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Source: *New Phytologist*, Vol. 163, No. 3 (Sep., 2004), pp. 499-505
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Rapid report

Leaf respiratory CO_2 is ^{13}C-enriched relative to leaf organic components in five species of C_3 plants

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Received: *18 May 2004*
Accepted: *14 June 2004*

Summary

- Here, we compared the carbon isotope ratios of leaf respiratory CO_2 ($\delta^{13}C_R$) and leaf organic components (soluble sugar, water soluble fraction, starch, protein and bulk organic matter) in five C_3 plants grown in a glasshouse and inside Biosphere 2. One species, *Populus deltoides*, was grown under three different CO_2 concentrations.
- The Keeling plot approach was applied to the leaf scale to measure leaf $\delta^{13}C_R$ and these results were compared with the $\delta^{13}C$ of leaf organic components.
- In all cases, leaf respiratory CO_2 was more ^{13}C-enriched than leaf organic components. The amount of ^{13}C enrichment displayed a significant species-specific pattern, but the effect of CO_2 treatment was not significant on *P. deltoides*.
- In C_3 plant leaves, ^{13}C-enriched respiratory CO_2 appears widespread. Among currently hypothesized mechanisms contributing to this phenomenon, non-statistical carbon isotope distribution within the sugar substrates seems most likely. However, caution should be taken when attempting to predict the $\delta^{13}C$ of leaf respiratory CO_2 at the ecosystem scale by upscaling the relationship between leaf $\delta^{13}C_R$ and $\delta^{13}C$ of leaf organic components.

Key words: C_3 plants, carbon isotope, dark respiration, fractionation, Keeling plot.

New Phytologist (2004) **163**: 499–505

© *New Phytologist* (2004) **doi**: 10.1111/j.1469-8137.2004.01153.x

Introduction

It is well known that carbon isotope discrimination takes place during plant photosynthetic CO_2 fixation, resulting in all higher plants being depleted in ^{13}C in organic carbon relative to atmospheric CO_2. The models of ^{13}C fractionation in photosynthesis have been well established (Farquhar *et al.*, 1982). By contrast, studies on the carbon isotope ratio of CO_2 generated by dark respiration ($\delta^{13}C_R$) are limited. Although a possible isotope effect during dark respiration might significantly influence the carbon isotope signature of plants and other components of an ecosystem, fewer studies have focused on determining the magnitude of this potential effect and the results appear contradictory (O'Leary, 1981; Lin & Ehleringer, 1997; Duranceau *et al.*, 1999, 2001).

Studies on the carbon isotopic effects during respiration trace back half a century. Historically, carbon isotope discrimination during respiration was considered to be negeligible

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(O'Leary, 1981; Farquhar *et al.*, 1982, 1989; Flanagan & Ehleringer, 1998). Early experimental studies observed that $\delta^{13}C_R$ is very close (approx. ±1‰) to bulk carbon in some geminating crop seedlings (Baertschi, 1953; Smith, 1971). More recently (Lin & Ehleringer, 1997) cultured mesophyll protoplasts of bean and corn leaves with carbohydrates of known isotopic ratios as the only carbon source and found no significant differences between $\delta^{13}C_R$ and $\delta^{13}C$ of the substrate in either species.

Still, other studies suggest that respiratory CO_2 of plants can be remarkably ^{13}C-enriched or ^{13}C depleted (4–5‰ more positive or −4–8‰ more negative) in comparison with whole plant or leaf $\delta^{13}C$ (Smith, 1971; Troughton *et al.*, 1974). Recently, Duranceau *et al.* (1999, 2001) and Ghashghaie *et al.* (2001) compared $\delta^{13}C$ of leaf respiration and leaf organic components in beans, tobacco, and sunflower. They report a 3–6‰ ^{13}C-enrichment in respiratory CO_2 compared to sucrose, the assumed substrate of dark respiration. Although this hypothesis was not tested in other species, they concluded that carbon isotope fractionation during dark respiration was widespread in C_3 plants. In C_4 plants, Henderson *et al.* (1992) found that the $\delta^{13}C$ of dry matter was more negative than that predicted by the discrimination occurring during CO_2 uptake and partly attribute the difference to an isotope effect during dark respiration. Furthermore, $\delta^{13}C_R$ can change daily or seasonally (Park & Epstein, 1961; Jacobson *et al.*, 1970; Damesin & Lelarge, 2003) and can be influenced by environmental or physiological factors (temperature, respiratory quotient, etc., Tcherkez *et al.*, 2003).

Despite the growing contradictory evidence, the assumption that carbon fractionation in dark respiration is negligible is widely applied in ecological and physiological studies (Flanagan & Ehleringer, 1998; Yakir & Sternberg, 2000; Ehleringer *et al.*, 2002). At the ecosystem scale, the concept of ecosystem ^{13}C discrimination ($\Delta^{13}C_e = (\delta^{13}C_{trop} - \delta^{13}C_R)/(1 + \delta^{13}C_R)$, or $\Delta^{13}C_e = (\delta^{13}C_{atm} - \delta^{13}C_R)$), has recently been used to partition NEE (Net Ecosystme Exchange) into photosynthesynthetic and respiratory components (Bowling *et al.*, 2001), by assuming that the $\delta^{13}C_R$ should reflect the ^{13}C signature of total organic carbon in the ecosystem (Buchmann *et al.*, 1997; Yakir & Sternberg, 2000). Likewise, the $\delta^{13}C$ of organic carbon in leaf, soil, and litter were used to estimate the $\delta^{13}C_R$ generated by each components (Lin *et al.*, 1999, 2001). If the $\delta^{13}C_R$ does not correctly reflect the $\delta^{13}C$ of the respiration substrate pool, the conclusions of these studies will need to be reconsidered and modified accordingly.

Additional uncertainties regarding the use of $\delta^{13}C_R$ as a tool for understanding ecosystem scale processess arise from several other factors. For example, initial studies focused on seedlings or tubers and subsequent leaf scale studies were conducted in only a few crop species. In addition, plant materials were subjected to a CO_2-free environment in all previous studies, which may itself influence leaf $\delta^{13}C_R$ (O'Leary, 1981). Clearly, much more detailed information on the species effects, and ultimately the mechanisms are needed to gain insight into ecosystem level processes. In this study, we applied a Keeling plot approach on the leaf scale to measure leaf $\delta^{13}C_R$ (Fessenden & Ehleringer, 2003) and studied its relationship with $\delta^{13}C$ of leaf soluble sugar, water soluble fraction, starch, protein, and bulk organic carbon of five C_3 plants. Our primary goal was to test the hypothesis (1) that $\delta^{13}C_R$ will be the same as major substrates (assumed to be soluble sugar), an assumption that underlies many current ecological studies, neglecting the carbon isotope effect in respiration. If hypothesis (1) is rejected, we further hypothesize (2) the differences between $\delta^{13}C_R$ and $\delta^{13}C$ leaf organic components is not species-specific and (3) will not be significantly influenced by growth CO_2 level (in *Populus deltoides*).

Materials and Methods

Plant materials

We studied five C_3 plants, which are among the most abundant species in the Tropical Rain Forest (TRF) and Intensive Forest Biome (IFB) of Biosphere 2 (a 1.29 hectare glass enclosed research facility in Oracle, AZ, USA). This leaf scale study was also designed to provide background information for further investigation on the respiratory isotope effect at mesocosom scale within Biosphere 2. Among 4 tropical species we studied, *Musa paradisiacal* (tree), *Coffea arabica* (shrub), and *Epipremumn pinnatum* (vine) were grown in a glasshouse (the demonstrate lab or DL), while *Clitoria racemosa* (tree), was grown in the TRF biome. *Populus deltoides* (IFB monoculture), a temperate tree species, was grown in three CO_2 concentrations: close to ambient in the DL, 800 p.p.m. in the IFB mid bay (MB) and 1200 p.p.m. in west bay (WB). In the IFB, tank supplied CO_2 with a very low $\delta^{13}C$ (*c.* −28‰) was used to maintain elevated CO_2 concentrations. All plants grew under a natural photoperiod and night time temperatures of 23–28°C depending on the location.

Air sampling

All air samples were collected between 20 : 00 h to 00 : 30 h, when plants were in natural darkness. One to several healthy, intact, visually mature (well expanded and with developed cuticle) leaves were sealed in an opaque respiration chamber (modified from a mylar balloon) with a small fan to ensure air mixing. The chamber was connected to a closed loop gas exchange system including a pump, a CO_2 infrared gas analyzer (LI-6200, Licor, Inc., Lincoln, NE, USA), a desiccant tube containing magnesium perchlorate and six 100 mL flasks (Fig. 1a). The entire system was 10–15 L to hold the largest leaf (*Epipremumn pinnatum*) in our study and was checked for leakage before each sampling by exhaling on all connections. The airflow rate was approx. 1 L^{-1} per min. Ambient air was

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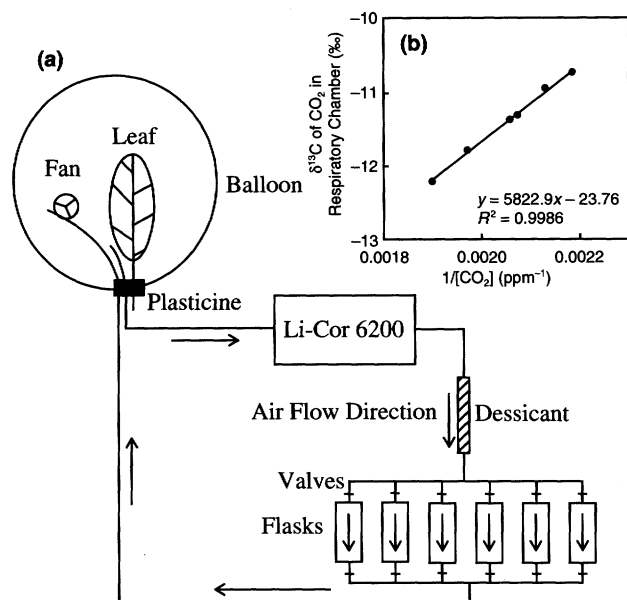

Fig. 1 Leaf scale Keeling plot apparatus and result. (a) Diagram of sampling apparatus used in this study. (b) The result of one *Musa paradisiacal* leaf ($R^2 = 0.9986 y = 5822.9x - 23.76$, geometric mean regression). The scale of *x*-axis (0.0023 – 0.0018) is equivalent to [CO_2] of 435–555 p.p.m. In all measurements, R2 is > 0.95 and in most cases close to 0.99.

pumped through the entire system before closure and then allowed to circulate for 10–15 min to ensure adequate mixing before sampling. The air samples were collected in sequence by closing both stopcocks on a flask for each 15–20 p.p.m. CO_2 increment. Humidity was not strictly controled in our study.

We compared our leaf-level Keeling plot method with a traditional CO_2 free chamber connected to the Li-6200 photosynthesis system and found that two methods yielded similar results in leaf $\delta^{13}C_R$ (±0.5%) when the incubation chamber was well sealed. However, the incubation method often gave much more scatter results with same plant leaf than leaf–level Keeling plot approach.

Leaf sampling and chemical extraction

After air sampling, half of the leaf material contained within the cuvette was immediately frozen in liquid nitrogen and then stored in −20°C freezer for subsequent extraction of carbohydrate and protein. The remaining leaf material was dried in a 60°C oven for carbon isotope analysis of bulk leaf organic matter.

A subsample of 0.1–1 g of leaf material was used for soluble sugar and starch extraction. For each 0.1 g of sample, 1 mL of deionized water was added and the mixture was ground in a chilled mortar and pestle. The resulting extract was kept at 0°C for 20 min before centrifugation at 12 000 **g** for 10 min. The supernatant containing the soluble fraction was then boiled for 3 min and centrifuged again as already described (Duranceau *et al.*, 1999). The water soluble fraction was then mixed with Dowex-50 (H^+) and Dowex-1 (Cl^-) resins in sequence to remove amino acids and organic acids, respectively. The eluate has been shown to have a carbon isotope composition representative of leaf soluble sugars (Brugnoli *et al.*, 1988). The pellets were washed in ethanol (80% v:v) at 80°C to eliminate chlorophyll and then suspended twice in 6 mol/L HCl at 5°C (1 h each) to solubilize the starch. After adding methanol (4× by volume), the supernanent was kept at 5°C overnight and starch precipited was desiccated in a freeze dryer (Damesin & Lelarge, 2003, with a few modifications). Proteins were extracted by boiling the supernanent of grounded leaf tissue (in 2% NaCl; 10 000 **g** 15 min) for 30 min (Jacobson *et al.*, 1970). The precipitant was dried overnight in a desiccator at room temperature. All products from these extractions were kept at −20°C until carbon isotope analyses were performed. According to the references mentioned above, fractionation of carbon isotopes did not occur during the extraction processes.

Carbon isotope analysis

The carbon isotope ratios in delta notation were expressed as $\delta^{13}C$ (‰) = $[R_{sample}/R_{standard} - 1] \times 1000$, where R is the molar ratio of $^{13}C/^{12}C$. $\delta^{13}C_R$ was measured in an Isochrom isotope-ratio mass spectrometer (Fison Instrument Inc., Manchester, UK) at Biosphere 2 Center (B2C). $\delta^{13}C$ of the leaf organic components was analyzed either at B2C or with an Europa 20/20 continuous flow (CF) isotope ratio mass spectrometer (IRMS) coupled with an ANCA NT combustion system at Lamont-Doherty Earth Observatory (PDZ-Europa, Cheshire, UK). NIST sucrose was used as the standard for intermachine calibration. All $\delta^{13}C$ values are expressed relative to Pee Dee Belemnite (PDB).

The mixing model of Keeling (1958, 1961) was used to calculate the isotope ratio of CO_2 respired by a leaf:

$$\delta^{13}C_{cham} = [CO_{2\,atm}] \times (\delta^{13}C_{atm} - \delta^{13}C_R)/[CO_{2\,cham}] + \delta^{13}C_R$$

where [CO_2] is the concentration of CO_2 and δ is the stable isotope ratio of CO_2. The subscripts cham, atm and R represent the air within the chamber, the air in experimental atmosphere and respiratory CO_2, respectively. Geometric mean regressions were used to establish the linear relationship between $\delta^{13}C_{cham}$ and $1/[CO_{2\,cham}]$ (Pataki *et al.*, 2003) and the intercept at the *Y* axis is the $\delta^{13}C$ value of leaf respiratory CO_2 (Fig. 1b).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to test the species effects on the possible differences of $\delta^{13}C$ between the respiratory CO_2 and leaf organic components. Effects were

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considered to be significant at the 0.05 probability level. In addition, A student's *t*-test was used for multiple comparisons among *P. deltioides* grown in three CO_2 concentrations to evaluate the effects of CO_2 treatments.

Linear regressions were used to analyze the relationship between $\delta^{13}C_R$ and $\delta^{13}C$ of the leaf organic components of all samples or averages of each species/treatment combination. Data from similar leaf-level studies (Duranceau *et al.*, 1999, 2001; Ghashghaie *et al.*, 2001; Tcherkez *et al.*, 2003), which had three C_3 crop species in three sets of environmental or genetic treatments, were included in the regression. We assume that the average $\delta^{13}C$ of all sugars and water soluble materials (soluble sugar and organic acids) analyzed in those studies are equivalent to the 'soluble sugar' and 'water soluble fraction' in our study.

Results

The isotopic signatures of the measured pools exhibited a similar pattern for all five species and the three CO_2 treatments for *P. Deltoides*. In each case, leaf $\delta^{13}C_R$ was the most positive, followed by the $\delta^{13}C$ of starch (except *M. paradisiacal*), while the $\delta^{13}C$ of the bulk organic matter was the lightest (Fig. 2). The amount of ^{13}C-enrichment in leaf $\delta^{13}C_R$ was 3.5–5.9‰ relative to soluble sugar (Table 1), the assumed major substrate for dark respiration. Compared with the water soluble fraction, starch, and bulk organic matter, the amount of ^{13}C enrichment in respiratory CO_2 was 2.7–5.2‰, 1.4–4.2‰, and 4.1–6.9‰, respectively, depending on species (Table 1). During the sampling period, leaves released < 0.001 g carbon, which should not

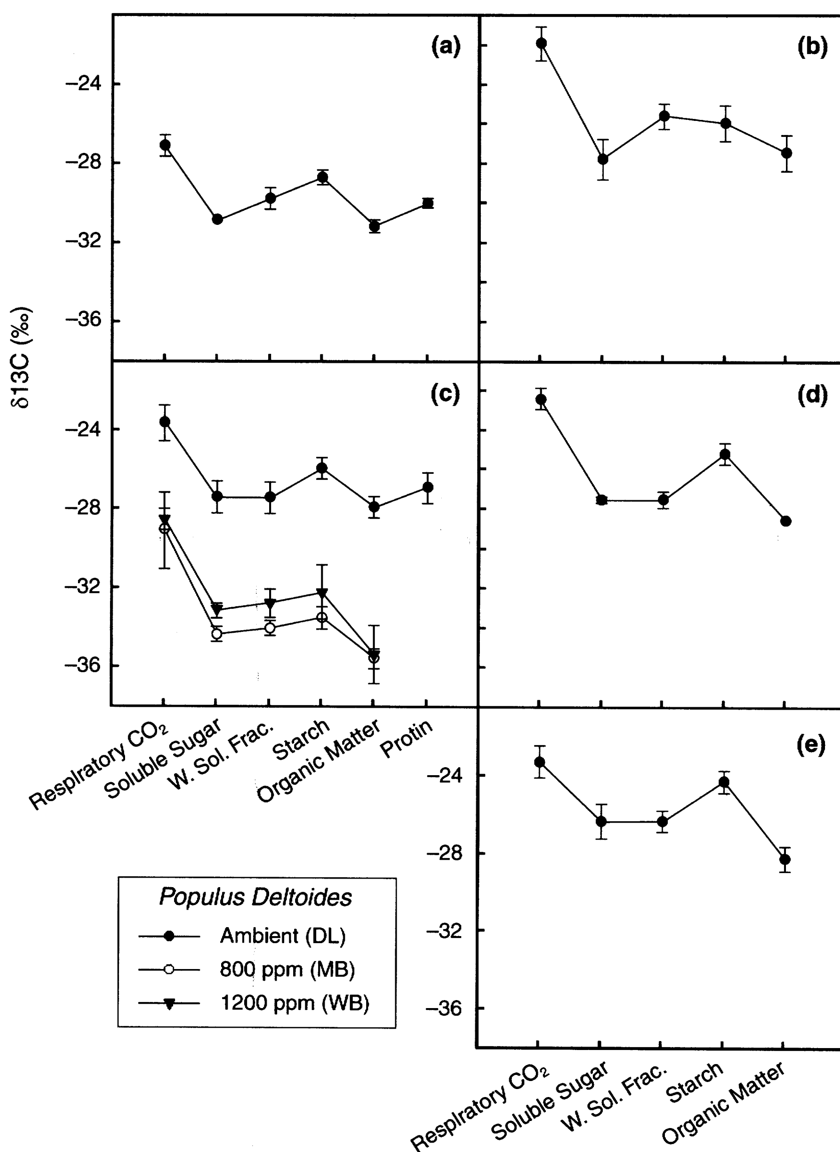

Fig. 2 Leaf $\delta^{13}C_R$ and leaf organic components in five species; (a) *Epipremumn pinnatu* (b) *Musa paradisiacal* (c) *Populus deltoides* Bartr (in 3 growth $[CO_2]$) (d) *Coffea arabica* (e) *Clitoria racemosa*. Values are mean ± SEM among leaves (n = 3–6).

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Table 1 The amount of ^{13}C enrichment in respiratory CO_2 (‰) in comparison with leaf organic components, shown by mean ± SEM (n = 3–6).

Species	Soluble Sugar	Water Soluble Fraction	Starch	Protein	Organic Matter
P. deltoides Bartr. (Ambient CO_2)	3.83 ± 0.37	3.81 ± 0.52	2.32 ± 0.37	3.30 ± 0.98	4.27 ± 0.53
P. deltoides Bartr. (800 p.p.m.)	5.24 ± 1.68	4.90 ± 1.73	4.39 ± 1.37	–	6.46 ± 1.73
P. deltoides Bartr. (1200 p.p.m.)	4.62 ± 0.86	4.25 ± 1.24	3.70 ± 1.80	–	6.83 ± 1.8
M. paradisiaca	5.86 ± 0.16	3.71 ± 0.29	4.06 ± 0.46	–	5.55 ± 0.47
C. arabica	5.12 ± 0.67	5.11 ± 0.68	2.77 ± 0.52	–	6.14 ± 0.57
E. pinnatum	3.74 ± 0.51	2.67 ± 0.16	1.91 ± 0.83	2.88 ± 0.33	4.05 ± 0.55
C. racemosa	3.40 ± 0.39	3.49 ± 0.46	1.28 ± 0.24	–	5.16 ± 0.36
ANOVA of Species Effect	P = 0.008	P = 0.049	P = 0.020	–	P = 0.066

One way ANOVA results of the species effect of ambient grown plants are listed in the last row (P < 0.05 are considered significant). In *P. deltoides*, ^{13}C enrichnments in respiratory CO_2, relative to all organic components are not significantly influenced by the growth $[CO_2]$ (multicomparison with student's t-tests, P = 0.10–0.87).

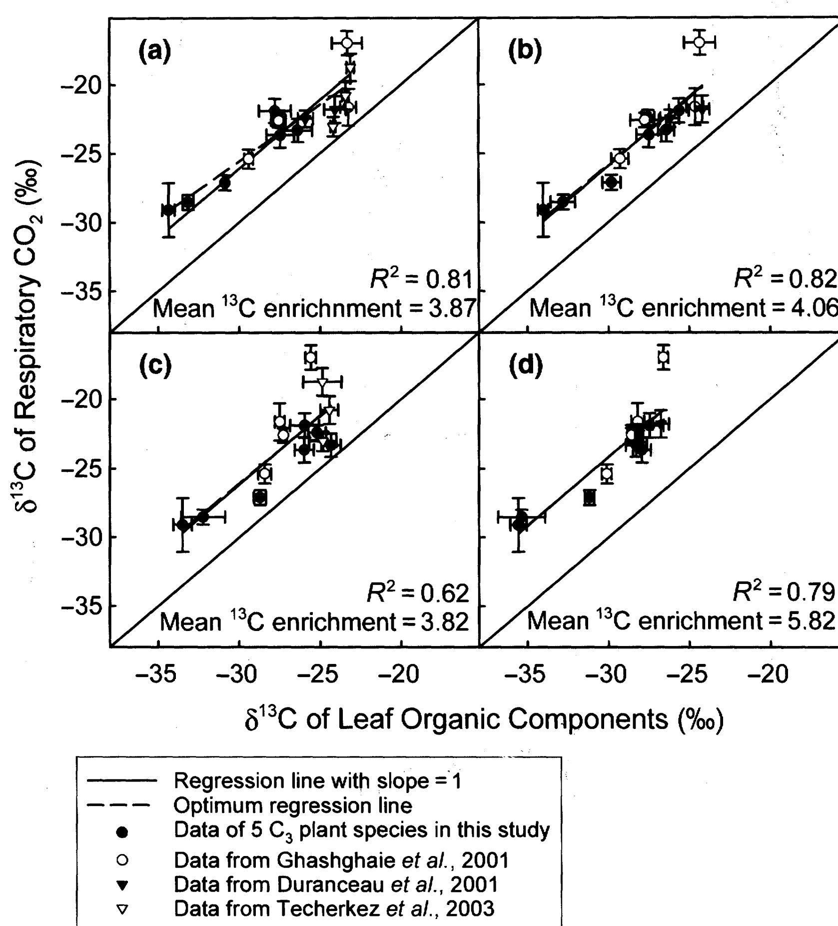

Fig. 3 Correlation between leaf $\delta^{13}C_R$ (y) and $\delta^{13}C$ of leaf organic components (a, soluble sugar; b, water soluble fraction; c, starch; d, organic matter). Data from previous similar studies at leaf scale are also included. R^2 of the regression line (in dashes) and the mean of ^{13}C-enrichment are shown. For all organic components compared, the slopes of linear regression are close to 1 (solid line, P = 0.08–0.96). Each point represents a data set from a single species or treatment, shown as mean ± SEM among leaves.

influence the $\delta^{13}C$ of remaining organic component pools significantly.

The amount of ^{13}C enrichment in respiratory CO_2 relative to soluble sugar, water soluble fraction and starch each had a significant species effect (Table 1). This effect was apparent but not so significant in the bulk leaf organic matter (P = 0.066). The effect of CO_2 treatment in *P. deltoides* was not significant. Although leaf respiratory ^{13}C-enrichment in the DL was smaller than that in the IFB in average, the differences were not statistically significant (Table 1), partly due to the large variation of leaf $\delta^{13}C_R$ in the MB and WB of the IFB.

The correlation between leaf $\delta^{13}C_R$ and $\delta^{13}C$ of the leaf organic components was highly significant (P < 0.01). On average across all species and treatments, the leaf respiratory CO_2 was 3.8‰ to 5.8‰ more positive than the four leaf organic components (Fig. 3) and in all cases, the slope of

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regression line was close to 1 (*F*-test to compare actual slope and 1, $P = 0.08–0.98$).

Discussion

Our results obtained with the leaf scale Keeling plot method are comparable to previous studies using a CO_2 free respiration chamber (Park & Epstein, 1961; Jacobson *et al.*, 1970; Smith, 1971; Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Damesin & Lelarge, 2003; Tcherkez *et al.*, 2003). O'Leary (1981) pointed out that the CO_2 free environment might influence stomatal conductance and the extent of anapleurotic respiratory CO_2 refixation, but the effect on leaf $\delta^{13}C_R$ has not been experimental evaluated. The 'leaf Keeling plot' approach maintains the leaf under CO_2 concentrations closer to their initial growth conditions (not more than 150 p.p.m. above background level, which is higher than the natural atmosphere in our study because of the plant and soil respiration in the glasshouse). In addition, the small air sampling flask (100cc) used is signficantly more convenient for field measurement in remote areas. However, because leaf $\delta^{13}C_R$ of C_4 plant is similar to surrounding ambient CO_2 (−7‰ to −15‰ vs. −8‰), the change in the $\delta^{13}C$ within the respiration chamber CO_2 is not large enough (Pataki *et al.*, 2003) to use the leaf-scale Keeling plot method with C_4 plants directly (data not shown).

In all five C_3 species we studied, respiratory CO_2 was more ^{13}C-enriched than the leaf organic components. Compared with soluble sugar, leaf $\delta^{13}C_R$ was 3.5‰ to 5.9‰ more positive, which is consistent with the observation of Ghashghaie *et al.* (2001) and Duranceau *et al.* (1999, 2001) in crop plants. Therefore, the results led us to reject hypothesis (1) and conclude that a 3‰ to 6‰ ^{13}C-enrichment relative to major respiratory substrate is widespread in leaf respiratory CO_2 from C_3 plants. The statistical significance found in the species is a basis for rejecting hypothesis (2) as well. Interestingly, in *P. Detoides*, the growth CO_2 concentration did not influence the pattern of ^{13}C enrichment to a signifcant extent, supporting our hypothesis (3). We attribute the large variation in the amount of ^{13}C enrichment in 800 p.p.m. and 1200 p.p.m. CO_2 concentration to the variable CO_2 environment in the IFB, which had diurnal $[CO_2]$ fluctuation of up to 300 p.p.m. and varible tank CO_2 injections. The variable background air CO_2 isotope signature could increase the isotopic heterogeneity in the substrate pool and ultimately, the variation in leaf $\delta^{13}C_R$.

Tcherkez *et al.* (2003) concluded that leaf $\delta^{13}C_R$ in C_3 plants is determined by (1) the carbon source used for respiration (2) possible isotope effects of respiratory enzymes, and (3) non-statistical distribution of ^{13}C in glucose. It is difficult to justify that an isotopically heavier respiratory substrate was used to any significant extent in addition to the pools measured here (particularly in light of the good correlations between putative substrates and $\delta^{13}C_R$; Fig. 3). Also, previous studies on mesophyll protoplasts (Lin & Ehleringer, 1997) indicated that fractionation likely does not occur in the main stream of respiratory enzyme reactions (glycolysis and TCA cycle). Instead, our synthesis of current results suggest that the non-statistical distribution of ^{13}C in sugars (Rossmann *et al.*, 1991) is the most reasonable explanation for ^{13}C-enriched respiratory CO_2. In dark respiration, the C-3 and C-4 of carbon atoms of glucose are ^{13}C-enriched (−20.9‰ in average), and are released early in glycolysis. The other 4 carbon atoms are isotopically lighter (−27.1‰ in average) and can enter secondary metabolisms through the TCA cycle. Thus a greater contribution of the C-3 and C-4 carbon atoms to respired CO_2 would result in the isotopically heavier $\delta^{13}C_R$ we observed. Based on the results of Rossmann *et al.* (1991), we estimate that a 3‰ enrichment in ^{13}C of respiratory CO_2 requires that 82% of the respired carbon be drived from C-3 and C-4. Although this number is possible, it is critical to conduct a more complete carbon budget for the entire leaf in future studies. Experiments similar to those of Lin and Ehleringer (1997), culturing protoplast in substrates labeled with ^{13}C on certain carbon atoms, may directly illustrate the origin of respiratory CO_2.

The concept of discrimination, defined as $\Delta = (\delta_{source} - \delta_{product})/\delta_{source} - 1$, applies to reactions with distinguishable source and product. However, in dark respiration, diverse substrates can be oxidized and a variety of compounds can be produced. Generation of CO_2 is only one branch of the overall metabolic network and it is not clear how an enzyme isotope effect (e.g. pyruvate dehydrogenase, Deniro & Epstein, 1977) may have influence the overall $\delta^{13}C_R$. Therefore we suggest that more suitable terminology is needed the ^{13}C enrichment of leaf respiratory CO_2 in C_3 plants.

The strong correlation between leaf $\delta^{13}C_R$ and $\delta^{13}C$ of leaf organic components suggest that in C_3 plants the amount of ^{13}C enrichment is limited within a narrow range based on the similar mechanism involved. Therefore, it is possible to scale up leaf level results to predict leaf $\delta^{13}C_R$ at the ecosystem level. For example, the average ^{13}C-enrichment in leaf $\delta^{13}C_R$ relative to bulk organic matter (5.8‰, Table 1) can be used to approximate a whole C_3 canopy. Obviously, a rigorous estimate of ecosystem scale effects would require more species specific data, as well as the effect of environmental factors (temperature, moisture, etc.) on the amount of ^{13}C enrichment in respiratory CO_2.

Synthesizing the currently available data, we found that significant inconsistency occurs in the results between the leaf and ecosystem levels. In most cases, $\delta^{13}C$ of soil respiration, a much larger component than foliar respiration (Law *et al.*, 2001), is more positive than ecosystem respiration, indicating relatively ^{13}C-depleted vegetation $\delta^{13}C_R$. The scale difference may originate from the fact that, leaf respiration has fairly homogenous substrates and generates CO_2 relatively 'instantaneously'; while at the ecosystem level, photo-assimilated carbon is released over much longer timescales as a 'lagged

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and prolonged' flux, which reflect more hetreogenous pools. Clearly, caution must be taken when predicting vegetation $\delta^{13}C_R$ by scaling up leaf level results. At the leaf scale, further studies are required to understand (1) long-term patterns (ontogenetic, seasonal and annual) of leaf $\delta^{13}C_R$ (2) the contribution and (3) representativeness of leaf respiration to total plant respiration. Alternatively, direct comparisons of $\delta^{13}C$ of assimilated and respired CO_2 at the ecosystem level could also provide critical information to create more robust models.

Acknowledgements

We thank Joost van Haren and Sara Green for technical assistance with the isotopic measurements. We also acknowledge Joost van Haren's effort in testing the protocol of leaf scale Keeling plot. Josslyn B. Shapiro and Dr O. R. Anderson are thanked for helpful comments on an earlier version of this manuscript. This work was supported in part by Biosphere 2 Center of Columbia University and by a grant from the Packard Foundation (DLP 998306 to G.L. & K.L.G.). G.L. was also supported in part by the 'Bairen Project' program of the Chinese Academy of Sciences.

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