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RESEARCH IN CONTEXT

Directional change in leaf dry matter δ¹³C during leaf development is widespread in C₃ plants

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• **Background and aims** The stable carbon isotope ratio of leaf dry matter ($\delta^{13}C_p$) is generally a reliable recorder of intrinsic water-use efficiency in C₃ plants. Here, we investigated a previously reported pattern of developmental change in leaf $\delta^{13}C_p$ during leaf expansion, whereby emerging leaves are initially ¹³C-enriched compared to mature leaves on the same plant, with their $\delta^{13}C_p$ decreasing during leaf expansion until they eventually take on the $\delta^{13}C_p$ of other mature leaves.
• **Methods** We compiled data to test whether the difference between mature and young leaf $\delta^{13}C_p$ differs between temperate and tropical species, or between deciduous and evergreen species. We also tested whether the developmental change in $\delta^{13}C_p$ is indicative of a concomitant change in intrinsic water-use efficiency. To gain further insight, we made online measurements of ¹³C discrimination ($\Delta^{13}C$) in young and mature leaves.
• **Key Results** We found that the $\delta^{13}C_p$ difference between mature and young leaves was significantly larger for deciduous than for evergreen species (−2.1 ‰ vs. −1.4 ‰, respectively). Counter to expectation based on the change in $\delta^{13}C_p$, intrinsic water-use efficiency did not decrease between young and mature leaves; rather, it did the opposite. The ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) was significantly higher in young than in mature leaves (0.86 vs. 0.72, respectively), corresponding to lower intrinsic water-use efficiency. Accordingly, instantaneous $\Delta^{13}C$ was also higher in young than in mature leaves. Elevated c_i/c_a and $\Delta^{13}C$ in young leaves resulted from a combination of low photosynthetic capacity and high day respiration rates.
• **Conclusion** The decline in leaf $\delta^{13}C_p$ during leaf expansion appears to reflect the addition of the expanding leaf's own ¹³C-depleted photosynthetic carbon to that imported from outside the leaf as the leaf develops. This mixing of carbon sources results in an unusual case of isotopic deception: less negative $\delta^{13}C_p$ in young leaves belies their low intrinsic water-use efficiency.

Key words: Carbon isotope ratio, intercellular CO_2 concentration, leaf development, water-use efficiency.

INTRODUCTION

Leaf demography can play an important role in controlling canopy gas exchange in both deciduous and evergreen forest trees (Beringer *et al.*, 2015; Wu *et al.*, 2016, 2017). When young leaves emerge, they typically have negative or low photosynthetic rates, which then increase as the photosynthetic apparatus matures (Choinski *et al.*, 2003; Cernusak *et al.*, 2006, 2009*a*). Leaf expansion and development also probably involve changes in water-use efficiency, and understanding such changes could be helpful for understanding how forests will respond to climate change, especially where the response involves changes in leaf demography and/or phenology (Wu *et al.*, 2016; Cernusak, 2020).

Measurements of stable carbon isotope ratios in leaf dry matter ($\delta^{13}C_p$) provide a valuable tool for assessing variation in water-use efficiency in C₃ plants (Farquhar and Richards, 1984). Such measurements have been used to assess water-use efficiency variation

among crop cultivars (Richards *et al.*, 2002; Rebetzke *et al.*, 2008), among species of wild plants (Marshall and Zhang, 1994; Cernusak *et al.*, 2007) and among functional groups with different ecological strategies (Brooks *et al.*, 1997; Guehl *et al.*, 2004). The association between leaf $\delta^{13}C_p$ and water-use efficiency derives from the relationship between discrimination against ¹³C during photosynthetic CO_2 assimilation ($\Delta^{13}C$) and the ratio of the intercellular to ambient CO_2 concentrations (c_i/c_a) (Farquhar *et al.*, 1982*b*; Farquhar and Richards, 1984). In the simplest form, the model relating $\Delta^{13}C$ to c_i/c_a can be expressed as:

$$\Delta^{13}C \approx a_s + \left(\overline{b} - a_s\right)\frac{c_i}{c_a} \qquad (1)$$

where a_s is the ¹³C/¹²C fractionation during diffusion through the stomatal pore (4.4 ‰) and $\overline{b}$ is discrimination against ¹³CO₂ by carboxylating enzymes. Equation (1) can also be written for the $\delta^{13}C$ of plant tissue ($\delta^{13}C_p$), such that:

$$\delta^{13}C_p \approx \delta^{13}C_a - a_s - \left(\bar{b} - a_s\right)\frac{c_i}{c_a} \qquad (2)$$

where $\delta^{13}C_a$ is the $\delta^{13}C$ of CO_2 in air surrounding the plant. The link to water-use efficiency comes about through the term c_i/c_a. Intrinsic water-use efficiency can be defined as A/g_s, where A is the net CO_2 assimilation rate of a leaf and g_s is the stomatal conductance to water vapour:

$$\frac{A}{g_s} \approx \frac{c_a\left(1 - \frac{c_i}{c_a}\right)}{1.6} \qquad (3)$$

where the factor 1.6 in the denominator represents the ratio of stomatal conductance for water vapour to that for CO_2. Equation (3) shows that A/g_s can be expected to increase if c_i/c_a decreases, assuming no change in c_a.

In a more complete form of the model, $\Delta^{13}C$ can be expressed as (Farquhar *et al.*, 1982*b*; Farquhar and Cernusak, 2012; Busch *et al.*, 2020):

$$\Delta^{13}C \approx \frac{1}{1-t}\left(a_b\frac{c_a - c_s}{c_a} + a_s\frac{c_s - c_i}{c_a}\right) + $$
$$\frac{1+t}{1-t}\left(a_m\frac{c_i - c_c}{c_a} + b\frac{c_c}{c_a} - e\frac{\mathcal{R}_d}{A}\frac{c_c}{c_a} - f\frac{\Gamma^*}{c_a}\right) \qquad (4)$$

where a_b is the $^{13}C/^{12}C$ fractionation during diffusion of CO_2 through the boundary layer (2.9 ‰), and a_m is that for dissolution and diffusion from the intercellular air spaces to the sites of carboxylation in the chloroplasts (1.8 ‰). The term b is here taken as fractionation by Rubisco (∼29 ‰), e is fractionation during day respiration and f is fractionation during photorespiration. R_d is the rate of day respiration and Γ^* is the CO_2 compensation point in the absence of day respiration. The terms c_s and c_c are the CO_2 concentrations at the leaf surface and at the sites of carboxylation, respectively. The term t is a ternary correction factor, defined as $t \approx E/(2g_c)$, where E is transpiration rate and g_c is leaf conductance to CO_2 (Farquhar and Cernusak, 2012). Equation (4) is thought to include most processes that would impact upon $\Delta^{13}C$ if it were measured instantaneously, for example using the online technique in which the $\delta^{13}C$ of CO_2 is measured in air entering and exiting a gas exchange cuvette (Evans *et al.*, 1986).

Comparison of eqns (1) and (4) shows that there are a number of processes in the more complete model for $\Delta^{13}C$ that have been neglected from the simplified expression. These include the drawdown in CO_2 concentration from c_i to c_c, fractionations associated with day respiration and photorespiration, and the ternary correction, for example. The simplified expression in eqn (2) is most often applied to $\delta^{13}C_p$ measured in plant dry matter, most commonly in leaves or wood. On the other hand, the more complete eqn (4) is most often applied to instantaneous measurements of $\Delta^{13}C$, when parameters determined by accompanying gas exchange measurements can be applied in combination, with the objective of solving for the conductance from c_i to c_c, termed mesophyll conductance. When eqn (2) is applied to plant dry matter, there can be additional considerations that may become important. Two such examples are post-photosynthetic fractionations, in which the carbon retained in the dry matter of a given tissue takes on a $\delta^{13}C$ different from

that of the photosynthate used to construct the tissue (Badeck *et al.*, 2005), and developmental effects, whereby ontogenetic changes in $\Delta^{13}C$ through time will be reflected in the integrated $\delta^{13}C$ signal of the accumulated carbon in the tissue (Cernusak *et al.*, 2009*a*).

The practical benefit of eqn (2) is that if the terms $\delta^{13}C_a$, a_s and $\bar{b}$ are known, then $\delta^{13}C_p$ can be used to estimate c_i/c_a and therefore A/g_s from only a measurement of the carbon isotope ratio of a dried leaf, for example. For plants grown outdoors, CO_2 in the atmosphere is generally well mixed and $\delta^{13}C_a$ can be approximated as −8 ‰, although it can be more negative beneath dense forest canopies (Buchmann *et al.*, 2002). Also, there is a decreasing trend in $\delta^{13}C_a$ due to fossil fuel combustion, such that the average tropospheric value for 2018 was −8.5 ‰ (Keeling *et al.*, 2017). The term a_s is assumed to have a value of 4.4 ‰, based on diffusivities of $^{13}CO_2$ and $^{12}CO_2$ in static air (Farquhar *et al.*, 1989*b*). When eqn (2) was first developed and applied to $\delta^{13}C_p$ of mature leaves, $\bar{b}$ was estimated to have a value of 27 ‰ (Farquhar *et al.*, 1982*a*). This is smaller than *in vitro* estimates of discrimination by Rubisco of about 29–30 ‰ (McNevin *et al.*, 2007). This is expected because $\bar{b}$ has lumped within it the additional processes shown in eqn (4). Furthermore, there also exists the possibility in C_3 plants that some carboxylation can take place by phosphoenolpyruvate carboxylase, which would lower the combined fractionation factor for Rubisco plus that enzyme (Farquhar and Richards, 1984; Raven and Farquhar, 1990). Interestingly, if one calculates $\bar{b}$ using typical parameter values for these additional processes, one would expect a value even smaller than 27 ‰, for example in the range from 21 to 24 ‰ (Ubierna and Farquhar, 2014). Thus, it appears that the empirically determined value of 27 ‰ for $\bar{b}$ for mature leaf tissue probably includes additional effects to those shown in eqn (4), such as post-photosynthetic fractionation or integration through time of a developmental change in photosynthetic discrimination.

The original estimate for $\bar{b}$ of 27 ‰ has been shown over the years to be reasonably general for mature leaf tissue, and $\delta^{13}C_p$ has accordingly been shown for the most part to be a reliable indicator of variation in c_i/c_a, and therefore of intrinsic water-use efficiency (Farquhar *et al.*, 1982*b*, 1989*a*; Cernusak *et al.*, 2013; Cernusak, 2020). An interesting exception to this general rule is that young, expanding leaves typically have less negative $\delta^{13}C_p$ than mature leaves of the same individual, while at the same time also having higher c_i/c_a (Evans, 1983; Francey *et al.*, 1985; Terwilliger *et al.*, 2001; Cernusak *et al.*, 2009*a*). This is opposite to the expectation based on eqn (2), which would predict that a leaf with higher c_i/c_a should have a lower, or more negative, $\delta^{13}C_p$. If widespread, this would establish an intriguing exception to the general relationship between $\delta^{13}C_p$ and intrinsic water-use efficiency, and it may also give insight into why $\bar{b}$ estimated for mature leaves takes on a value somewhat higher than expected.

We had two objectives in this paper. The first was to compile data from both published and unpublished sources to determine (1) how widespread the $\delta^{13}C_p$ difference between mature and young leaves is, and (2) whether this difference shows dependence on temperate vs. tropical climate, or on whether the plant is deciduous or evergreen. Our second objective was to place the change in $\delta^{13}C_p$ from young to mature leaves into the

context of the value of $\overline{b}$, which has been estimated for mature leaves. We hypothesized that a general decrease in $\delta^{13}C_p$ during leaf expansion could partly explain why $\overline{b}$ appears to be somewhat larger than expected based on comparison of eqns (1) and (4). To gain insight into the physiological processes at work, we measured instantaneous $\Delta^{13}C$ in both young and mature leaves, along with c_i/c_a. We then built upon previous work to develop a theoretical basis for why $\delta^{13}C_p$ decreases during leaf expansion in C_3 plants.

METHODS

We compiled published and unpublished data to test whether the extent of the decline in leaf $\delta^{13}C_p$ during leaf expansion differs by biome or by deciduousness. We categorized study locations as either temperate or tropical, depending on geographical location. In addition, we categorized species as either evergreen or deciduous based on notes in the published papers, or botanical descriptions of the species. Previously unpublished data were included for *Ficus insipida* and *Swietenia macrophylla* grown as seedlings at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Field Facility, Gamboa, Panama [9°07′N, 79°42′W; 28 m above sea level a.s.l.)], and for *Toona ciliata* grown in a glasshouse at the Australian National University in Canberra, Australia (35°17′S, 149°7′E; 577 m a.s.l.). The seedlings of *T. ciliata* were obtained from north-east Queensland, and were therefore categorized as tropical. Although this species is deciduous, we considered it evergreen for our analysis because the seedlings had not experienced a leaf shedding event before sampling, and the canopy had not therefore been reconstructed from stored carbohydrate, which we assumed was an important process for $\delta^{13}C_p$ of leaves of deciduous species.

We similarly surveyed published literature for measurements of c_i/c_a in young and mature leaves. We also included in this compilation values of c_i/c_a for young and mature leaves of *F. insipida*, *S. macrophylla* and *T. ciliata* from the locations described above. We made gas exchange measurements of *F. insipida* and *S. macrophylla* with a Li-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). The chamber was illuminated with 1200 µmol photosynthetically active radiation m^{-2} s^{-1}, provided by an artificial light source (6400-02B LED, Li-Cor Inc.). Leaf temperature averaged 30.8 ± 0.5 °C (mean ± s.d.) during measurements, leaf-to-air vapour pressure difference averaged 0.9 ± 0.3 kPa, and the CO_2 concentration in the chamber averaged 378 ± 22 µmol mol^{-1}. We measured leaves of different ages within a plant on the same day, and we also made repeated measurements of the same leaves as they expanded from one day to the next. These gas exchange measurements were made between 13 and 19 October 2009.

We measured online carbon isotope discrimination ($\Delta^{13}C$) on young and mature leaves of *T. ciliata* at the Australian National University between 12 and 20 April 2012. These measurements were made by coupling a Li-6400 portable photosynthesis system with a tunable diode laser spectrometer (TGA100A, Campbell Scientific Inc., Logan, UT, USA). The calibration and measurement protocol was similar to that described previously (Tazoe *et al.*, 2011; Evans and von Caemmerer, 2013). $\Delta^{13}C$ was calculated from the carbon isotope ratio of CO_2 entering and exiting the leaf chamber, along with CO_2 concentrations

of dry air, according to the equation presented by Evans *et al.* (1986). The irradiance during measurements was 1500 µmol photosynthetically active radiation m^{-2} s^{-1} supplied with an artificial light source (6400-02B LED, Li-Cor Inc.); average leaf temperature was 26.9 ± 0.2 °C; average leaf-to-air vapour pressure difference was 1.1 ± 0.2 kPa; and average CO_2 concentration in the sample chamber was 380 ± 3 µmol mol^{-1}.

Leaves measured for gas exchange in both Panama and at the Australian National University were collected, and their leaf area was determined using a leaf area meter (Li-3100, Li-Cor Inc.). They were then oven dried at 70 °C, weighed, and thereafter ground to a fine powder and measured for the $\delta^{13}C$ of their dry mass as described previously (Cernusak *et al.*, 2003, 2009*b*).

We used a paired *t*-test to compare $\delta^{13}C$ values between mature and young leaves. We then calculated the difference between mature and young leaf $\delta^{13}C$ for each species, and used two-way ANOVA to test for effects on this difference of climate and deciduousness. A similar analysis was conducted for c_i/c_a. Statistical analyses were conducted in R (R Core Team, 2019).

RESULTS

In our literature survey, we found paired $\delta^{13}C$ values for young and mature leaves of 54 species. Of these, 36 were from tropical environments and 18 from temperate environments, and we designated 18 of the species as evergreen and 36 as deciduous (Table 1). The $\delta^{13}C$ values for mature leaves ranged from −31.3 to −25.8 ‰, and those for young leaves ranged from −30.8 to −23.0 ‰ (Fig. 1). A paired *t*-test indicated that young leaf $\delta^{13}C$ was significantly less negative than mature leaf $\delta^{13}C$ (t = 13.2, $P < 0.0001$, $n = 54$). Values for the difference between mature and young leaf $\delta^{13}C$ ranged from −4.2 to 0.4 ‰, with a mean of −1.9 ‰. Two-way ANOVA indicated that the $\delta^{13}C$ difference between mature and young leaves did not vary significantly between tropical and temperate climates (t = 1.6, P = 0.11, $n = 54$). However, whether a species was deciduous or evergreen had a significant effect on the mature-to-young leaf $\delta^{13}C$ difference (t = 2.1, $P < 0.05$, $n = 54$). The mean $\delta^{13}C$ difference for deciduous species was −2.1 ‰ and that for evergreen species was −1.4 ‰, with median and quartiles shown in Fig. 2. The interaction effect between climate and deciduousness was not significant (t = −1.4, P = 0.18, $n = 54$).

For instantaneous measurements of c_i/c_a, we compiled data for 25 woody C_3 species for which we could locate paired values for young and mature leaves. We again designated these species as either deriving from temperate or tropical environments, and as either deciduous or evergreen (Table 2). The instantaneous c_i/c_a for young leaves was significantly higher than that for mature leaves (t = 6.4, $P < 0.0001$, $n = 25$), with mean values of 0.86 and 0.72, respectively (Fig. 3). For the difference in instantaneous c_i/c_a between mature and young leaves, we did not detect significant variation between temperate and tropical species (t = 1.0, P = 0.31, $n = 25$), nor between deciduous and evergreen species (t = 1.5, P = 0.15, $n = 25$).

The measurements that we made in *S. macrophylla*, *F. insipida* and *T. ciliata* were consistent with these overall trends. The instantaneous values of c_i/c_a were higher in young than in mature leaves, and $\delta^{13}C$ was less negative in young

TABLE 1. *Stable carbon isotope ratios of young and mature leaves* ($\delta^{13}C_p$; ‰) *for plants that use the C₃ photosynthetic pathway.*

Species	Young leaf δ^{13}C	Mature leaf δ^{13}C	Difference, mature–young	Deciduousness	Geography	Reference
Acer grandidentatum	−26.8	−28.2	−1.4	Deciduous	Temperate	Leavitt and Long (1985)
Acer negundo	−27.9	−30.1	−2.2	Deciduous	Temperate	Tipple and Ehleringer (2018)
Acer rubrum	−25.2	−28.1	−2.9	Deciduous	Temperate	Lowdon and Dyck (1974)
Acer rubrum	−27.1	−29.6	−2.5	Deciduous	Temperate	Suh and Diefendorf (2018)
Acer saccharum	−28.7	−30.6	−1.9	Deciduous	Temperate	Suh and Diefendorf (2018)
Acrocomia aculeata	−26.9	−27.8	−0.9	Evergreen	Tropical	Holtum and Winter (2005)
Albizia adinocephala	−27.1	−29.1	−2	Evergreen	Tropical	Holtum and Winter (2005)
Anacardium excelsum	−24.6	−25.9	−1.3	Deciduous	Tropical	Holtum and Winter (2005)
Anacardium excelsum	−25.4	−26.7	−1.3	Deciduous	Tropical	Terwilliger (1997)
Annona spraguei	−26.7	−27.8	−1.1	Deciduous	Tropical	Holtum and Winter (2005)
Antirrhoea trichantha	−26.0	−28.9	−2.9	Deciduous	Tropical	Holtum and Winter (2005)
Astronium graveolens	−28.0	−27.6	0.4	Deciduous	Tropical	Holtum and Winter (2005)
Betula occidentalis	−28.5	−29.7	−1.2	Deciduous	Temperate	Tipple and Ehleringer (2018)
Beureria cumanensis	−26.2	−27.8	−1.6	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Capparis aristiguetae	−27.3	−31.2	−3.9	Evergreen	Tropical	Sobrado and Ehleringer (1997)
Castilla elastica	−26.0	−28.0	−2	Deciduous	Tropical	Holtum and Winter (2005)
Cecropia insignis	−27.2	−29.1	−1.9	Evergreen	Tropical	Terwilliger (1997)
Cecropia longipes	−23.7	−26.3	−2.6	Deciduous	Tropical	Terwilliger (1997)
Cecropia longipes	−24.9	−26.2	−1.3	Deciduous	Tropical	Terwilliger *et al.* (2001)
Cecropia longipes	−25.3	−26.1	−0.8	Deciduous	Tropical	Holtum and Winter (2005)
Cecropia peltata	−24.8	−27.5	−2.7	Evergreen	Tropical	Terwilliger (1997)
Combretum apiculatum	−25.7	−27.7	−2	Deciduous	Tropical	February and Higgins (2016)
Cordia alliadora	−26.7	−29.4	−2.7	Deciduous	Tropical	Terwilliger (1997)
Coursetia arborea	−26.0	−28.2	−2.2	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Didymopanax morototoni	−26.3	−28.0	−1.7	Evergreen	Tropical	Holtum and Winter (2005)
Enterolobium cyclocarpum	−27.1	−28.7	−1.6	Deciduous	Tropical	Holtum and Winter (2005)
Fagus crenata	−25.9	−29.8	−3.9	Deciduous	Temperate	Han *et al.* (2016)
Fagus sylvatica	−24.1	−28.1	−4	Deciduous	Temperate	Damesin and Lelarge (2003)
Fagus sylvatica	−24.5	−27.3	−2.8	Deciduous	Temperate	Keitel *et al.* (2003)
Fagus sylvatica	−24.5	−26.0	−1.5	Deciduous	Temperate	Helle and Schleser (2004)
Ficus insipida	−23.8	−25.8	−2	Evergreen	Tropical	Holtum and Winter (2005)
Ficus insipida	−28.5	−30.5	−2	Evergreen	Tropical	This paper
Larix sibirica	−23.0	−25.8	−2.8	Deciduous	Temperate	Li *et al.* (2007)
Lonchocarpus dipteroneurus	−25.5	−27.4	−1.9	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Luehea seemannii	−27.3	−27.6	−0.3	Evergreen	Tropical	Holtum and Winter (2005)
Luehea seemanii	−27.3	−28.2	−0.9	Evergreen	Tropical	Terwilliger (1997)
Morisonia americana	−29.8	−30.1	−0.3	Evergreen	Tropical	Sobrado and Ehleringer (1997)
Picea crassifolia	−25.5	−27.1	−1.6	Evergreen	Temperate	Li *et al.* (2019)
Pithecellobium dulce	−26.7	−29.3	−2.6	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Populus angustifolia	−30.8	−31.3	−0.5	Deciduous	Temperate	Tipple and Ehleringer (2018)
Pseudobombax septenatum	−26.4	−27.5	−1.1	Deciduous	Tropical	Holtum and Winter (2005)
Quercus ilex	−26.2	−26.4	−0.2	Evergreen	Temperate	Damesin *et al.* (1998)
Quercus pubescens	−23.9	−26.5	−2.6	Deciduous	Temperate	Damesin *et al.* (1998)
Salix interior	−23.6	−26.7	−3.1	Deciduous	Temperate	Le Roux-Swarthout *et al.* (2001)
Sassafras albidum	−26.0	−29.5	−3.5	Deciduous	Temperate	Suh and Diefendorf (2018)
Siparuna guianensis	−29.8	−30.3	−0.5	Evergreen	Tropical	Vitoria *et al.* (2016)
Spondias mombin	−25.1	−27.4	−2.3	Deciduous	Tropical	Holtum and Winter (2005)
Swietenia macrophylla	−28.3	−30.4	−2.1	Evergreen	Tropical	This paper
Terminalia sericea	−23.4	−27.6	−4.2	Deciduous	Tropical	February and Higgins (2016)
Toona ciliata	−25.8	−26.7	−0.9	Evergreen	Tropical	This paper
Ulmus americana	−28.3	−30.1	−1.8	Deciduous	Temperate	Suh and Diefendorf (2018)
Urera caracasana	−28.0	−28.7	−0.7	Evergreen	Tropical	Terwilliger *et al.* (2001)
Xylopia sericea	−29.8	−30.3	−0.5	Evergreen	Tropical	Vitoria *et al.* (2016)
Zuelania guidonia	−27.7	−30.1	−2.4	Deciduous	Tropical	Holtum and Winter (2005)

than in mature leaves. Figure 4 shows δ¹³C of bulk leaf material plotted against instantaneous c_i/c_a for these three species for young and mature leaves. For a given instantaneous c_i/c_a, there is a clear separation between δ¹³C of young and mature leaves, with that of young leaves being about 2 ‰ less negative than that of mature leaves. The relationship between mature leaf δ¹³C and instantaneous c_i/c_a is similar to that originally reported by Farquhar *et al.* (1982a), having the form $\delta^{13}C_p = \delta$

¹³$C_a − a_s − (\bar{b} − a_s)c_i/c_a$, with $a_s = 4.4$ ‰ and $\bar{b} = 27$ ‰. Thus, the coefficients that we observed for the linear relationship between mature leaf δ¹³C and c_i/c_a match reasonably well those originally observed for mature leaves of *Phaseolus vulgaris*, *Avicennia marina* and *Aegiceras corniculatum* (Farquhar *et al.*, 1982a). However, the relationship that we observed for young leaves shows a consistent offset from this well-established relationship (Fig. 4).
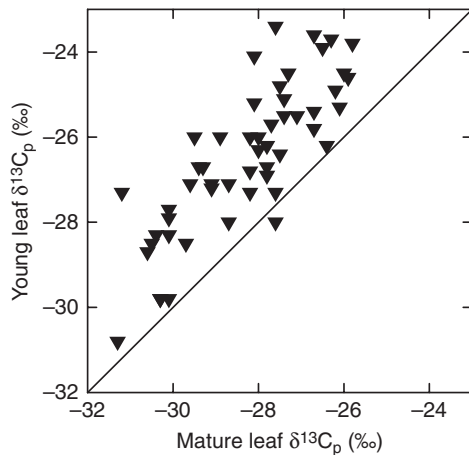
FIG. 1. Stable carbon isotope ratios of young vs. mature leaf dry matter ($\delta^{13}C_p$) for the species shown in Table 1. The solid line shows the one-to-one line.

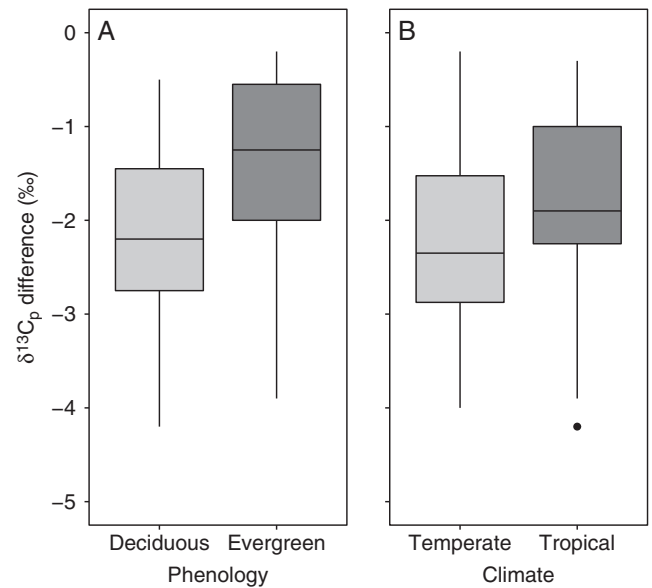FIG. 2. Boxplots showing the $\delta^{13}C_p$ difference between mature and young leaf dry matter as a function of phenology (A), and whether the trees grew in temperate or tropical climate zones (B). The $\delta^{13}C$ difference was calculated as mature leaf $\delta^{13}C_p$ minus young leaf $\delta^{13}C_p$ for the same species. Further details of the dataset are given in Table 1.

Figure 5 shows instantaneous observations of c_i/c_a for *S. macrophylla*, *F. insipida* and *T. ciliata* plotted against leaf size, where leaf size in this case acts as a proxy for leaf age. For *S. macrophylla* and *F. insipida*, we quantified the relationship between leaf size and leaf age during leaf expansion. Leaves of both species required about 15 d to reach full expansion, and showed a sigmoidal relationship between days after emergence and area as a proportion of the fully expanded area. For all three species in Fig. 5, there is a non-linear relationship between c_i/c_a and leaf size, which can be approximated by an exponential decay function. The smallest leaves that we could measure showed the highest values of c_i/c_a, and as the leaves expanded, c_i/c_a declined until it stabilized at values typical of mature leaves on the same plant.

On-line measurements of $\Delta^{13}C$ are plotted against simultaneously measured c_i/c_a in young and mature leaves of *T. ciliata* in Fig. 6. For these instantaneous measurements, the relationship between $\Delta^{13}C$ and c_i/c_a was similar between young and mature leaves, although the range of c_i/c_a covered by young leaves only partly overlapped with the range covered by mature leaves. Analysis of covariance indicated that the interaction term between leaf age (young or mature) and c_i/c_a did not significantly improve the prediction of $\Delta^{13}C$, and with this interaction term removed, the main effect of leaf age also did not improve the prediction of $\Delta^{13}C$ compared to a model with only an intercept and c_i/c_a. Thus, a simple linear model could be fitted to all the data in Fig. 6 ($R^2 = 0.94$, $P < 0.0001$, $n = 18$). This model had a slope of 38.7 ‰. This indicates that a developmentally driven decrease in c_i/c_a of 0.1 in a young leaf of *T. ciliata* will be accompanied by a decrease in instantaneous $\Delta^{13}C$ of 3.9 ‰.

DISCUSSION

Our survey of literature data (Table 1) confirms that there is a general pattern for C$_3$ plants in which leaf $\delta^{13}C_p$ decreases as leaves expand. This pattern has been previously observed by a number of authors for selected species (see references in Table 1). Following seminal work relating carbon isotope discrimination to c_i/c_a (Farquhar *et al.*, 1982*a, b*), it was also recognized that this developmental change in leaf dry matter $\delta^{13}C$

is opposite to that which would be expected based on the relationship between instantaneous $\Delta^{13}C$ and c_i/c_a (Evans, 1983; Francey *et al.*, 1985; Evans *et al.*, 1986; Terwilliger, 1997, 2001; Cernusak *et al.*, 2009*a*). Our literature survey further establishes the generality of this observation (Table 2).

Evans (1983) and Francey *et al.* (1985) put forth an explanation for the divergence of leaf $\delta^{13}C_p$ and c_i/c_a during leaf expansion based on the following sequence of logic: (1) the structural carbon in a leaf comprises a mixture, some of which is imported from source leaves in the phloem or from storage in buds or woody tissues, and some of which is fixed by the leaf's own photosynthesis; (2) in an emerging leaf, the mixture will primarily comprise carbon imported from source leaves or from storage; (3) source leaves are likely to be mature leaves, operating at their typical c_i/c_a; (4) the young, expanding leaf will have higher c_i/c_a than these mature leaves; (5) higher c_i/c_a in the young, expanding leaf will cause the carbon fixed by its own photosynthesis to have more negative $\delta^{13}C$ than that imported from mature leaves or from storage; (6) this will cause the expanding leaf's $\delta^{13}C_p$ to become progressively more negative as it adds more of its own photosynthate to the structural carbon being laid down during leaf expansion; and (7) finally, when the leaf reaches maturity, it will go on to export carbon which it fixes at lower c_i/c_a and therefore with less negative $\delta^{13}C$ than the mixture which comprises its own structural carbon.

The results that we present here help to strengthen and reinforce this mechanistic explanation for the developmental change in leaf $\delta^{13}C_p$ that occurs during leaf expansion in C$_3$ plants. We demonstrated that for those instances in which c_i/c_a was measured in both young and mature leaves of the same species, the young leaves had significantly higher c_i/c_a than the mature leaves. In some instances, the young leaves had c_i/c_a greater than unity, indicating a net CO$_2$ efflux from the leaves (Table 2;

TABLE 2. *The ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) for young and mature leaves of plants that use the C_3 photosynthetic pathway.*

| Species	Young leaf c_i/c_a	Mature leaf c_i/c_a	Difference, mature–young	Deciduousness	Geography	Reference
Anthocephalus chinensis	0.89	0.63	−0.26	Evergreen	Tropical	Cai *et al.* (2005)
Beureria cumanensis	0.71	0.74	0.03	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Byrsonima sericea	0.90	0.69	−0.21	Deciduous	Tropical	Vitoria *et al.* (2016)
Cecropia longipes	1.09	0.78	−0.31	Deciduous	Tropical	Terwilliger *et al.* (2001)
Coursetia arborea	0.77	0.71	−0.06	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Eucalyptus delegatensis	0.85	0.82	−0.03	Evergreen	Temperate	Cernusak (2020)
Eucalyptus miniata	1.11	0.83	−0.28	Evergreen	Tropical	Cernusak *et al.* (2006)
Eucalyptus pauciflora	0.88	0.81	−0.07	Evergreen	Temperate	Cernusak (2020)
Eucalyptus tetrodonta	1.03	0.82	−0.21	Evergreen	Tropical	Cernusak *et al.* (2006)
Ficus insipida	0.89	0.87	−0.02	Evergreen	Tropical	This paper
Lagarostrobos franklinii	0.70	0.60	−0.1	Evergreen	Temperate	Francey *et al.* (1985)
Litsea dilleniifolia	0.80	0.72	−0.08	Evergreen	Tropical	Cai *et al.* (2005)
Litsea pierrei var. *semois*	0.82	0.70	−0.12	Evergreen	Tropical	Cai *et al.* (2005)
Lonchocarpus dipteroneurus	0.82	0.71	−0.11	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Luehia seemanii	0.99	0.86	−0.13	Deciduous	Tropical	Terwilliger (1997)
Morisonia americana	0.78	0.76	−0.02	Evergreen	Tropical	Sobrado and Ehleringer (1997)
Pachysandra terminalis	0.58	0.56	−0.02	Evergreen	Temperate	Yoshie and Kawano (1986)
Persea americana	0.77	0.60	−0.17	Evergreen	Tropical	Terwilliger (1997)
Pithecellobium dulce	0.69	0.72	0.03	Deciduous	Tropical	Sobrado and Ehleringer (1997)
Quercus douglasii	0.72	0.50	−0.22	Deciduous	Temperate	Xu and Baldocchi (2003)
Siparuna guianensis	1.03	0.70	−0.33	Evergreen	Tropical	Vitoria *et al.* (2016)
Swietenia macrophylla	0.95	0.82	−0.13	Evergreen	Tropical	This paper
Toona ciliata	0.81	0.68	−0.13	Evergreen	Tropical	This paper
Urera caracasana	0.92	0.79	−0.13	Evergreen	Tropical	Terwilliger *et al.* (2001)
Xylopia sericea	0.89	0.64	−0.25	Evergreen	Tropical	Vitoria *et al.* (2016)
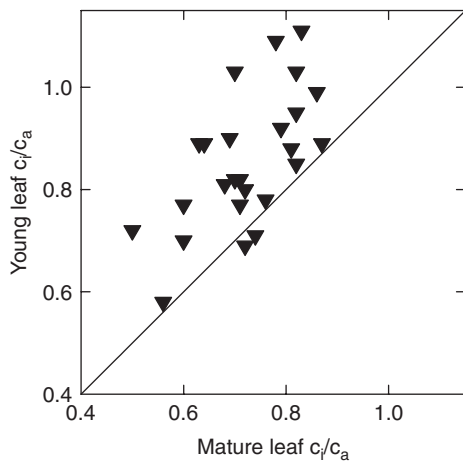
FIG. 3. Instantaneous measurements of the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) for young leaves plotted against those for mature leaves of the same species. Data are detailed in Table 2. The solid line shows the one-to-one line.

FIG. 4. The stable carbon isotope ratio of the dry matter ($δ¹³C_p$) of young and mature leaves plotted against instantaneous measurements of the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) in the same leaves. The solid line represents the theoretical relationship according to the model of Farquhar *et al.* (1982a). The broken lines represent least-squares regressions through the mature and young leaves. The regression equation for mature leaves is $δ¹³C_p = −10.4 − 23.8(c_i/c_a)$ ($R^2 = 0.90$), and that for young leaves is $δ¹³C_p = −7.8 − 22.3(c_i/c_a)$ ($R^2 = 0.64$).

Fig. 3). Why should young, expanding leaves have higher c_i/c_a than mature leaves? Figure 7A shows net CO_2 assimilation rates for young and mature leaves of two *Eucalyptus* species, for which CO_2 responses curves (A–c_i) were also measured (Cernusak, 2020). Analysis of the A–c_i curves provides insight
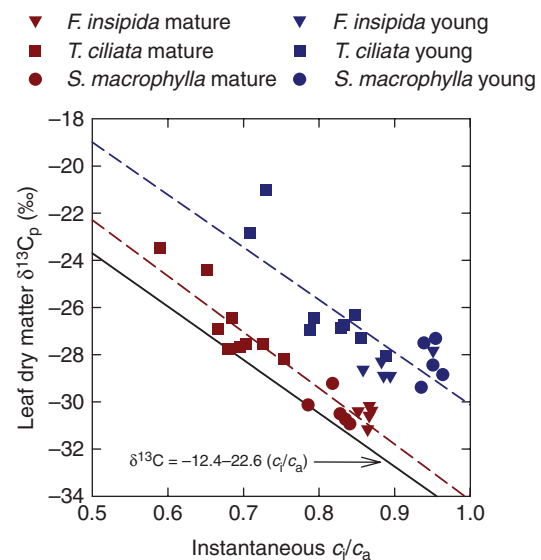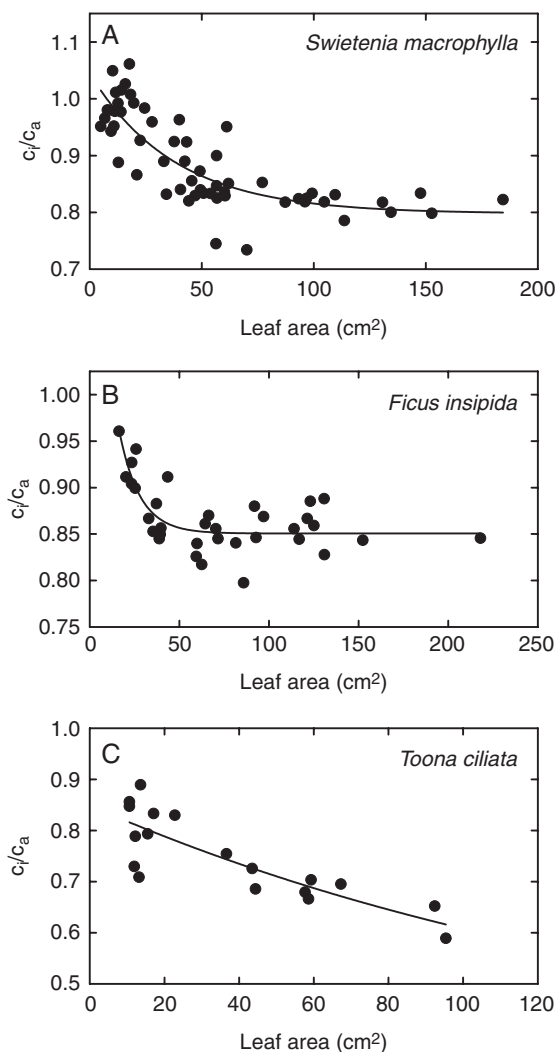
FIG. 5. Instantaneous measurements of the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) plotted against leaf area for three tropical tree species. Here, leaf area is a proxy for leaf age.
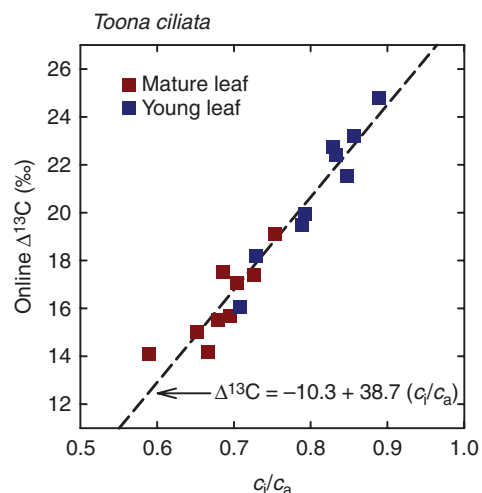

FIG. 6. Online measurements of carbon isotope discrimination ($\Delta^{13}C$) plotted against the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) measured at the same time; measurements were made in both young and mature leaves of *Toona ciliata*. The broken line shows a least squares regression through the data described by the equation shown at the bottom of the panel.

into why c_i/c_a is higher in young than in mature leaves. Young leaves have both lower photosynthetic capacity, represented by the maximum Rubisco carboxylation rate, V_{cmax} (Fig. 7B), and a higher day respiration rate, R_d (Fig. 7C). The combination of these two factors leads to less CO_2 being removed from the intercellular air spaces by carboxylation in the chloroplasts of the young leaves, due both to the lower carboxylation velocity and because more CO_2 is being fed into the chloroplasts by mitochondrial CO_2 release, which means less will come from the intercellular air spaces (Ubierna *et al.*, 2019). The patterns for net CO_2 assimilation, V_{cmax} and R_d can be approximated as exponential functions of leaf area, where leaf area is again taken as a proxy for leaf age, similar to the pattern of c_i/c_a change with leaf area (Fig. 5).

We showed, for the first time to our knowledge, that the instantaneous $\Delta^{13}C$ observed in young, expanding leaves is larger than that in mature leaves of the same plant, consistent with expectation based on their higher c_i/c_a (Fig. 6). The slope of the relationship between $\Delta^{13}C$ and c_i/c_a for this dataset, which

includes both young and mature leaves, was rather steep, although within expectations if one were to apply the more complete eqn (4) and assume mesophyll resistance remained in a fixed proportion to stomatal resistance. It indicated a change in $\Delta^{13}C$ of almost 4 ‰ for each 0.1 change in c_i/c_a. The mean difference in c_i/c_a between young and mature leaves from our literature survey was 0.13. Applying the $\Delta^{13}C$–c_i/c_a relationship that we observed for *T. ciliata* to this difference would predict a mean difference of about 5 ‰ in instantaneous $\Delta^{13}C$ between young and mature leaves; that is, young leaves would fix carbon depleted in ^{13}C by 5 ‰ compared to mature leaves. This would seem to make quite plausible the suggestion that young leaves can add enough of their own photosynthate to their structural carbon during leaf expansion to shift their overall $\delta^{13}C_p$ by about 2 ‰.

Figure 6 suggests that the relationships between $\Delta^{13}C$ and c_i/c_a for both young and mature leaves are similar, whereas Fig. 4 shows that the relationships between leaf $\delta^{13}C_p$ and c_i/c_a differ for young and mature leaves. The sequence of logic detailed above implies that the relationship between mature leaf $\delta^{13}C_p$ and c_i/c_a should reflect the legacy of the contribution to $\delta^{13}C_p$ of photosynthesis while the leaves were expanding, with this process having added carbon with more negative $\delta^{13}C$. If this is the case, we could expect the general relationship between mature leaf $\delta^{13}C_p$ and c_i/c_a established by Farquhar *et al.* (1982*a*), and subsequently confirmed in numerous other species (Farquhar *et al.*, 1989*a*; Cernusak *et al.*, 2013; Cernusak, 2020), to differ from instantaneous relationships between $\Delta^{13}C$ and c_i/c_a. The relationship observed for mature leaf dry matter $\delta^{13}C_p$ is the one represented by eqn (2) with $a_s = 4.4$ ‰ and $\bar{b} = 27$ ‰.

Our expectation is that this relationship, based on mature leaf $\delta^{13}C_p$, should appear to show a larger $\Delta^{13}C$ for a given c_i/c_a due to the ~2 ‰ depletion of $\delta^{13}C_p$ that occurs during leaf expansion than would be shown for instantaneous $\Delta^{13}C$ in mature leaves. Figure 8 shows example data for instantaneous $\Delta^{13}C$ and c_i/c_a for a dataset recently collected for mature leaves of grey
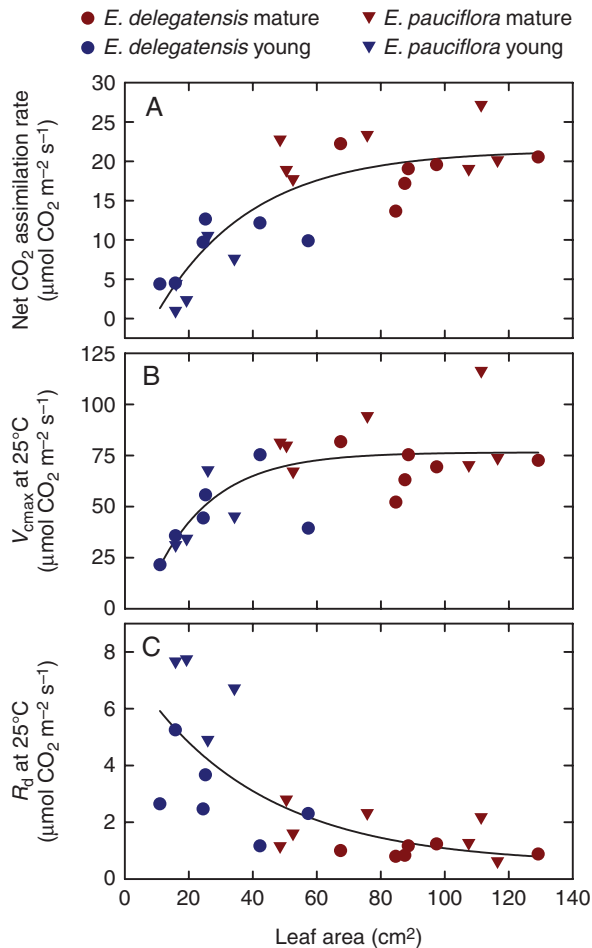
FIG. 7. Changes in net photosynthesis rate (A), maximum rate of carboxylation by Rubisco (B) and day respiration rate (C) as a function of leaf area for two *Eucalyptus* species. Here, leaf area represents a proxy for leaf age. These data were presented previously by Cernusak (2020), and details of the measurements are given there.

FIG. 8. Instantaneous measurements of carbon isotope discrimination ($\Delta^{13}C$) plotted against the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) measured concurrently. Data are for mature leaves of wild type plants of *Populus × canescens* from the dataset published by Cernusak *et al.* (2019). The solid line in the graph represents a general, empirical relationship between $\Delta^{13}C$ measured in mature leaf dry matter and c_i/c_a (Farquhar *et al.*, 1982a).

poplar, *Populus × canescens* (Cernusak *et al.*, 2019) and the relationship developed for mature leaf $\delta^{13}C_p$ (Farquhar *et al.*, 1982a). The relationship for mature leaf $\delta^{13}C_p$ is indeed offset compared to the instantaneous measurements, with $\Delta^{13}C$ from mature leaf $\delta^{13}C_p$ appearing to be larger for a given c_i/c_a than that for the instantaneous measurements. This pattern of the relationship for mature leaf $\delta^{13}C_p$ lying above observations of instantaneous $\Delta^{13}C$ for a given c_i/c_a is common to other datasets as well (Evans *et al.*, 1986; von Caemmerer and Evans, 1991). It is also consistent with $\Delta^{13}C$ inferred from measurements of mature leaf dry matter being larger than instantaneous observations of $\Delta^{13}C$ at c_i/c_a representative of growth conditions (Evans *et al.*, 1986; von Caemmerer and Evans, 1991). This latter difference for the mature leaves of *T. ciliata* in the present study averaged 3 ‰.

Is there other experimental evidence to support the sequence of logic given above to explain changes in leaf $\delta^{13}C_p$ with leaf expansion? One kind of experiment is to examine leaves in which photosynthesis did not occur during leaf expansion to see if they show different mature leaf $\delta^{13}C_p$ than
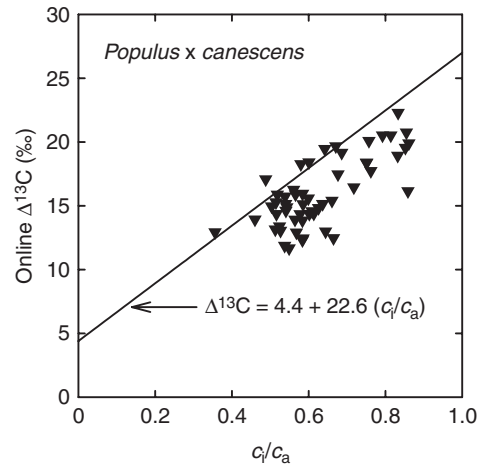
those in which photosynthesis did occur. Evans (1983) allowed leaves of *Triticum aestivum* and *Phaseolus vulgaris* to expand in darkness and therefore in the absence of photosynthesis. He found that dark-expanded leaves were consistently less negative in $\delta^{13}C$ than light-expanded leaves by 1 ‰. Terwilliger and Huang (1996) applied an inhibitor of carotene synthesis to allow chlorophyll-free leaves of *Lycopersicon esculentum* to grow alongside photosynthetic leaves on the same plant. They found that mature leaf $\delta^{13}C_p$ of the leaves that expanded without photosynthesis was 1–3 ‰ less negative than adjacent, simultaneously produced leaves that expanded with photosynthesis. Both of these experiments support the idea that leaf $\delta^{13}C_p$ becomes more negative during leaf expansion due to the progressive addition of the leaf's own photosynthate to its structural carbon, with this photosynthate being fixed at relatively high c_i/c_a and therefore more negative $\delta^{13}C$.

We found that deciduous species tended to have a larger $\delta^{13}C_p$ difference between mature and young leaves compared with evergreen species. We can think of two possible explanations for this. First, there could be additional fractionation associated with putting carbon into and taking it out of storage in deciduous species, such that the carbon that is used to reconstruct the canopy after a leafless period is less negative in $\delta^{13}C$ than the carbon that would be donated directly by mature, source leaves. There is some evidence in the literature to suggest that processes associated with carbon storage do lead to such ^{13}C enrichment of the stored carbon pool (Gleixner *et al.*, 1993, 1998; Damesin and Lelarge, 2003; Offermann *et al.*, 2011). Second, it is possible that in deciduous species the contribution of the expanding leaf's own photosynthesis to the mature leaf structural carbon is larger, due to lack of mature, source leaves exporting carbon at the time of new leaf emergence and expansion. Further research would be useful to help clarify the mechanism or mechanisms that lead deciduous species to have larger $\delta^{13}C_p$ differences between mature and young leaves than evergreen species.

The widespread nature of developmental depletion of $\delta^{13}C_p$ in leaves of C_3 plants and the explanation reiterated here, but put forth previously (Evans, 1983; Francey *et al.*, 1985; Evans *et al.*, 1986; Cernusak *et al.*, 2009*a*), have a number of important implications for interpreting leaf $\delta^{13}C_p$. First, the average $\delta^{13}C_p$ of C_3 leaves globally is probably depleted by about 2 ‰ compared to the $\delta^{13}C$ of global gross primary production of C_3 plants. This can be seen, for example, in stems and roots having less negative $\delta^{13}C$ than leaves (Badeck *et al.*, 2005; Cernusak *et al.*, 2009*a*). This is a detail which may be useful to include in ¹³C-constrained global carbon cycle models (Francey *et al.*, 1995; Lloyd *et al.*, 1996; Battle *et al.*, 2000; Kaplan *et al.*, 2002; Keeling *et al.*, 2017). Second, our analysis suggests that reconstruction of c_i/c_a from mature leaf $\delta^{13}C_p$ should be carried out using the relationship which is shown in eqn (2) above, with $a_s = 4.4$ ‰ and $\bar{b} = 27$ ‰ (Farquhar *et al.*, 1982*a*, *b*). If one instead uses a more complete model which has been parameterized based on observations of how instantaneous $\Delta^{13}C$ relates to c_i/c_a, this will cause c_i/c_a to be overestimated. This is because such a parameterization would not include the legacy effect of leaf expansion which causes $\Delta^{13}C$ measured in mature leaf dry matter to be larger than instantaneous $\Delta^{13}C$ measured in the same leaves. An example of this can be seen in a recent analysis in which the prediction of c_i/c_a with eqn (2) was made along with the prediction of a more complete model based on instantaneous $\Delta^{13}C$, with both compared to c_i/c_a measured by gas exchange (Bloomfield *et al.*, 2019). In that example, the latter prediction overestimated the observed c_i/c_a. Third, the extent of depletion in $\delta^{13}C_p$ which occurs between young leaves and mature leaves may provide useful insight into the dynamics of carbon sources for leaf expansion, in terms of import from source leaves and storage vs. the leaf's own photosynthesis (Terwilliger *et al.*, 2001).

Finally, one of the most useful applications of leaf $\delta^{13}C_p$ for C_3 plants is as an indicator of intrinsic water-use efficiency (Farquhar and Richards, 1984; Farquhar *et al.*, 1989*a*). The relationship between intrinsic water-use efficiency and mature leaf $\delta^{13}C_p$ has generally been observed to be robust (Farquhar *et al.*, 1989*a*; Cernusak *et al.*, 2013; Cernusak, 2020). However, our analysis suggests that for young vs. mature leaves of C_3 plants, higher $\delta^{13}C_p$ should not be interpreted as an indication of higher intrinsic water-use efficiency. Indeed, the pattern of change for intrinsic water-use efficiency as C_3 leaves expand is just the opposite.

ACKNOWLEDGMENTS

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