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Viewpoint:

Why are non-photosynthetic tissues generally ^{13}C enriched compared with leaves in C_3 plants? Review and synthesis of current hypotheses

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Abstract. Non-photosynthetic, or heterotrophic, tissues in C_3 plants tend to be enriched in ^{13}C compared with the leaves that supply them with photosynthate. This isotopic pattern has been observed for woody stems, roots, seeds and fruits, emerging leaves, and parasitic plants incapable of net CO_2 fixation. Unlike in C_3 plants, roots of herbaceous C_4 plants are generally not ^{13}C-enriched compared with leaves. We review six hypotheses aimed at explaining this isotopic pattern in C_3 plants: (1) variation in biochemical composition of heterotrophic tissues compared with leaves; (2) seasonal separation of growth of leaves and heterotrophic tissues, with corresponding variation in photosynthetic discrimination against ^{13}C; (3) differential use of day *v.* night sucrose between leaves and sink tissues, with day sucrose being relatively ^{13}C-depleted and night sucrose ^{13}C-enriched; (4) isotopic fractionation during dark respiration; (5) carbon fixation by PEP carboxylase; and (6) developmental variation in photosynthetic discrimination against ^{13}C during leaf expansion. Although hypotheses (1) and (2) may contribute to the general pattern, they cannot explain all observations. Some evidence exists in support of hypotheses (3) through to (6), although for hypothesis (6) it is largely circumstantial. Hypothesis (3) provides a promising avenue for future research. Direct tests of these hypotheses should be carried out to provide insight into the mechanisms causing within-plant variation in carbon isotope composition.

Additional keywords: diel cycle, heterotrophic tissue, PEP-carboxylase, refixation, respiration.

Introduction

Seventy years have passed since the first report of significant variation in nature of the relative abundance of the stable carbon isotopes, ^{12}C and ^{13}C (Nier and Gulbransen 1939). Such variation was subsequently reported to exist among plant taxa, and among plants growing in different environments (Wickman 1952). In the time since these early reports, analysis of stable carbon isotope ratios has provided many valuable insights into plant physiology. One of the first applications of $^{13}C/^{12}C$ measurements in this context was to aid identification of species using different photosynthetic pathways (C_3, C_4, and crassulacean acid metabolism) (Bender 1968). It is now well established that the $^{13}C/^{12}C$ ratio of organic

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material in plants using the C_3 photosynthetic pathway records information about the intercellular CO_2 concentration during photosynthesis (Farquhar *et al*. 1982). More recently, $^{13}C/^{12}C$ ratios in terrestrial plants and atmospheric CO_2 have been used to constrain carbon cycle models at both ecosystem (e.g. Lloyd *et al*. 1996) and global scales (e.g. Francey *et al*. 1995). Numerous other applications have been developed, and carbon isotope analysis is rapidly becoming a standard tool in environmental plant science.

Carbon isotope ratios are expressed relative to an international standard, using delta notation:

$$\delta_{Sample} = \frac{R_{Sample} - R_{Std}}{R_{Std}}, \quad (1)$$

where δ_{Sample} is defined by this relationship, R_{Sample} is the $^{13}C/^{12}C$ ratio of the sample, and R_{Std} is the $^{13}C/^{12}C$ ratio of the standard. The δ_{Sample} is typically multiplied by 1000 to avoid working with very small numbers; it is, thus, expressed as parts per thousand (‰). The internationally accepted standard against which delta values are expressed is CO_2 prepared from a Cretaceous belemnite from the PeeDee formation in South Carolina, USA. This standard was reported to have a $^{13}C/^{12}C$ ratio of 0.0112372 (Craig 1957), and is referred to as PDB.

Despite tremendous progress over the past few decades in understanding what causes variation in carbon isotope ratios of terrestrial plants (Dawson *et al*. 2002), there are patterns in nature for which the proposed physiological mechanisms have been little tested. One such pattern is the tendency for heterotrophic, or non-photosynthetic, plant tissues to be enriched in ^{13}C compared with the leaves that supply them with photosynthate. This pattern was first reported by Craig (1953), who observed that the branch wood of several tree species was enriched in ^{13}C compared with leaves. A paired *t*-test of Craig's data shows that the mean $\delta^{13}C$ of branch wood, at –25.8‰, was significantly less negative than that of leaves, at –26.8‰ ($P < 0.01$, $n = 7$). The observation that branch or stem wood is generally enriched by between 1 and 3‰ compared with leaves of the same branch or tree has been repeated many times and for many species since Craig's initial observation (e.g. Leavitt and Long 1982, 1986; Francey *et al*. 1985; Schleser 1992; Yoder *et al*. 1994; Panek and Waring 1997; Guehl *et al*. 1998; Martinelli *et al*. 1998; Pate and Arthur 1998; Miller *et al*. 2001; Cernusak *et al*. 2005). In a compilation of published data for 116 observations of $\delta^{13}C$ in leaves and woody stems, Badeck *et al*. (2005) calculated that woody stems were on average enriched in ^{13}C by 1.9‰ compared with leaves.

Figure 1 shows an example of $\delta^{13}C$ values for branch wood and leaves distal to that section of branch for two temperate tree species sampled in Idaho, USA. Samples were collected at varying heights in the crowns, such that there was a gradient from less negative $\delta^{13}C$ at the tops of the crowns, to more negative $\delta^{13}C$ at the bases of the crowns. These data demonstrate two points: first, that the branch wood in both species is enriched in ^{13}C compared with leaves of the same branch; and second, that the magnitude of the leaf-wood $\delta^{13}C$ difference can vary with crown depth, but appears to do so in a species-specific way. Thus, for *Populus tremuloides* Michx., a deciduous angiosperm tree, the $\delta^{13}C$ difference between leaves and branch wood was largest at the top of the crown and smallest at the base. In contrast, *Pinus monticola* Dougl., an evergreen conifer, showed little variation in the $\delta^{13}C$ difference between leaves and wood with relative crown depth (Fig. 1).

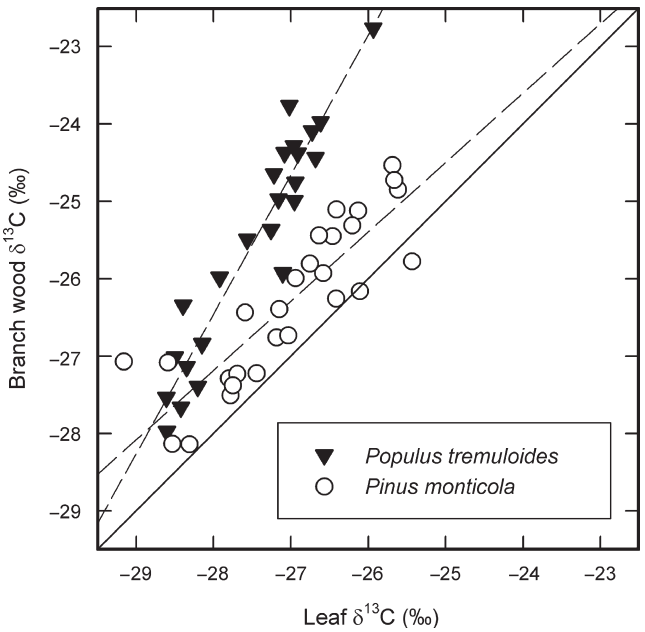

Fig. 1. Carbon isotope composition of branch wood plotted against that of leaves from the same branch for an evergreen (*Pinus monticola*) and a deciduous (*Populus tremuloides*) tree species growing in Idaho, USA. Samples were collected at varying crown depths, with associated variation in $\delta^{13}C$ of both leaves and wood, in 1998 and 1999 (LA Cernusak and JD Marshall, unpubl. data). The solid line is the one-to-one line, and the dashed lines are least-squares regression lines for each species.

Similar to woody stems, roots also tend to be enriched in ^{13}C compared with leaves in C_3 plants (e.g. Park and Epstein 1960; Gebauer and Schulze 1991; Handley *et al*. 1993; Ineson *et al*. 1995; Syvertsen *et al*. 1997; Scartazza *et al*. 1998; Brugnoli and Farquhar 2000; Hobbie and Colpaert 2004). In their compilation of published data, including 78 observations in C_3 plants, Badeck *et al*. (2005) observed that roots were enriched in ^{13}C compared with leaves by an average of 1.1‰. Figure 2 shows $\delta^{13}C$ of leaves, stems, and roots for seedlings of seven C_3 tropical tree species (Cernusak *et al*. 2007). These data again demonstrate that the $\delta^{13}C$ of stems and roots tends to be enriched compared with that of leaves, and that the extent of enrichment varies among species.

Further, roots in C_4 plants tend to show similar or slightly depleted $\delta^{13}C$ compared with leaves of the same plant (Hobbie and Werner 2004). For example, the two tissues showed an average difference of only 0.1‰ for 10 observations (Badeck *et al*. 2005). In the experiment for which data are shown in Fig. 2, *Saccharum spontaneum* L., a C_4 grass, was grown along-side seedlings of seven C_3 tree species (Cernusak *et al*. 2007). In that experiment, root dry matter of the C_4 grass was depleted, rather than enriched, in ^{13}C compared with leaves of the same plant ($P < 0.01$, $n = 7$); mean root $\delta^{13}C$ was –11.8‰ and mean leaf $\delta^{13}C$ was –10.6‰.

In addition to stems and roots, seeds and fruits also tend to be ^{13}C enriched compared with leaves in C_3 plants (e.g. Yoneyama and Ohtani 1983; Farquhar and Richards 1984; Scartazza *et al*. 1998; Yoneyama *et al*. 1998, 2000; Barbour *et al*. 2000;

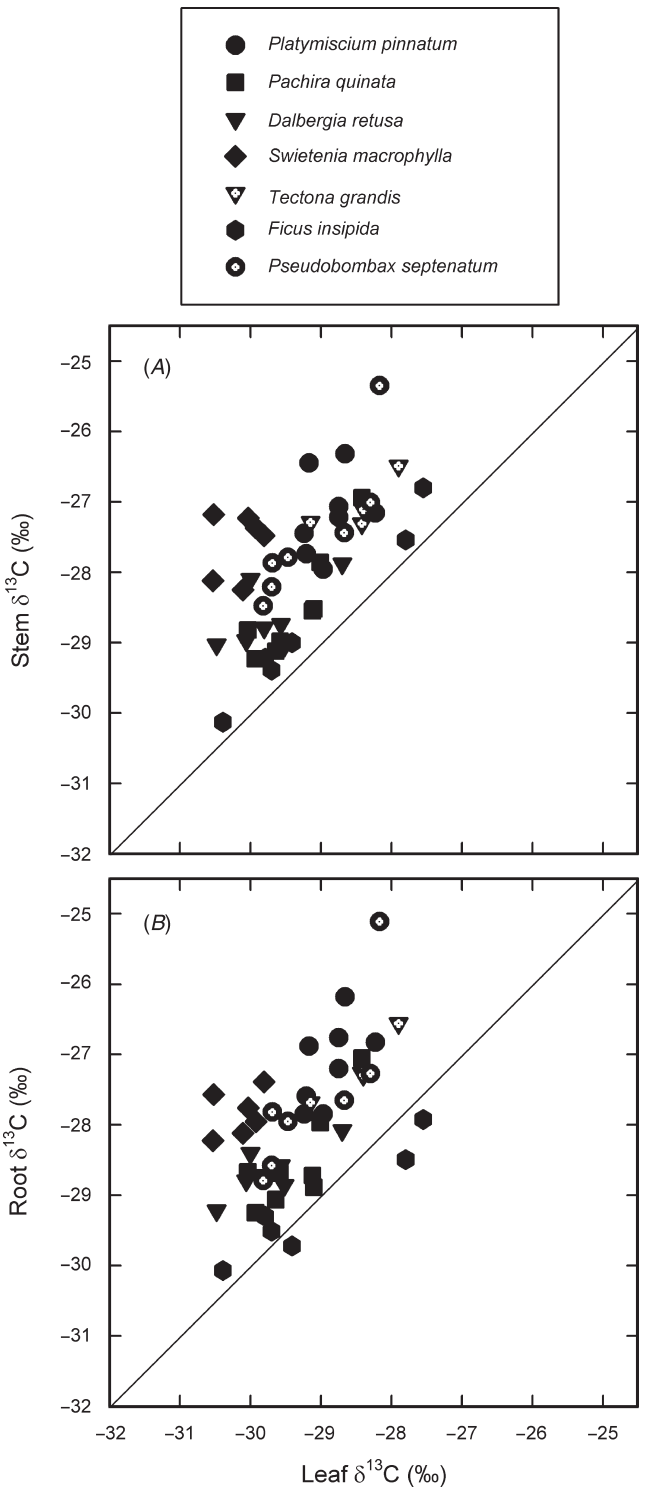

Fig. 2. (*A*) Stem, and (*B*) root $\delta^{13}C$ plotted against leaf $\delta^{13}C$ of the same plant for seedlings of seven tropical tree species grown during the rainy season in the Republic of Panama. Data presented in this figure were originally published by Cernusak *et al*. (2007). Solid lines are one-to-one lines.

Behboudian *et al*. 2000; Brugnoli and Farquhar 2000; Cernusak *et al*. 2002). Few data are available for seeds and fruits for comparison in C_4 plants; however, it would appear that corn kernels are typically enriched by ~1.5‰ compared with corn leaves (Lowdon 1969; Gleixner *et al*. 1993).

Holoparasitic plants, which are heterotrophic plants that derive their carbon parasitically from a photosynthetic host plant, tend to be enriched by between 1.0 and 1.5‰ compared with leaves of their hosts for parasitic angiosperms (e.g. de la Harpe *et al*. 1981; Ziegler 1994; Cernusak *et al*. 2004*b*). The only known parasitic gymnosperm (*Parasitaxus ustus* (Vieill.) de Laub.) is also enriched in ^{13}C compared with leaves of its host, although the mechanism of enrichment may differ from that of parasitic angiosperms (Feild and Brodribb 2005).

Finally, young, emerging leaves of C_3 plants, for which growth may be mostly heterotrophic, tend to be ^{13}C-enriched compared with fully-expanded leaves (e.g. Lowdon and Dyck 1974; Leavitt and Long 1985; Terwilliger 1997; Damesin *et al*. 1998; Le Roux-Swarthout *et al*. 2001*b*; Terwilliger *et al*. 2001; Damesin and Lelarge 2003; Holtum and Winter 2005; Li *et al*. 2007). No data of this type are available for C_4 plants.

A mechanistic understanding of variation in $\delta^{13}C$ between leaves and non-photosynthetic tissues in C_3 plants would be useful for several applications. For example, measurements of leaf dry matter $\delta^{13}C$ are often employed in ecophysiological, agricultural, and silvicultural investigations to indicate variation in the ratio of intercellular to ambient CO_2 partial pressures, p_i/p_a, during photosynthesis. The p_i/p_a in turn correlates with plant water-use efficiency, assuming similar leaf-to-air vapour pressure difference and respiratory carbon use among experimental plants (Farquhar and Richards 1984; Seibt *et al*. 2008). Assuming p_a is known or can be estimated, p_i can be calculated, and the resulting value employed in analyses aimed at predicting optimal stomatal control in relation to leaf area and nitrogen content (Farquhar *et al*. 2002; Wright *et al*. 2003). For both of these applications, it is important to know whether the leaf $\delta^{13}C$ signal or that of heterotrophic tissues provides a better representation of p_i/p_a during photosynthesis. Additionally, any systematic ^{13}C fractionation between leaves and heterotrophic tissues should be included in ecosystem and global carbon cycle models if $\delta^{13}C$ measurements are to be used to constrain carbon fluxes (Bowling *et al*. 2008). Studies of carbon flow through food webs based on $\delta^{13}C$ analyses would also benefit from taking into account systematic $\delta^{13}C$ differences between leaves and heterotrophic tissues, as would tree ring-based studies of historical variation in carbon isotope discrimination (Gessler *et al*. 2008). However, without mechanistic information about what causes $\delta^{13}C$ divergence between leaves and heterotrophic tissues, it is difficult to incorporate such a process effectively into any modelling framework.

To summarise, ^{13}C enrichment of heterotrophic tissues relative to leaves is a widespread phenomenon in C_3 plants. The extent of enrichment can vary depending on tissue, species, and possibly environment. Further, C_4 plants tend to show no enrichment, or even a depletion, of ^{13}C in roots compared with leaves. Because C_3 plants are responsible for ~80% of terrestrial net primary production (Still *et al*. 2003), an understanding of the ^{13}C enrichment of heterotrophic tissues is important for $\delta^{13}C$ applications at plant, ecosystem, and global scales.

In the following sections, we review various hypotheses proposed to explain the ^{13}C enrichment of heterotrophic tissues,

and their supporting evidence. The hypotheses are summarised in Table 1. Figure 3 shows a simplified representation of carbon flow through a C_3 plant, and indicates the position in the flow network at which each hypothesis operates. It should be noted at the outset that these hypotheses are not mutually exclusive, and there may be multiple mechanisms that contribute to the general tendency for heterotrophic tissues to be ^{13}C-enriched compared with leaves. Our intention is that this review should complement other recent reviews relating to this topic (Hobbie and Werner 2004; Badeck *et al*. 2005; Bowling *et al*. 2008) by providing a framework of multiple working hypotheses that can be used to direct new experimental work aimed at unravelling the physiological mechanisms underlying heterotrophic ^{13}C enrichment. We include here two hypotheses that were not covered in the earlier reviews [hypotheses (3) and (6) below]. To set the context for the description of the hypotheses, we begin by briefly reviewing photosynthetic fractionation of carbon isotopes in C_3 and C_4 plants.

Carbon isotope discrimination during photosynthesis

Several authors have developed models to describe carbon isotope fractionation during C_3 photosynthesis (Vogel 1980; O'Leary 1981; Farquhar *et al*. 1982). The most widely applied of these defines discrimination against ^{13}C (Δ) as follows (Farquhar *et al*. 1982; Farquhar and Richards 1984; Hubick *et al*. 1986):

$$\Delta = a - d + (b - a)\frac{p_i}{p_a}, \qquad (2)$$

Table 1. Summary of the six hypotheses proposed to explain ^{13}C enrichment of heterotrophic tissues compared with leaves

Hypothesis	Description	Evidence in favour	Evidence opposed	Testable prediction
1	Variation in biochemical constituents between leaves and heterotrophic tissues accounts for $\delta^{13}C$ differences	Different biochemical fractions show consistent offsets in $\delta^{13}C$ (e.g. cellulose is ^{13}C-enriched, lignin is ^{13}C-depleted)	A single constituent, cellulose, showed $\delta^{13}C$ differences between organs	Leaves have larger concentrations of compounds relatively depleted in ^{13}C than heterotrophic tissues, or *vice versa*
2	Seasonal separation of growth of leaves *v.* heterotrophic tissues is associated with shifts in ^{13}C discrimination of photosynthesis	In many ecosystems leaf, stem, and root growth occur at different times and under different environmental conditions	In experiments with simultaneous leaf, stem, and root growth, associated $\delta^{13}C$ variations were still observed	New leaves and heterotrophic tissues faithfully record the $\delta^{13}C$ of carbon delivered to them in phloem sap, and phloem sap $\delta^{13}C$ faithfully reflects photosynthetic ^{13}C discrimination
3	Diel variation in $\delta^{13}C$ of growth substrate (sucrose) is coupled to diel patterns in growth rate; sucrose is ^{13}C-enriched at night and ^{13}C-depleted during the day	Diel variation in $\delta^{13}C$ of phloem sap sucrose was observed; contrasting diel growth patterns between leaves and heterotrophic tissues were also observed	Species that apparently do not show contrasting diel patterns in growth between leaves and heterotrophic tissues still show $\delta^{13}C$ differences between organs	(1) Blocking export of carbon from a leaf increases leaf $\delta^{13}C$ at a given photosynthetic ^{13}C discrimination; (2) non-starch-producing plants show no $\delta^{13}C$ difference between leaves and heterotrophic tissues
4	Fractionation during dark respiration causes ^{13}C depletion of leaves and/or ^{13}C enrichment of heterotrophic tissues	Dark respired CO_2 in leaves is typically ^{13}C-enriched compared with leaf organic material, and root-respired CO_2 tends to be ^{13}C-depleted	Dark respiration from woody tissues also tends to be ^{13}C-enriched compared with organic material	Environmental or genetic variation altering the $\delta^{13}C$ difference between respiratory CO_2 and organic material alters the $\delta^{13}C$ difference between leaves and heterotrophic tissues
5	Heterotrophic tissues have a proportionally larger production and retention of PEP carboxylase-derived organic molecules than leaves	Carbon fixed by PEP carboxylase is ^{13}C-enriched compared with source CO_2; PEP carboxylase activity was higher in heterotrophic tissues than in leaves	Some sink tissues have shown similar $\delta^{13}C$ to carbon delivered to them in phloem sap, suggesting a very modest input from PEP carboxylase	Fluxome analyses suggest that PEP carboxylase-derived compounds make a larger contribution to biosynthetic processes in heterotrophic tissues than in leaves
6	Developmental variation in photosynthetic discrimination against ^{13}C results in accumulation of ^{13}C-depleted organic material during expansion of young leaves	Higher values of p_i/p_a were observed in expanding leaves compared with mature leaves	Evidence also suggests a ^{13}C enriching mechanism in roots	Photosynthetic discrimination against ^{13}C is higher in expanding than mature leaves, and the amount of carbon fixed under elevated discrimination is sufficient to affect $\delta^{13}C$ of total leaf carbon

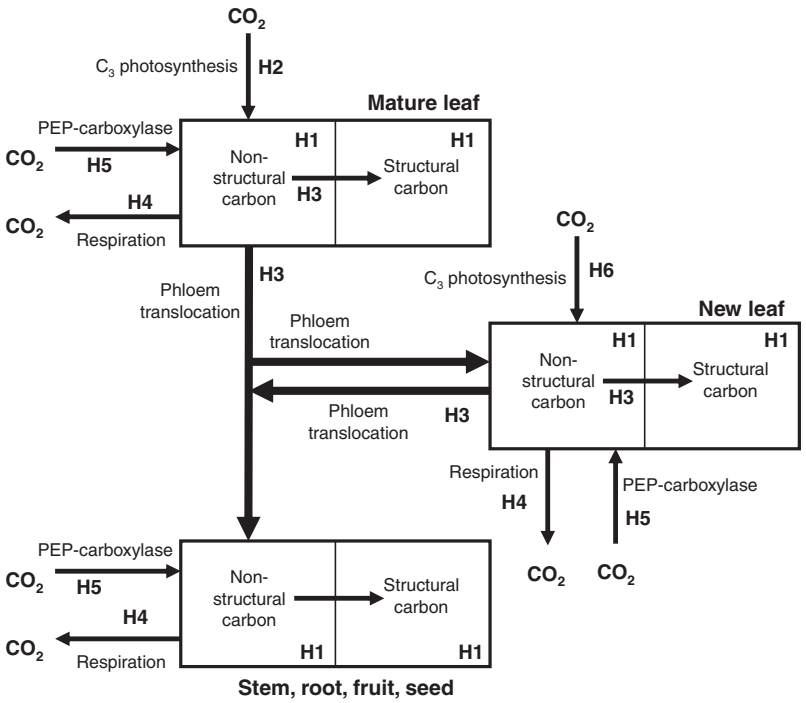

Fig. 3. A simplified representation of carbon flow through a C_3 plant. The abbreviations H1 through H6 refer to hypotheses (1) through to (6), described briefly below, and in more detail in Table 1 and in the text. These hypotheses aim to provide a mechanistic explanation for why heterotrophic plant tissues tend to be ^{13}C-enriched compared with leaves. The position of each abbreviation shows where in the carbon flow pathway each hypothetical mechanism operates. Hypothesis summaries are as follows: H1, variable biochemical composition; H2, seasonal separation of growth; H3, day *v.* night sucrose utilisation; H4, respiratory fractionation; H5, carbon fixation by PEP carboxylase; and H6, developmental variation in photosynthetic discrimination against ^{13}C.

where a is the discrimination against ^{13}C during diffusion of CO_2 through stomatal pores (4.4‰), b is the discrimination against ^{13}C associated with carboxylation by Rubisco (~29‰), p_i/p_a is the ratio of intercellular to ambient CO_2 partial pressures, and d is a composite term that takes into account discriminations associated with CO_2 transfer in the boundary layer, dissolution of CO_2 and liquid-phase diffusion, photorespiration, and dark respiration (Farquhar *et al*. 1989). In some applications, the term d is omitted from Eqn (2), in which case a lower value for b is typically employed (~27‰) to account for the decrease in Δ associated with the various fractionation processes summarised by d. The discrimination, Δ, is defined with respect to CO_2 in air as

$$\Delta = \frac{R_a - R_p}{R_p}, \qquad (3)$$

where R_a is the $^{13}C/^{12}C$ ratio of CO_2 in air, and R_p is that of carbon taken up by the plant during photosynthesis. The capital delta (Δ) in Eqn (3) relates to the lower case delta in Eqn (1) as

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p}, \qquad (4)$$

where δ_a is the $\delta^{13}C$ of CO_2 in air, and δ_p is that of carbon taken up by the plant during photosynthesis.

Several models have also been proposed to describe Δ during C_4 photosynthesis (Reibach and Benedict 1977; Hattersley 1982; Peisker 1982; Deléens *et al*. 1983; Farquhar 1983; Peisker and Henderson 1992). We review here the expression derived by Farquhar (1983):

$$\Delta = a + (b_4 + b_3\varphi - s\varphi - a)\frac{p_i}{p_a}, \qquad (5)$$

where b_4 and b_3 are fractionations associated with carboxylation by phosphoenolpyruvate (PEP) carboxylase (–5.7‰) and Rubisco (~29‰), respectively, ϕ is the proportion of CO_2 released by decarboxylation that subsequently leaks out of the bundle sheath, and s is the fractionation associated with this leakage, which may be near 1.8‰ (Farquhar 1983; Henderson *et al*. 1992). This Δ model for C_4 photosynthesis is also simplified, in that fractionations caused by CO_2 transfer in the boundary layer and between the intercellular air spaces and the mesophyll cells have been omitted (Farquhar 1983).

The different processes of C_3 and C_4 photosynthesis, thus, have differing discriminations against ^{13}C associated with them. This results in large differences in $\delta^{13}C$ of organic material associated with C_3 and C_4 plants when grown under conditions of similar δ_a. This provided the original basis for using $\delta^{13}C$ measurements to identify C_4 *v.* C_3 plants. In addition to the large difference in Δ between C_3 and C_4 plants, the two also

show very different dependencies of Δ on p_i/p_a. For C_3 photosynthesis, the $\Delta - p_i/p_a$ relationship typically has a slope ranging from ~20 to 25‰, indicating an increase in Δ of 2.0‰ to 2.5‰ for each 0.1 increase in p_i/p_a. If Eqn (2) is fitted to experimental data (without d), this corresponds to predicted b values ranging from ~24 to 29‰ (Farquhar *et al*. 1989; Brugnoli and Farquhar 2000). In contrast, the Δ for C_4 plants typically shows a weakly negative relationship with p_i/p_a, with slopes ranging from ~0 to –5‰, indicating a decrease in Δ of 0.0 to 0.5‰ for each 0.1 increase in p_i/p_a (Farquhar *et al*. 1989; Brugnoli and Farquhar 2000).

Hypotheses to explain ^{13}C enrichment in heterotrophic tissues

Hypothesis 1: Variation in biochemical composition

One possible explanation for the widespread difference in $\delta^{13}C$ between leaves and heterotrophic tissues of the same plant is that the two differ in biochemical composition (Table 1; Fig. 3). Different biosynthetic pathways can result in variation in the carbon isotope composition of their respective end products (Gleixner and Schmidt 1997; Hobbie and Werner 2004). As a result, some biochemical constituents of plant dry matter show consistent offsets in $\delta^{13}C$. For example, lipids (Park and Epstein 1961) and lignin (Wilson and Grinsted 1977) are generally depleted in ^{13}C compared with whole organic material by 3 to 8‰, and 1 to 4‰, respectively; however, cellulose tends to be enriched in ^{13}C compared with whole organic material by 1 to 2‰ (Olsson *et al*. 1972; Leavitt and Long 1982; Badeck *et al*. 2005). Thus, if leaves and non-photosynthetic organs differed consistently in their biochemical composition, this might explain the widespread difference in $\delta^{13}C$ of whole tissues.

This hypothesis is based on two parts: first, that individual biochemical constituents, such as lignin and cellulose for example, maintain the same isotopic composition across different tissue types; and second, that the relative amounts of these isotopically distinct constituents vary among tissue types. For example, this hypothesis would be supported if lignin from both leaves and roots had the same $\delta^{13}C$, which was in turn isotopically depleted compared with whole tissue, and leaves had a larger concentration of lignin than roots. Thus, one test of this hypothesis is to analyse $\delta^{13}C$ of a single biochemical constituent of both leaves and heterotrophic tissues. Such analyses have most commonly focussed on cellulose, a primary constituent of plant organic material. Leavitt and Long (1982) analysed $\delta^{13}C$ of cellulose extracted from both leaves and wood of juniper trees, and found that the leaf/wood $\delta^{13}C$ difference for cellulose generally mirrored that observed for whole tissues. Similar results have been observed for cellulose in leaves and wood of other tree species (Francey *et al*. 1985; Panek and Waring 1997; Guehl *et al*. 1998), and for cellulose in emerging *v.* fully-expanded leaves (Terwilliger *et al*. 2001).

Thus, available evidence does not support the first part of this hypothesis, because the observations cited above demonstrate a divergence in $\delta^{13}C$ between leaves and non-photosynthetic organs for a single biochemical constituent. Whereas variation in biochemical composition of different tissues can potentially result in $\delta^{13}C$ differences, the observation that a single biochemical constituent differs consistently in $\delta^{13}C$ between leaves and heterotrophic tissues suggests that there is an additional process or processes at work. Furthermore, Badeck *et al*. (2005) noted that variation in $\delta^{13}C$ among biochemical constituents in and of itself cannot cause divergence of whole-tissue $\delta^{13}C$, so long as total mass is conserved for each tissue. For example, if a particular biochemical component is ^{13}C-depleted due to $^{13}C/^{12}C$ fractionating steps associated with its biosynthetic pathway, there will necessarily be a simultaneous ^{13}C enrichment of the other compounds derived from the precursor molecules. Thus, a net addition or loss of carbon with $\delta^{13}C$ different from the average for the whole tissue must occur in order for variation in whole-tissue $\delta^{13}C$ to be associated with variation in biochemical composition. We note, however, that such addition or loss need not necessarily occur as a result of carboxylating or decarboxylating reactions. Many other processes could potentially cause selective loss or addition of compounds with $\delta^{13}C$ different from the whole-tissue mean. Some examples are fine root turnover, litter fall, abrasion of leaf waxes, organic acid efflux from roots, emission of volatile organic compounds, sugar secretions from extra-floral nectaries, and uptake of amino acids from the soil.

Hypothesis 2: Seasonal separation of growth

A second possible explanation for the widespread enrichment of ^{13}C in heterotrophic tissues compared with leaves is that carbon used for synthesis of the two takes place at different times during the growing season, with corresponding differences in photosynthetic discrimination against ^{13}C (Table 1; Fig. 3). According to this hypothesis, growth of heterotrophic tissues in C_3 plants would take place at a time of lesser photosynthetic discrimination against ^{13}C than growth of new leaves; the variation in Δ would result from environmental constraints on p_i/p_a, such as increased soil water deficit or atmospheric vapour pressure deficit. Examples of such a pattern would be for seasonally ephemeral plant communities (Smedley *et al*. 1991) or cereal crops in temperate climates, where vegetative growth would take place in the spring, and storage organ or grain filling would take place later in the summer while soil moisture reserves were being exhausted. The Δ during vegetative growth would then be higher than during storage organ or grain filling phases due to the seasonal progression of drought stress.

There is some circumstantial support for this hypothesis for plants growing in seasonal environments. For example, seeds of *Lupinus angustifolius* L. plants growing in south-western Australia were observed to be ^{13}C-enriched by ~3‰ compared with leaves; however, the seed $\delta^{13}C$ closely matched that of phloem sap carbon sampled concurrently (Cernusak *et al*. 2002). This suggested that the seeds were faithfully recording the carbon isotope ratio of carbon delivered to them in phloem sap in the later stages of the growing season. A similar situation was observed for plantations of *Eucalyptus globulus* Labill, also growing in south-western Australia. Seasonal variations of ~4‰ were observed in the $\delta^{13}C$ of newly formed stem-wood dry matter. However, these variations could be entirely accounted for by considering corresponding variations in the $\delta^{13}C$ of phloem sap carbon delivered to the growing stems (Pate and Arthur 1998; Cernusak *et al*. 2005), and variation in $\delta^{13}C$ of phloem sap carbon was observed to correlate with variation in p_i/p_a of

canopy leaves in response to drought stress (Cernusak *et al*. 2003). However, these observations can only be considered as partial evidence, because the corollary assumption of the hypothesis, that leaf dry matter formed early in the growing season also faithfully recorded the $\delta^{13}C$ of concurrent phloem sap carbon, was not demonstrated.

Moreover, some other observations clearly cannot be explained by the hypothesis of seasonal separation of growth of leaves *v.* heterotrophic tissues. This is the case for the data presented in Fig. 2. In this experiment, seedlings of tropical tree species were grown for three to four months during the rainy season, and were maintained under well watered conditions (Cernusak *et al*. 2007). Initial seedling dry weights were less than 0.5 g, and final dry weights at harvest averaged 65 g. Leaves, stems, and roots grew concurrently throughout the experiment, such that there was no seasonal separation of growth of leaves *v.* heterotrophic tissues.

Hypothesis 3: Day v. *night translocation*

Tcherkez *et al*. (2004) and Gessler *et al*. (2008) have offered a third hypothesis to explain ^{13}C enrichment of heterotrophic plant tissues compared with leaves (Table 1; Fig. 3). The authors posit that a diel rhythm in leaf carbohydrate dynamics results in an isotopic partitioning between carbohydrate that is consumed within the leaf and that which is exported to sink organs, with the latter being preferentially associated with transitory starch degradation. Tcherkez *et al*. (2004) predicted a diel cycle in the $\delta^{13}C$ of phloem sap sucrose, in which carbohydrate exported during the night would be ^{13}C-enriched compared with that exported during the day. Such a diel oscillation would result from alternation between night-time export associated with transitory starch breakdown, producing sucrose relatively enriched in ^{13}C, and daytime export associated with sucrose biosynthesis from triose phosphates, producing sucrose relatively depleted in ^{13}C. Experimental results for *Ricinus communis* L. support the prediction of a day/night cycle (Gessler *et al*. 2008), as do observations of diel variation in $\delta^{13}C$ of twig phloem sap sucrose in *Pinus sylvestris* L. (Brandes *et al*. 2006) and *Eucalyptus delagatensis* R.T. Baker (Gessler *et al*. 2007). This diel cycle may cause divergence between $\delta^{13}C$ of leaves and heterotrophic tissues if sucrose consumption for growth within organs occurs preferentially during the day or night.

Growth processes can also be associated with diel cycles. In several C_3 species, leaf growth was primarily observed during the day (Walter and Schurr 2005), and, thus, may preferentially use relatively ^{13}C-depleted daytime sucrose. In contrast, roots do not show a diel growth cycle (Walter and Schurr 2005), and therefore may incorporate carbon that is relatively ^{13}C-enriched compared with that incorporated by leaves. It has been postulated that woody stem growth occurs mostly at night (Steppe *et al*. 2005; Saveyn *et al*. 2007), and it would, therefore, preferentially consume starch-derived sucrose. As a consequence, wood is expected to be ^{13}C-enriched.

Several recent reports have demonstrated depletion in the $\delta^{13}C$ of soluble sugars extracted from leaves compared with those extracted from stem tissues. For current year stems of *Fagus sylvatica* L., sucrose extracted from mature leaves had a $\delta^{13}C$ ~2‰ more negative than sucrose extracted from stem tissue, suggesting ^{13}C enrichment of exported sucrose relative to leaf sucrose (Damesin and Lelarge 2003). Similarly, $\delta^{13}C$ of sucrose extracted from potato leaves was observed to be ~2‰ more negative than sucrose extracted from tubers (Gleixner *et al*. 1998). Similar offsets in $\delta^{13}C$ of leaf soluble sugars compared with stem phloem sap sugars were also observed in *Fagus sylvatica* (Scartazza *et al*. 2004), *Pinus sylvestris* (Brandes *et al*. 2006), and *Eucalyptus delegatensis* (Gessler *et al*. 2007). These reports would be consistent with the day/night hypothesis if leaves were sampled during the day, such that sucrose extracted from leaves mainly represented that originating from triose phosphates, whereas sucrose extracted from stem phloem sap or heterotrophic tissues represented a mixture of that originating from both day and night export from leaves.

The translocation of carbohydrate from leaves to heterotrophic tissues may be associated with further fractionating processes, in addition to those associated with day/night effects. For example, a gradient of ^{13}C-enrichment from twig phloem sap to stem phloem sap was observed in *Fagus sylvatica* (Gessler *et al*. 2004) and *Pinus sylvestris* (Brandes *et al*. 2006). This enrichment was postulated to result from leakage of sucrose out of the phloem, and subsequent partial retrieval of a ^{13}C-enriched fraction (Gessler *et al*. 2007). Root soluble sugars were observed to be ^{13}C-enriched compared with soluble sugars in the stem in *Phaseolus vulgaris* L. (Badeck *et al*. 2005), whereas root soluble sugars were ^{13}C-depleted compared with phloem sap organic material in *Eucalyptus delegatensis* (Gessler *et al*. 2007). Thus, a complex phloem-trafficking pathway, which may differ among species, could further lead to variability in relative ^{13}C enrichment among sink organs. However, $\delta^{13}C$ of sucrose collected from petiole phloem sap did not differ significantly from that of leaves in *Phaseolus vulgaris* (Badeck *et al*. 2005). This latter result suggests that phloem loading, in and by itself, is not likely to be a fractionating process. Further research is necessary to identify potentially fractionating processes associated with the transfer of carbohydrates from source leaves to sink organs, and to elucidate how these processes may vary within and among species.

Hypothesis 4: Fractionation during respiration

A fourth hypothesis to explain ^{13}C enrichment in heterotrophic tissues relative to leaves is that respiratory processes cause divergence in $\delta^{13}C$ of the two tissues (Table 1; Fig. 3). This hypothesis would require either that leaves release CO_2 during respiration that is ^{13}C-enriched, thereby leaving behind ^{13}C-depleted carbon to be incorporated into leaf biomass, and/or that heterotrophic tissues release ^{13}C-depleted CO_2, thereby leaving behind ^{13}C-enriched carbon to be incorporated into their biomass.

Study of the carbon isotope composition of plant respiration is currently an active area of research. Thus far, and consistent with this hypothesis, results have suggested that dark-respired CO_2 is generally ^{13}C-enriched relative to whole tissues and/or respiratory substrates in leaves (Duranceau *et al*. 1999; Cernusak and Marshall 2001; Duranceau *et al*. 2001; Ghashghaie *et al*. 2001, 2003; Tcherkez *et al*. 2003; Ocheltree and Marshall 2004; Xu *et al*. 2004; Hymus *et al*. 2005; Prater *et al*.

2006; Barbour *et al*. 2007). In addition, it was recently shown that CO_2 evolved in darkness just after illumination was ^{13}C-enriched by more than 10‰ compared with organic material (Barbour *et al*. 2007). This peak in ^{13}C enrichment of leaf-respired CO_2 was assumed to be associated with the decarboxylation of organic acids, like malate, in a process known as light-enhanced dark respiration (LEDR) (Atkin *et al*. 2000). Malate has been shown to be relatively enriched in ^{13}C (Melzer and O'Leary 1987). Accordingly, in *Ricinus communis*, both the amount and the ^{13}C enrichment of CO_2 evolved during LEDR correlated closely with malate decarboxylation (Gessler *et al*. 2009). After the end of the post-illumination respiratory peak, when the respiration rate has stabilised and the Krebs cycle is fully activated, fragmentation fractionation is thought to occur during CO_2 release by pyruvate dehydrogenase. This leaves behind ^{13}C-depleted acetyl-coA that can be incorporated into fatty acids, known to be depleted in ^{13}C compared with sugars, or enter the TCA cycle (Ghashghaie *et al*. 2003). These results tend to support the suggestion that dark respiration in leaves causes a relative depletion in $\delta^{13}C$ of residual leaf organic material.

Some recent reports have also provided results for roots consistent with the respiration hypothesis. For three species of herbaceous plants, CO_2 respired in the dark by shoots was significantly enriched in ^{13}C compared with the respiratory substrate pool, whereas CO_2 respired by roots showed no enrichment (Klumpp *et al*. 2005); the difference between $\delta^{13}C$ of shoot- and root-respired CO_2 was sufficient to explain observed differences of 0.8 to 1.6‰ in whole-tissue $\delta^{13}C$ between roots and shoots. Thus, in this dataset, respiratory fractionation of carbon isotopes caused an apparent depletion of ^{13}C in leaf tissue, but not in root tissue. A similarly consistent result was recently observed for another herbaceous species, *P. vulgaris*, where $\delta^{13}C$ signatures for dark-respired CO_2 diverged between leaves and roots in 10-day-old plants, causing the characteristic ^{13}C enrichment of ~1‰ in root organic material relative to that of leaves (Bathellier *et al*. 2008). This pattern agreed with results presented previously for this species, in which root-respired CO_2 was ^{13}C-depleted when compared with root carbohydrates or total root organic material (Badeck *et al*. 2005). This also agrees with an analysis of metabolic fluxes, showing the larger prevalence of the pentose phosphate pathway, expected to produce ^{13}C-depleted CO_2, in roots as compared with leaves (Bathellier *et al*. 2009).

In contrast to the above cited reports, observations of respired CO_2 from woody tissues appear not to be consistent with the respiratory fractionation hypothesis. In woody tissues, dark-respired CO_2 was observed to be ^{13}C-enriched relative to whole tissues and/or respiratory substrates (Cernusak *et al*. 2001; Damesin and Lelarge 2003; Damesin *et al*. 2005; Brandes *et al*. 2006; Gessler *et al*. 2007; Maunoury *et al*. 2007), such that respiration in these tissues would cause a relative depletion of ^{13}C in residual organic material, rather than the characteristic enrichment. Thus, on the basis of currently available results, it is difficult to draw a generalised conclusion about the impact of respiration on $\delta^{13}C$ divergence between leaves and heterotrophic tissues, especially with regard to woody stem tissues. Careful quantification and comparison of the magnitude of ^{13}C enrichment of dark-respired CO_2 in leaves and woody tissues is necessary to determine what contribution, if any, the respiratory process makes to their characteristic $\delta^{13}C$ divergence.

Hypothesis 5: Carbon fixation by PEP carboxylase in heterotrophic tissues

As noted earlier, PEP carboxylase, the enzyme that catalyses addition of HCO_3^- to PEP, shows a discrimination relative to gaseous CO_2 of about –5.7‰, such that carbon fixation by this enzyme would be expected to result in the addition of ^{13}C-enriched organic material in C_3 plants. Thus, a fifth hypothesis to explain ^{13}C enrichment of heterotrophic tissues relative to leaves in C_3 plants is that PEP carboxylase fixes HCO_3^- at a greater rate in heterotrophic tissues than in leaves, thus causing ^{13}C enrichment of organic material in heterotrophic tissues (Table 1; Fig. 3). Several authors have favoured this hypothesis (Terwilliger and Huang 1996; Le Roux-Swarthout *et al*. 2000, 2001*a*; Terwilliger *et al*. 2001; Badeck *et al*. 2005).

In plants, PEP carboxylase activity contributes to biosynthetic functions of the TCA cycle. The TCA cycle is an important component of aerobic respiration in all eukaryotic cells. In plants, however, the TCA cycle also provides carbon skeletons for biosynthesis of organic acids and proteins. When TCA cycle intermediates are removed for biosynthetic reactions, for example during the conversion of inorganic nitrogen to glutamate, they must be replaced to ensure continued functioning of the TCA cycle. The enzyme PEP carboxylase functions to replenish the supply of TCA cycle intermediates by converting PEP to oxaloacetate, which can in turn be converted to malate. Malate can then enter the mitochondrion, where it subsequently becomes available for engagement in the TCA cycle. Such carbon fixation by PEP carboxylase has been termed anaplerotic carbon fixation, where anaplerotic is a word of Greek origin meaning 'to fill up'. The term emphasises the important role of this carbon fixation pathway in replenishing the supply of intermediate molecules for biosynthetic reactions.

The activity of PEP carboxylase was recently observed to be several-fold higher in current-year stems than in leaves of adult *Fagus sylvatica* trees (Berveiller and Damesin 2008), and about twice as high in roots as in leaves of *Ricinus communis* (Gessler *et al*. 2009). Such results lend support to the PEP carboxylase hypothesis.

However, it is uncertain what proportion of PEP carboxylase-derived molecules are subsequently integrated within cell metabolism. Under the assumption of no subsequent decarboxylation of PEP carboxylase-derived products, PEP carboxylase activity would be expected to cause an increase in $\delta^{13}C$ of total organic material. In contrast, if PEP carboxylase-derived molecules were subsequently decarboxylated, no net effect of such activity would be expected. For example, the latter case may occur when all of the oxaloacetate molecules synthesised by PEP carboxylase enter the TCA cycle as pyruvate and are consequently decarboxylated. However, this scenario is unlikely in roots that ordinarily show large concentrations of organic acids, such as malate. Inorganic carbon labelled with ^{14}C that was supplied to roots and stems of tobacco and celery accumulated as insoluble organic compounds within vascular tissues of these heterotrophic organs (Hibberd and Quick 2002). Thus, it is possible that PEP carboxylase activity and assimilation

of respiratory CO_2 from soil and plant tissue causes some ^{13}C enrichment of root and stem organic material through addition of ^{13}C-enriched organic acids that are then incorporated into cell metabolism and biosynthesis (Raven and Farquhar 1990; Badeck *et al*. 2005).

The potential influence of PEP carboxylase on $\delta^{13}C$ of hetertrophic tissues can be illustrated by assuming that ~10% of respired CO_2 is refixed by PEP carboxylase. Under such conditions, and assuming no subsequent decarboxylation of PEP carboxylase-derived acids, a ^{13}C-enrichment of near 0.5‰ would be expected in root organic material (Badeck *et al*. 2005). This calculation also assumes that the amount of carbon lost to respiration is equal to the amount of carbon incorporated into organic material. In the case of leaves, the synthesis of organic acids during the day may be compensated for by subsequent decarboxylation at the very beginning of the dark period, fueling the light-enhanced dark respiration rate, and thereby canceling the ^{13}C-enriching effect of the previous PEP carboxylations (Barbour *et al*. 2007). In that way, differential activity of PEP carboxylase and subsequent decarboxylation of organic acids may contribute to $\delta^{13}C$ divergence between leaves and heterotrophic tissues.

Hypothesis 6: Developmental variation in photosynthetic discrimination

A sixth hypothesis to explain ^{13}C enrichment of heterotrophic tissues compared with leaves is that leaves undergo a developmental shift in Δ during leaf expansion, such that young, expanding leaves fix carbon with a more negative $\delta^{13}C$ than mature leaves responsible for carbon export to heterotrophic tissues (Table 1; Fig. 3). According to this hypothesis, new leaves would begin their lives completely dependent on carbon donated by mature, source leaves. As soon as the young leaf gained a degree of photosynthetic competency, it would begin to fix CO_2, which would then be incorporated into its biomass as it continued to expand. However, CO_2 fixation in the young leaf would proceed at a higher p_i/p_a than CO_2 fixation in mature leaves, such that the young leaf would be adding carbon to its biomass at more negative $\delta^{13}C$ than the carbon being imported from other leaves. As discussed previously, C_3 photosynthesis shows a strong, linear dependence of Δ on p_i/p_a. This process would eventually manifest itself as a depletion of whole-tissue $\delta^{13}C$ in the new leaf, once it had fully expanded, relative to carbon imported during leaf expansion, and relative to carbon that it would go on to export as a mature leaf. Francey *et al*. (1985) favoured this hypothesis for explaining an observed $\delta^{13}C$ gradient from needle tip to first wood in branches of *Lagarostrobos franklinii* (Hook. F.) Quinn.

Ubierna Lopez and Marshall (2007) recently expanded upon this hypothesis to include not just net CO_2 fixation by young leaves, but also refixation of respired CO_2 before new leaves are capable of net CO_2 fixation. They further argued that photosynthetic refixation of respired CO_2 would continue following leaf maturity, whenever leaf irradiance was above zero but below the light compensation point for photosynthetic CO_2 uptake. Cernusak *et al*. (2001) described equations to predict Δ for photosynthetic refixation in woody tissues, based on Comstock's (2001) generalised description of isotope fractionation in branched pathways. The model can be applied to refixation in young leaves displaying net CO_2 efflux in the light:

$$\Delta_{Refix} = \left(1 - \frac{P_g}{R_d}\right)\left(\frac{R_d}{R_d + (R_d - P_g)\frac{p_a}{p_i - p_a}}\right) \times \left(b\frac{p_i}{p_i - p_a} - a - \Delta_D\frac{p_a}{p_i - p_a}\right), \quad (6)$$

where R_d is day respiration (μmol CO_2 m^{-2} s^{-1}), P_g is gross photosynthesis (μmol CO_2 m^{-2} s^{-1}), p_a and p_i are atmospheric and intercellular CO_2 partial pressures, respectively (μbar), b is ^{13}C discrimination by carboxylating enzymes (~29‰, assuming only Rubisco), a is ^{13}C discrimination during diffusion through stomata (4.4‰), and Δ_D is discrimination of day respiration with respect to atmospheric CO_2. The Δ_D can be calculated according to Eqn (2), assuming no fractionation during translocation of photosynthate from mature leaves to the refixing leaf, and no fractionation during day respiration. The term Δ_{Refix} is defined as $R_D/R_P - 1$, where R_D is $^{13}C/^{12}C$ of day respiration and R_P is $^{13}C/^{12}C$ of refixed photosynthate (Cernusak *et al*. 2001). The Δ_{Refix} can be defined in little delta terms as

$$\Delta_{Refix} = \frac{\delta_D - \delta_P}{1 + \delta_P}, \quad (7)$$

where δ_D is the $\delta^{13}C$ of day respiration and δ_P is the $\delta^{13}C$ of refixed photosynthate.

Thus, Δ_{Refix} essentially describes the depletion of ^{13}C in refixed photosynthate compared with that in the CO_2 produced by day respiration. The third term on the right side in Eqn (6) describes discrimination against ^{13}C caused by the carboxylating enzymes (assumed to be Rubisco), gaseous diffusion of CO_2, and Δ_D, which accounts for variation in the isotopic composition of the respiratory carbon. The Δ_D is defined as $R_a/R_D - 1$, where R_a is $^{13}C/^{12}C$ of atmospheric CO_2 and R_D is $^{13}C/^{12}C$ of day respiration. This composite term for isotopic discrimination is modified by two processes, represented by the first and second terms on the right side in Eqn (6). The first term describes the deviation from unity of the proportional refixation rate, P_g/R_d. When P_g equals R_d, the discrimination goes to zero, whereas the discrimination is at a maximum when P_g is very small compared with R_d. The second term on the right side in Eqn (6) accounts for the diffusion of atmospheric CO_2, with $\delta^{13}C$ independent of that of respired CO_2, into the intercellular air spaces. This term could also be written as $R_d/(R_d + g_t p_a/P)$, where g_t is total conductance to CO_2 of stomata plus boundary layer, and P is atmospheric pressure. Thus, the term is reduced from unity by the magnitude of $g_t p_a/P$, which describes the one-way flux of CO_2 from the atmosphere into the leaf. The importance of considering one-way fluxes in isotopic models has been discussed in detail elsewhere (Farquhar *et al*. 1993; Cernusak *et al*. 2004*a*; Farquhar and Cernusak 2005). A step-by-step derivation in Eqn (6) is provided in Part 3 of the Appendix by Cernusak *et al*. (2001). Eqn (6) does not account for any mixing of refixed photosynthate into the respiratory substrate pool. It is analogous to Eqn (2) without the term d, in that it ignores the drawdown in CO_2 partial pressure from p_i to p_c, and any effects of photorespiration.

Gas-exchange characteristics of leaves can vary during leaf expansion. Figure 4 shows measurements of p_i/p_a in expanding

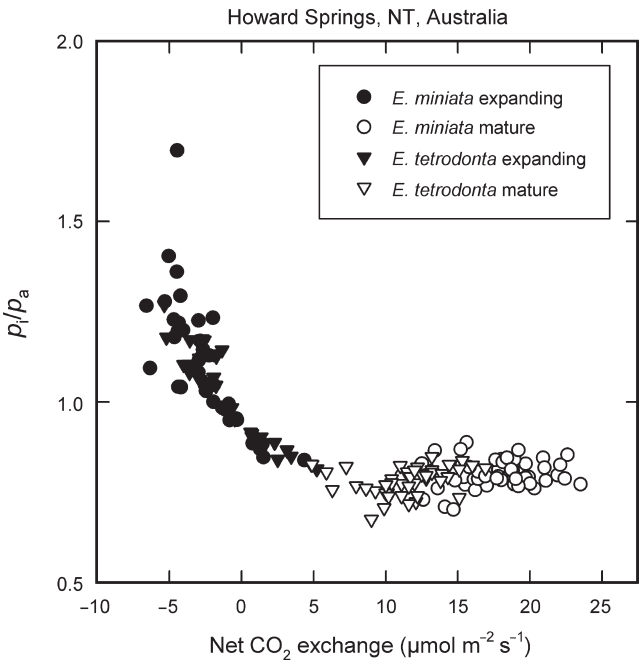

Fig. 4. Ratio of intercellular to ambient CO_2 partial pressures (p_i/p_a) plotted against the net CO_2 exchange rate for expanding and mature leaves of two *Eucalyptus* species sampled in the Northern Territory, Australia. Measurements were conducted at incident photon flux densities larger than 1000 μmol m^{-2} s^{-1}. Negative values of net CO_2 exchange indicate CO_2 efflux from illuminated leaves, and positive values indicate CO_2 uptake. Data presented in this figure were originally published by Cernusak *et al*. (2006).

and mature leaves of two *Eucalyptus* species, sampled with incident photon flux densities larger than 1000 μmol m^{-2} s^{-1} (Cernusak *et al*. 2006). The net photosynthetic rate, plotted on the *x*-axis, can be taken as a measure of the developmental stage of the expanding leaves. As leaf expansion proceeds, the photosynthetic competency of the leaf increases, and the net CO_2 exchange in sunlight trends from net CO_2 efflux (negative values in Fig. 4) to net CO_2 uptake (positive values in Fig. 4). This was similarly demonstrated for another myrtaceous species, *Corymbia gummifera* (Gaertn.) K.D. Hill & L.A.S. Johnson (Choinski *et al*. 2003). The net CO_2 exchange of expanding leaves in the light in Fig. 4 ranges from about –7 to 5 μmol CO_2 m^{-2} s^{-1}. The net CO_2 exchange in darkened leaves at similar leaf temperatures (~39°C) was about –8.0 μmol CO_2 m^{-2} s^{-1} (Cernusak *et al*. 2006). Assuming no change in respiration rate in the light and dark, these data can be used to make predictions of Δ_{Refix}. Thus, for a proportional refixation rate of 0.5, such that $R_d = 8$ μmol CO_2 m^{-2} s^{-1} and $P_g = 4$ μmol CO_2 m^{-2} s^{-1}, and with stomatal plus boundary layer conductance to CO_2 equal to 0.05 mol m^{-2} s^{-1} (Cernusak *et al*. 2006), Eqn (6) predicts a Δ_{Refix} of ~10‰. Thus, the carbon taken up by refixation would have a $\delta^{13}C$ ~10‰ more negative than the CO_2 produced by day respiration.

The data presented in Fig. 4 suggest that when expanding leaves of these two *Eucalyptus* species become capable of net CO_2 fixation in the light they will also photosynthesise at p_i/p_a values higher than those of mature leaves. This agrees with observations in other species (Francey *et al*. 1985; Terwilliger 1997; Terwilliger *et al*. 2001). The predicted difference in Δ in this case between expanding and mature leaves could be calculated with Eqn (2). Expanding leaves would be expected to fix CO_2 with a $\delta^{13}C$ more negative than that fixed by mature leaves by a difference of ~2.0–2.5‰ for each 0.1 increase in p_i/p_a.

Discussion

We have reviewed six hypotheses proposed to explain ^{13}C enrichment of heterotrophic tissues relative to leaves in C_3 plants (Table 1; Fig. 3). Each of these six hypothesised mechanisms can potentially contribute to the characteristic $\delta^{13}C$ divergence. In the case of hypotheses (1) and (2), variable biochemical composition and seasonal separation of growth, some situations can be identified where they do not appear to be operative. This suggests an additional process or additional processes that contribute to the $\delta^{13}C$ difference between leaves and non-photosynthetic tissues. However, there is clearly a need for more information about how different biosynthetic pathways influence $\delta^{13}C$ dynamics, and about seasonal variation in photosynthetic discrimination and associated changes in carbon allocation. We suggest that future research into all six hypotheses, focusing on testable predictions of each (Table 1), will provide valuable insights into sources of within-plant variation in $\delta^{13}C$.

It is worthwhile to ask whether the $\delta^{13}C$ divergence between leaves and heterotrophic tissues in C_3 plants arises primarily as the result of a ^{13}C-enriching process in the sink tissue, or as a result of a ^{13}C-depleting process in leaves. Available evidence would appear to support both of these possibilities. In the three studies in which respiratory fractionation appeared to cause $\delta^{13}C$ divergence between leaves and roots in herbaceous plants, the divergence was largely associated with ^{13}C-enriched respiration causing a ^{13}C depletion of leaf biomass, whereas the effect of ^{13}C-depleted respiration on root biomass was smaller (Badeck *et al*. 2005; Klumpp *et al*. 2005; Bathellier *et al*. 2008). Additionally, the observation that emerging leaves tend to be ^{13}C enriched and decline in $\delta^{13}C$ during leaf expansion further suggests a ^{13}C-depleting process. Finally, the observation that sink tissues tend to show similar $\delta^{13}C$ to concurrent phloem sap carbon argues against a marked ^{13}C-enriching process in heterotrophic tissues (Pate and Arthur 1998; Cernusak *et al*. 2002, 2005). However, there is also counterevidence to suggest that heterotrophic tissues can be subject to a ^{13}C-enriching process; $\delta^{13}C$ analyses of achlorophyllous, heterotrophic tobacco plants showed an enrichment of ~2‰ in plant organic material compared with the organic growth media (Terwilliger and Huang 1996; Le Roux-Swarthout *et al*. 2001*a*).

The theoretical prediction (Tcherkez *et al*. 2004) and recent experimental confirmation (Gessler *et al*. 2008) of a diel cycle in the $\delta^{13}C$ of phloem-exported carbon in C_3 plants, summarised under hypothesis (3), provides a promising avenue for future research. In addition to the day/night variation in $\delta^{13}C$ of exported carbohydrate, the diel explanation also requires a differential use of day sucrose *v.* night sucrose between leaves and heterotrophic tissues. Thus, if this is an important cause of ^{13}C enrichment of heterotrophic tissues compared with leaves, one would expect species that predominantly exhibit leaf growth during the night, such as *Helianthus annuus* L. (Boyer 1968) and *Glycine max* (L.)

Merr. (Bunce 1977), to show a smaller $\delta^{13}C$ divergence between leaves and heterotrophic tissues compared with species that primarily exhibit leaf growth during the day.

The absence of a $\delta^{13}C$ difference between leaves and roots in C_4 plants provides a distinct contrast to the pattern typical of C_3 plants. As noted by Hobbie and Werner (2004), further research could examine perennial, woody C_4 plants (Pearcy and Troughton 1975) to see whether their stem and root tissue shows ^{13}C enrichment compared with their leaves. We note that hypothesis (6), concerning developmental variation in Δ, could provide an explanation for such a difference between C_3 and C_4 plants, due to the contrasting dependencies of Δ on p_i/p_a for C_3 *v.* C_4 photosynthesis. If young, expanding C_4 leaves show higher values of p_i/p_a than mature leaves, this could result in the addition of relatively ^{13}C-enriched photosynthate to the growing leaf tissue, because Δ typically decreases with increasing p_i/p_a in C_4 leaves. Such a mechanism might explain the results for *Saccharum spontaneum* (Cernusak *et al*. 2007), described in the Introduction, in which leaf tissue was enriched in ^{13}C compared with roots.

There is little direct evidence in support of hypothesis (6), although there is experimental support for the idea that p_i/p_a is higher in expanding than in mature leaves (Francey *et al*. 1985; Terwilliger 1997; Terwilliger *et al*. 2001; Cernusak *et al*. 2006). However, the study by Terwilliger and Huang (1996) may be relevant. They grew tomato plants, in which paired leaves were either forced to grow heterotrophically by application of an inhibitor of photosynthesis, or allowed to develop normally. The normally developing photosynthetic leaves were 1 to 3‰ more negative than the adjacent, simultaneously produced non-photosynthetic leaves. These data can be interpreted as indicating depletion in ^{13}C in the normally developing leaves associated with the ability to photosynthesise during leaf expansion. Photosynthesis may have caused a decline in $\delta^{13}C$ due to refixation and/or net CO_2 fixation at higher p_i/p_a than that of mature leaves donating photosynthate for heterotrophic growth.

If hypothesis (6) plays an important role in determining $\delta^{13}C$ divergence between leaves and heterotrophic tissues, one might expect variation among species in the magnitude of the divergence to be associated with variation in leaf expansion characteristics. Species vary in their rates of leaf expansion (Moles and Westoby 2000; Schlindwein *et al*. 2006; Sun *et al*. 2006), and in their extent of photosynthetic activity during leaf expansion (Kursar and Coley 1992). Such variation might, thus, be associated with variation in the proportion of autotrophic *v.* heterotrophic carbon input to expanding leaves, and consequently with variation among species in the $\delta^{13}C$ difference between fully-expanded leaves and heterotrophic tissues.

Finally, it is worth pointing out that the potential mechanisms of ^{13}C enrichment of heterotrophic tissues summarised by hypotheses (1) through to (6) could have differing implications for some $\delta^{13}C$ applications. For example, hypotheses (2), (3) and (6) suggest that photosynthate is partitioned, with associated isotopic variation, either seasonally (hypothesis 2), between day and night (hypothesis 3), or ontogenetically (hypothesis 6). However, at the whole-plant scale, the mean $\delta^{13}C$ would still reflect a photosynthesis-weighted average of photosynthetic discrimination against ^{13}C. In contrast, hypotheses (4) and (5) would imply carbon losses (hypothesis 4) or additions (hypothesis 5) that would alter the whole-plant $\delta^{13}C$ from what it would otherwise have been as a result of photosynthetic ^{13}C discrimination. Hypothesis (1) could involve carbon losses, gains, or within-plant partitioning, with associated isotopic variation, such that it may or may not involve a departure from photosynthetic ^{13}C discrimination at the whole-plant scale. Thus, for applications that aim to interpret photosynthetic discrimination against ^{13}C, it could be important to differentiate between the various mechanisms potentially causing ^{13}C enrichment of heterotrophic tissues.

Conclusion

We have attempted to summarise a series of hypotheses aimed at explaining observations of ^{13}C enrichment in non-photosynthetic tissues compared with leaves in C_3 plants. The hypotheses concerning carbon isotope fractionation associated with diel carbohydrate dynamics, respiration, PEP carboxylation, and developmental shifts in Δ in expanding leaves all appear to be consistent with some available evidence; this evidence is, however, mostly circumstantial or partial in nature. Direct tests of these hypotheses will be required to advance our understanding of processes leading to $\delta^{13}C$ divergence between leaves and heterotrophic tissues. We emphasise that there may not exist a single, unifying explanation for this isotopic pattern; rather, it may be the net result of multiple, simultaneous processes, all of which contribute to a greater or lesser extent to the general tendency for heterotrophic tissues to be ^{13}C-enriched compared with leaves. Unravelling the relative contributions of these processes, and their associated environmental and physiological controls, will undoubtedly lead to a deeper understanding of plant physiology.

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