhizosphere respiration in Table 2. The difference in the measured $^{13}C$ fractionation may result from three factors. First, all these studies except three from our lab (Cheng 1996; Dijkstra and Cheng 2007; and this study) excluded rhizosphere microorganisms and thus an important component of root-derived $CO_2$—rhizomicrobial respiration. If rhizomicrobial respiration accounts for a significant proportion of rhizosphere respiration and $^{13}C$ fractionation differs between root respiration and rhizomicrobial respiration, then the measured $^{13}C$ fractionation during root respiration (e.g. Klumpp et al. 2005; Werth and Kuzyakov 2005) would be different than that during rhizosphere respiration (Cheng 1996; Dijkstra and Cheng 2007; this study). However, simultaneous measurements of both root respiration (e.g. without inoculation, maintain a microbe-free growth medium) and rhizosphere respiration (e.g. inoculate with native soil microorganisms that consume rhizodeposits) of the same species, under the same environment and using the same method, are required to directly test this hypothesis.

Second, many published studies conducted snapshot measurements (<30 min) of $\delta^{13}C$ value of root respiratory $CO_2$ based on a gas exchange system (Badeck et al. 2005; Schnyder and Lattanzi 2005) or a direct gas sampling system (Gessler et al. 2009; Wegener et al. 2010). Both $^{13}C$ values of respiratory substrates and the relative contribution of different metabolic pathways to $CO_2$ evolution may change within a day, therefore $^{13}C$ fractionation during plant respiration can change significantly on a diurnal basis, as shown in previous studies on $^{13}C$ fractionation during leaf respiration (Gessler et al. 2007; Sun et al. 2009; Wegener et al. 2010). Diurnal variations in $^{13}C$ fractionation during root respiration and rhizosphere respiration are probable, but direct experimental evidence is not yet available. In this study we measured $\delta^{13}C$ value of rhizospheric $CO_2$ integrated over a two-day period using a closed-circulation $CO_2$ trapping system (Cheng 1996; Cheng et al. 2003). The $CO_2$ trapping efficiency with this system was greater than 99%, eliminating preferential sorption of $^{13}CO_2$ vs. $^{12}CO_2$. The $CO_2$ from microbial respiration of the 10-g soil (with a $\delta^{13}C$ value of −26.65‰) used to inoculate the sand-perlite mixture with microorganisms was less than 1% of the $CO_2$ from rhizosphere respiration. Therefore, the possible contamination of the $\delta^{13}C$ value of root-derived $CO_2$ by the $\delta^{13}C$ value of soil-derived $CO_2$ is negligible (<0.1‰). The circulation system was carefully maintained to avoid any leakage of ambient air, and blanks (the same circulation system in Fig. 1 without the pot) were included to correct for possible contamination from carbonate in the NaOH stock solution and from sample handling (Cheng et al. 2003). Therefore, unlike the gas exchange system or the direct gas sampling system which measured $CO_2$ produced during a short time period (<30 min) used in previous studies (Bathellier et al. 2008; Gessler et al. 2007; Klumpp et al. 2005; Moyes et al. 2010), our closed-

**Table 2** The magnitude of carbon isotope fractionation during root respiration (method 1–3) or rhizosphere respiration (method 4) relative to root biomass in different plant species by different studies. For the six species in this study, values represent means of six replicates with standard error in parenthesis

| Plant species | $C_3$ or $C_4$ | Fractionation (‰) | Method* | Reference |
|---|---|---|---|---|
| *Eucalyptus delegatensis* | $C_3$ | 0.7~3.1 | 1 | Gessler et al. 2007 |
| *Ricinus communis* | $C_3$ | −4~−2 | 1 | Gessler et al. 2009 |
| *Halimium halimifolium* | $C_3$ | −2.4 | 1 | Wegener et al. 2010 |
| *Melissa officinalis* | $C_3$ | −0.2 | 1 | Wegener et al. 2010 |
| *Acer negundo* | $C_3$ | 9.0 | 1 | Moyes et al. 2010 |
| *Phaseolus vulgaris* | $C_3$ | −1.5 | 2 | Badeck et al. 2005 |
| *Phaseolus vulgaris* | $C_3$ | −2~0 | 2 | Bathellier et al. 2008 |
| *Medicgo sativa* | $C_3$ | −3.7~−1.5 | 2 | Klumpp et al. 2005 |
| *Helianthus annuus* | $C_3$ | −2.0~−0.5 | 2 | Klumpp et al. 2005 |
| *Lolium perenne* | $C_3$ | −5.4 | 2 | Klumpp et al. 2005 |
| *Lolium perenne* | $C_3$ | −5.5~−3.5 | 2 | Schnyder and Lattanzi 2005 |
| *Paspalum dilatatum* | $C_4$ | −5.0~−0.5 | 2 | Schnyder and Lattanzi 2005 |
| *Zea mays* | $C_4$ | −0.7~0.3 | 3 | Werth and Kuzyakov 2005 |
| *Triticum aestivum* | $C_3$ | −0.2 | 4 | Cheng 1996 |
| *Pinus ponderosa* | $C_3$ | −1.5~0.4 | 4 | Dijkstra and Cheng 2007 |
| *Glycine max* | $C_3$ | −1.7 (0.16) | 4 | This study |
| *Helianthus annuus* | $C_3$ | −1.0 (0.26) | 4 | This study |
| *Triticum aestivum* | $C_3$ | −0.9 (0.17) | 4 | This study |
| *Amaranthus tricolor* | $C_4$ | −7.0 (0.24) | 4 | This study |
| *Sorghum bicolor* | $C_4$ | −6.5 (0.24) | 4 | This study |
| *Zea mays* | $C_4$ | −3.7 (0.36) | 4 | This study |

1—direct gas sampling (excised roots incubated in tubes or chambers), <30 min

2—gas exchange system (intact roots still attached to shoots incubated in cuvettes or chambers), <30 min

3—closed-circulation $CO_2$ trapping system (intact roots still attached to roots in nutrient solution), 4 days

4—closed-circulation $CO_2$ trapping system (intact roots still attached to roots in sand-perlite with nutrient solution, inoculated with native soil microorganisms), 2–3 days

circulation $CO_2$ trapping system rendered a more integrative measurement of $^{13}C$ fractionation during rhizosphere respiration.

Third, different species may also contribute to the difference in measured $^{13}C$ fractionation in addition to different $CO_2$ sources (root respiration vs. rhizosphere respiration) and methods of $CO_2$ measurement (snapshot measurement vs. integrative measurement) discussed above. Our results of $^{13}C$ fractionation during rhizosphere respiration of three $C_3$ species (soybean 1.7‰, sunflower 1.0‰ and wheat 0.9‰) fall in the range of previous results of $^{13}C$ fractionation during root or rhizosphere respiration of same or similar $C_3$ species (*Phaseolus vulgaris* 0~2‰, sunflower 0.5~2.0‰, wheat 0.2‰), but are significantly lower than previous results of $^{13}C$ fractionation during root respiration of other $C_3$ species (*Lolium perenne* 3.5~5.4‰, *Paspalum dilatatum* 0.5~5‰ and *Ricinus communis* 2~4‰, Table 2). Note that two recent field studies (Gessler et al. 2007; Moyes et al. 2010) found $^{13}C$-enrichment (up to 9‰) of root respiration of two woody species (*Eucalyptus delegatensis* and *Acer negundo*) compared to root biomass, a result that differs markedly from those of laboratory studies. The short-term direct gas sampling method used in these two studies (incubation of excised roots in a root chamber for a few minutes) may partly contribute to this difference. In addition, our results of $^{13}C$ fractionation during rhizosphere respiration of three $C_4$ species (maize 3.7‰, sorghum 6.5‰ and amaranthus 7.0‰) were significantly higher than those of three $C_3$ species in this study (0.9~1.7‰). Only two

studies have reported data on $^{13}C$ fractionation during root respiration in $C_4$ species (Table 2). Using a gas exchange system to measure the $\delta^{13}C$ value of respiratory $CO_2$ from roots grown in quartz sand, Schnyder and Lattanzi (2005) reported 0.5~5.0‰ $^{13}C$-depletion (depending on growth temperature) in root respiration relative to root biomass in *Paspalum dilatatum*. However, using a closed-circulation $CO_2$ trapping system to measure the $\delta^{13}C$ value of respiratory $CO_2$ from roots grown in nutrient solution, Werth and Kuzyakov (2005) reported a very small difference in $\delta^{13}C$ value (<1‰) between root respiration and root biomass in maize. Therefore, although the relative $^{13}C$ fractionation during rhizosphere respiration between $C_3$ plants and $C_4$ plants needs further investigation, our data clearly showed higher $^{13}C$ fractionation during rhizosphere respiration in the three $C_4$ plants (amaranthus, sorghum, and maize) than in the three $C_3$ plants (soybean, sunflower, and wheat).

## Possible mechanisms for $^{13}C$ fractionation during rhizosphere respiration

Rhizosphere respiration has two components—root respiration and rhizomicrobial respiration. First, *root respiration* may be $^{13}C$-depleted relative to root biomass (Gessler et al. 2009; Klumpp et al. 2005; Schnyder and Lattanzi 2005; but see Gessler et al. 2007 and Moyes et al. 2010). A number of hypotheses have been advanced to explain this depletion in root respiration compared to likely respiratory substrates. The pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCA, or the Krebs cycle), and phosphoenolpuruvate carboxylase reaction (PEPC, or the refixation of $CO_2$ by phosphoenolpuruvate carboxylase) all release $^{13}C$-depleted $CO_2$ relative to the original substrate glucose, while PDC (pyruvate dehydrogenation complex) releases $^{13}C$-enriched $CO_2$ relative to glucose (Barbour and Hanson 2009; Wingate 2008). The relative allocation of carbon to the different pathways in roots may lead to different carbon isotope fractionation in root respiration among different species or under different environmental conditions (Bathellier et al. 2009; Gessler et al. 2009; Wegener et al. 2010).

Second, *rhizomicrobial respiration*, or the respired $CO_2$ from microorganisms utilizing root-derived substrates in the rhizosphere, may have different $\delta^{13}C$ values than root biomass. The fractionation during rhizomicrobial respiration may occur during two processes: (1) root-derived carbon compounds in the rhizosphere (or rhizodeposition) may have different $\delta^{13}C$ values than root biomass (Werth and Kuzyakov 2005); and (2) microbially respired $CO_2$ may be different in $\delta^{13}C$ value with the root-derived carbon substrates taken up and utilized by them (Fernandez and Cadisch 2003; Henn and Chapela 2000; Mary et al. 1992). The first process is mainly determined by the chemical composition of rhizodeposition, which is a mixture of different compounds (Lynch and Whipps 1990) that may differ significantly in $\delta^{13}C$ value (Bowling et al. 2008). Few studies have compared the $^{13}C$ values of individual compounds or overall rhizodeposits with bulk root tissue. The second process is controlled by many factors: temperature, isotopic distribution within the substrates, chemical nature of the substrates, metabolic pathways of carbon, and physiological conditions of microbial growth (Fernandez et al. 2003; Werth and Kuzyakov 2010). Despite more studies on this process than the first process, the results remain inconsistent among studies (e.g. significant depletion in Blair et al. 1985 and Mary et al. 1992; not significant in Ehleringer et al. 2000 and Ekblad et al. 2002) and change over time within studies (e.g. Fernandez et al. 2003; Schweizer et al. 1999). There have been some studies on fractionation during root respiration (Table 2), but no studies are available on fractionation during rhizomicrobial respiration, mainly because it is difficult to collect root-derived carbon substrates for $^{13}C$ measurement and concurrently measure the $\delta^{13}C$ values of substrate and respired $CO_2$ for microbial respiration (Werth and Kuzyakov 2010). The $^{13}C$ depletion in rhizosphere respiration relative to root biomass observed in this study may be partly caused by the $^{13}C$ depletion in rhizomicrobial respiration, particularly during the microbial uptake and utilization of root-derived substrates, in addition to the $^{13}C$ depletion in root respiration relative to root biomass or respiratory substrate as discussed above.

Why does the $^{13}C$ fractionation differ significantly between the three $C_3$ plants and the three $C_4$ plants in this study? Although the exact mechanisms of this phenomenon are unknown at this point and need further investigation, here we point out two possible mechanisms. First, during root respiration, plants with different photosynthetic pathways (e.g. $C_3$ vs. $C_4$)

may differ in the relative contribution of different metabolic pathways to root respired $CO_2$ (Bathellier et al. 2009; Gessler et al. 2009; Wegener et al. 2010), which can lead to different $^{13}C$ values of $CO_2$ from root respiration. However, direct comparisons of carbon substrate allocation to different root metabolic pathways between $C_3$ and $C_4$ plants are lacking. Second, during rhizomicrobial respiration, differences in $^{13}C$ distribution in sugars derived from $C_3$ vs. $C_4$ plants (Rossmann et al. 1991) and the possible different fractionation during fungi uptake of sugars derived from $C_3$ vs. $C_4$ plants (Henn and Chapela 2000), may further contribute to different $^{13}C$ values of $CO_2$ from rhizomicrobial respiration. As only three $C_3$ and $C_4$ plants are included in this study, considering the large variation in $^{13}C$ fraction during root respiration among species (Table 2), we urgently need more studies to determine whether the different $^{13}C$ fractionation during rhizosphere respiration is species-specific or photosynthesis-pathway-specific.

## Implications for partitioning soil respiration

Our results showed relatively small and consistent differences in $\delta^{13}C$ values between root biomass and rhizospheric $CO_2$ in sunflower, soybean and wheat (0.9~1.7‰), but the differences were relatively large in maize, sorghum and amaranthus (3.7~7.0‰). This has important implications for partitioning total soil respiration ($C_t$) into root-derived ($C_r$) and soil-derived ($C_s$) components using isotope mixing models: $C_r = C_t\ (\delta^{13}C_s - \delta^{13}C_t) / (\delta^{13}C_s - \delta^{13}C_r)$, $C_s = C_t - C_r$, where $\delta^{13}C_t$, $\delta^{13}C_r$, and $\delta^{13}C_s$ are $\delta^{13}C$ values of $C_t$, $C_r$, and $C_s$ respectively (Cheng 1996). For example, if we grow a $C_3$ plant (e.g. sunflower) in a $C_4$ soil in an ambient air (−8‰), assume that soil-derived $CO_2$ has a $\delta^{13}C$ value of −14‰ ($\delta^{13}C_s$=−14‰), root biomass has a $\delta^{13}C$ value of −28‰, and total soil respiration has a $\delta^{13}C$ value of −21‰ ($\delta^{13}C_t$=−21‰). If we use $\delta^{13}C$ value of root biomass for $\delta^{13}C$ value of root-derived $CO_2$ ($\delta^{13}C_r$=−28‰), rhizosphere respiration would be 50% of total soil respiration. However, if we consider the $^{13}C$-depletion in rhizosphere respiration relative to root biomass by 1~7‰ and use $\delta^{13}C$ value of −29~−35‰ for $\delta^{13}C_r$, rhizosphere respiration would be 46.7~33.3% of total soil respiration (Fig. 2a). Therefore, for $C_3$ plants grown in $C_4$ soils, not accounting for the $^{13}C$-depletion in rhizosphere respiration relative to root biomass would slightly overestimate *root-derived* autotrophic respiration and underestimate *soil-derived* heterotrophic respiration. This suggests that previous estimates of positive rhizosphere priming effect for $C_3$ plants grown in $C_4$ soils (i.e. higher SOM decomposition rate in planted soil than in unplanted soil; Cheng et al. 2003) was conservative, because considering the $^{13}C$-depletion in rhizosphere respiration relative to root biomass

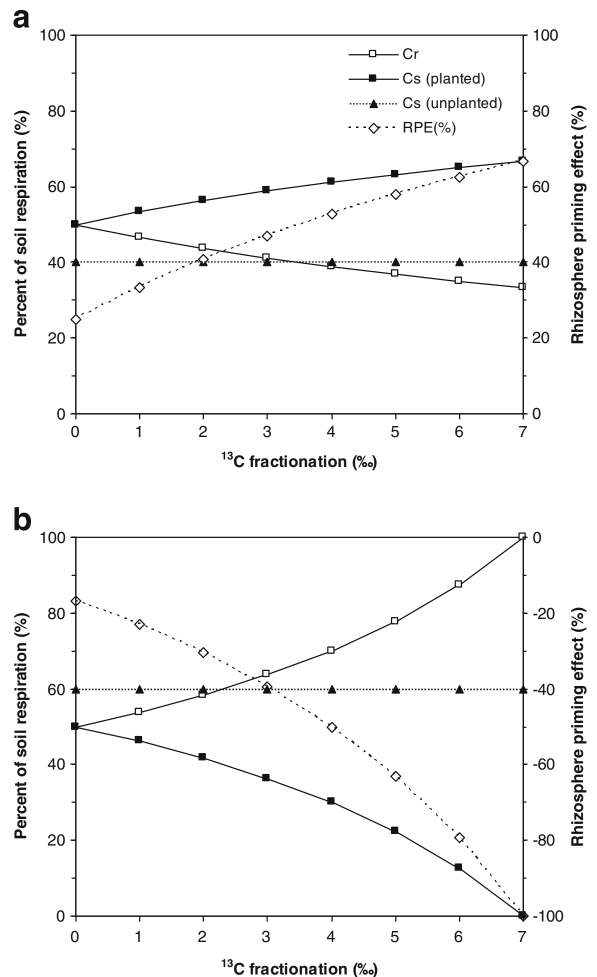


**Fig. 2** A sensitivity analysis to show how the $^{13}C$-depletion during rhizosphere respiration relative to root biomass may affect the partitioning of total soil respiration ($C_t$) into root-derived ($C_r$) and soil-derived ($C_s$) components as well as the rhizosphere priming effect (RPE). $C_r = C_t\ (\delta^{13}C_s - \delta^{13}C_t) / (\delta^{13}C_s - \delta^{13}C_r)$, $C_s = C_t - C_r$, RPE = [$C_s$(planted) − $C_s$(unplanted)] / $C_s$(unplanted) X 100%. **a** Grow a $C_3$ plant in $C_4$-plant-derived soil, assume $\delta^{13}C$(root)=−14‰, $\delta^{13}C_s$(unplanted)=−28‰, $\delta^{13}C_t$=−21‰, $C_t$=100, and $C_s$(unplanted)=40. **b** Grow a $C_4$ plant in $C_3$-plant-derived soil, assume $\delta^{13}C$(root)=−28‰, $\delta^{13}C_s$(unplanted)=−14‰, $\delta^{13}C_t$=−21‰, $C_t$=100, and $C_s$(unplanted)=60

would yield a higher rate of SOM decomposition in the presence of plants and thus a higher, positive rhizosphere priming effect (Fig. 2a). This situation is also applicable to the cases of $C_3$ plants labeled with $^{13}C$-depleted $CO_2$ and grown in native $C_3$ soils (Dijkstra et al. 2006; Zhu and Cheng 2010). The influence of the $^{13}C$-depletion of rhizospheric $CO_2$ on the $CO_2$ partitioning would be reduced if shoot $\delta^{13}C$ value was used as the end-member for rhizospheric $CO_2$, because the isotopic fractionation between shoots and rhizospheric $CO_2$ was smaller, especially for wheat (Table 1, Fig. 1).

Additionally, in the case of growing a $C_4$ plant (e.g. maize) in a $C_3$ soil, neglecting $^{13}C$ fractionation during rhizosphere respiration will result in substantial underestimation of root-derived $CO_2$, and overestimation of soil-derived $CO_2$ in planted treatments. To illustrate this point, assume that $\delta^{13}C_s$=−28‰, $\delta^{13}C_t$=−21‰, and $\delta^{13}C$ value of root biomass is −14‰. $C_r$ (rhizosphere respired $CO_2$) would be 50% of $C_t$ without figuring in the $^{13}C$ fractionation, but 53.8~100% of $C_t$ if 1~7‰ $^{13}C$ fractionation is included in the calculation (Fig. 2b). For example, Fu and Cheng (2002) showed that the SOM decomposition rates in a $C_3$ grassland soil planted with sorghum and amaranthus were 9% and 5% lower than in the unplanted soil. If the $^{13}C$ depletion in rhizosphere respiration relative to root biomass is used in a recalculation, the rate of SOM decomposition in the presence of plants would be much lower, and thus the negative rhizosphere priming effect would be much stronger (Fig. 2b).

The $^{13}C$ depletion in rhizosphere respiration relative to root biomass also has implications in partitioning ecosystem respiration ($R_{eco}$) into different components (aboveground plant respiration—$R_{above}$, rhizosphere respiration—$R_{rhizo}$, and microbial respiration—$R_{mic}$). In natural systems without significant land use change, microbial respiration tends to be slightly $^{13}C$-enriched compared to rhizosphere and aboveground plant respiration, thus a three-source mixing model can be used to partition $R_{eco}$ into $R_{above}$, $R_{rhizo}$ and $R_{mic}$ (Tu and Dawson 2005). If we account for the $^{13}C$-depletion in $R_{rhizo}$ relative to root biomass, the fraction of microbial respiration ($f_{micro}$) in total ecosystem respiration will be higher than original estimate assuming no fractionation in rhizosphere respiration. This suggests that neglecting the $^{13}C$ depletion in rhizosphere respiration relative to root biomass would underestimate the heterotrophic component of soil respiration ($R_{mic}$) and thus overestimate net ecosystem production (NEP = NPP (net primary production) – $R_{mic}$). Moreover, in studies that partition NEP into GPP (gross primary production) and $R_{eco}$ at ecosystem (e.g. Bowling et al. 2001) and global (e.g. Fung et al. 1997) scales using carbon isotopes, the isotopic composition of NEP, GPP and $R_{eco}$ are either directly measured by eddy covariance combined with flask sampling (for NEP/NEE) and Keeling plots (for $R_{eco}$) or indirectly estimated using theoretical models (for GPP). Therefore, accounting for $^{13}C$ deletion in rhizosphere respiration relative to root biomass will not affect these results.

## Conclusions

In conclusion, rhizospheric $CO_2$ was $^{13}C$-depleted (by up to 7‰) relative to root biomass in the six species studied. It appears that the $^{13}C$ fractionation during rhizosphere respiration relative to root biomass ($\Delta C_r$) did not change significantly between two measurement periods or growth stages, but varied significantly among species and particularly physiological groups ($C_3$ vs. $C_4$). Three $C_3$ plants (wheat, sunflower and soybean) showed small and consistent $^{13}C$-depletion in rhizosphere respiration (0.9~1.7‰), while three $C_4$ plants (maize, sorghum and amaranthus) had relatively large $^{13}C$-depletion in rhizosphere respiration (3.7~7.0‰) compared to root biomass. Whether the fractionation is photosynthetic-pathway-specific or simply species-specific should be tested with future research with more $C_3$ and particularly $C_4$ species. The mechanisms leading to $\Delta C_r$ remain unclear at this point. Further studies are needed to understand: (1) $\Delta C_r$ in other species, particularly woody species, and under field conditions; (2) ontogenetic, diurnal and seasonal changes in $\Delta C_r$; and (3) responses of $\Delta C_r$ to changes in environmental and physiological factors (e.g. temperature, respiratory quotient). The new and emerging technologies (e.g. tunable diode laser absorption spectroscopy or TDLAS, and cavity ring-down spectroscopy or CRDS) that provide instantaneous and continuous field measurements of $\delta^{13}C$ of $CO_2$ from plant (leaf, stem or root) or microbial respiration in combination with specially-designed chambers (e.g. Wingate et al. 2010) is particularly recommended. Nevertheless, our findings demonstrate that the $^{13}C$ fractionation in

rhizosphere respiration relative to root biomass should be seriously considered in future studies involving carbon flux partitioning using natural abundance carbon isotope methods (Baggs 2006; Kuzyakov 2006).

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