1 Carbon Isotopes and Water Use Efficiency in C₄ Plants

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10 Abstract

11 Drought is a major agricultural problem worldwide. Therefore, selection for
12 increased water use efficiency (WUE) in food and biofuel crop species will be an
13 important trait in plant breeding programs. The leaf carbon isotopic composition
14 ($\delta^{13}\text{C}_{\text{leaf}}$) has been suggested to serve as a rapid and effective high throughput
15 phenotyping method for WUE in both C_3 and C_4 species. This is because WUE, leaf
16 carbon discrimination ($\Delta^{13}\text{C}_{\text{leaf}}$), and $\delta^{13}\text{C}_{\text{leaf}}$ are correlated through their relationships
17 with intercellular to ambient CO_2 partial pressures (C_i/C_a). However, in C_4 plants,
18 changing environmental conditions may influence photosynthetic efficiency (bundle-
19 sheath leakiness) and post-photosynthetic fractionation that will potentially alter the
20 relationship between $\delta^{13}\text{C}_{\text{leaf}}$ and C_i/C_a . Here we discuss how these factors influence the
21 relationship between $\delta^{13}\text{C}_{\text{leaf}}$ and WUE, and the potential of using $\delta^{13}\text{C}_{\text{leaf}}$ as a
22 meaningful proxy for WUE.

Drought - a major, widespread agricultural problem

Drought in both rain-fed and irrigated agricultural land is a worldwide constraint to crop production. Therefore, plant-breeding programs are working to develop new high yielding crop varieties that can cope with these conditions [1,2]. In recent years breeding programs have undergone rapid advancements in molecular and genomics techniques [3,4]; however, high-throughput phenotyping of complex traits related to water use efficiency (WUE) is lagging behind [5,6]. For example, traditional phenotyping for WUE requires laborious and costly measurements that are not feasible on a large numbers of individual plants. Therefore, few crops have been bred for WUE, particularly in C₄ species [7]. However, as outlined below, the carbon isotope signature of leaves ($\delta^{13}\text{C}_{\text{leaf}}$) may be a potential WUE proxy for high-throughput phenotyping of C₄ plants.

Defining water use efficiency (WUE)

Agricultural WUE is related to the harvestable yield relative to the amount of water used by the crop. Simplistically, this can be demonstrated by the fact that crop yield can be defined by whole plant transpiration efficiency (TE_{plant}), the total water used by the crop, and the harvest index (HI) as [8]:

$$\text{Yield} = \text{TE}_{\text{plant}} \times \text{Water Use} \times \text{HI} \quad \text{Equation 1.}$$

Where TE_{plant} is defined as the total harvestable yield for a given amount of transpired water and is an important component of agricultural WUE and yield (see Box 1). TE_{plant} is a complex trait that is partly driven by leaf level TE (TE_{leaf}), the net rate of CO₂ assimilation (A_{net}) relative to the rate of transpiration (E). Where A_{net} and E are linked by stomatal conductance (g_s) through

$$A_{\text{net}} = g_s \text{CO}_2 (C_a - C_i), \quad E = g_s \text{H}_2\text{O} (e_i - e_a) \quad \text{Equations 2, 3}$$

and

$$\text{TE}_{\text{leaf}} = \frac{A}{E} = \frac{C_a(1 - C_i/C_a)}{1.6(e_i - e_a)} \quad \text{Equation 4}$$

(see Box 2 for term definitions). These simple equations describe how A_{net} , g_s and the water vapor pressure deficient (VPD) between the leaf and the surrounding air influence TE_{leaf} . However, the intrinsic TE ($\text{TE}_{\text{intrinsic}}$)

$$TE_{\text{intrinsic}} = \frac{A_{\text{net}}}{g_s} = \left(1 - \frac{C_i}{C_a}\right) \quad \text{Equation 5}$$

is a better indicator of genotypic differences in WUE because it is not driven by variation in VPD, which is determined by leaf temperature and the relative humidity of the surrounding air [9]. Values of A_{net} and g_s drive $TE_{\text{intrinsic}}$ in opposite directions but both influence C_i/C_a . For example, greater A_{net} can decrease C_i/C_a through increased CO_2 fixation, while greater g_s will increase C_i/C_a by increasing CO_2 supply coming into the leaf. From an agricultural perspective it is desirable to take advantage of all water available in a field. Therefore, increasing TE_{plant} by reducing g_s is not advantageous if the net result is maintaining yield while water remains unused in the field. Instead it is better to increase TE_{plant} by improving A_{net} relative to g_s to obtain higher yields while using all of the available water [9].

Relationship between $\Delta^{13}\text{C}$, $\delta^{13}\text{C}_{\text{leaf}}$ and $TE_{\text{intrinsic}}$

The link between $\delta^{13}\text{C}_{\text{leaf}}$ and C_i/C_a is driven by leaf CO_2 discrimination ($\Delta^{13}\text{C}_{\text{leaf}}$), which can be estimated as

$$\Delta^{13}\text{C}_{\text{leaf}} = \frac{(\delta_s - \delta_p)}{(1 + \delta_p)} \quad \text{Equation 6}$$

where δ_p and δ_s represent the carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) relative to a standard of leaf material and the CO_2 available to the leaf, respectively. Compared to C_3 plants, the relationship between $\Delta^{13}\text{C}_{\text{leaf}}$ and C_i/C_a is diminished in C_4 plants and is less variable in response to growth conditions or between genotypes [11,12]. For example, $\delta^{13}\text{C}_{\text{leaf}}$ of most C_4 species fall between -12 and -15 ‰, while $\delta^{13}\text{C}_{\text{leaf}}$ of most C_3 plants varies between -23 and -32 ‰ [12,13]. However, even in C_4 plants there are well defined fractionation steps associated with CO_2 diffusion through the stomata, the biochemistry of CO_2 fixation by Rubisco, HCO_3^- fixation by phosphoenolpyruvate carboxylase (PEPC), mesophyll conductance to CO_2 (g_m), carbonic anhydrase activity, bundle-sheath leakiness (ϕ), and C_i/C_a that influence $\Delta^{13}\text{C}_{\text{leaf}}$ [11,12,14]. Therefore, measurements of $\delta^{13}\text{C}_{\text{leaf}}$ in C_4 leaves have been used as proxy to estimate changes in C_4 photosynthetic efficiency and $TE_{\text{intrinsic}}$ in response to various growth conditions. However, to be an effective proxy requires detailed understanding of the factors influencing $\delta^{13}\text{C}_{\text{leaf}}$. Below we discuss several of the primary mechanisms driving $\delta^{13}\text{C}_{\text{leaf}}$ in C_4 plants and the potential use of $\delta^{13}\text{C}_{\text{leaf}}$ for estimating $TE_{\text{intrinsic}}$.

Leaf CO₂ discrimination ($\Delta^{13}C_{leaf}$)

Several fractionation factors associated with CO₂ diffusion and fixation by photosynthetic enzymes influence $\Delta^{13}C_{leaf}$. For example, in C₄ plants $\Delta^{13}C_{leaf}$ is strongly determined by C_i/C_a and the efficiency of the CO₂ concentrating mechanism (ϕ ; leakiness) [11,14]. $\Delta^{13}C_{leaf}$ forms an inverse relationship with C_i/C_a when ϕ is below 0.37, but has an opposite relationship if ϕ is higher [11,12,14]. However, as will be discussed in the next section, C₄ photosynthesis is well coordinated through the metabolic flux between the C₄ and C₃ cycles and when ϕ is estimated with direct measurements of photosynthesis and leaf CO₂ isotope exchange it is relatively constant between species and under various growth conditions [See Box 3; 11,12,14-18]. The relationship of $\Delta^{13}C_{leaf}$ in C₄ plants with C_i/C_a can be explained with a simplified model as:

$$\Delta^{13}C_{leaf} = a + (b_4 + \phi(b_3 - s) - a) C_i/C_a \quad \text{Equation 7}$$

where a , b_4 , b_3 , ϕ , s , C_i , and C_a are defined in Box 1. By parameterizing these factors under various conditions, $\Delta^{13}C_{leaf}$ can be primarily attributed to variation in C_i/C_a . Although this equation is a simplified version of the original model described by Farquhar (1984), it incorporates the major fractionation factors influencing $\delta^{13}C$.

Bundle-sheath leakiness

Bundle-sheath leakiness (ϕ) describes the efficiency of the CO₂-concentrating mechanism by the leak rate of CO₂ from the bundle sheath relative to the rate of CO₂ supplied by the C₄ cycle [11,14]. Changes in ϕ can have a strong influence on $\Delta^{13}C_{leaf}$ because it controls the extent of Rubisco discrimination, which has a large fractionation factor. Therefore, ϕ increases $\Delta^{13}C_{leaf}$ at a given C_i/C_a , controlling the relationship between $\Delta^{13}C_{plant}$ and C_i/C_a [11,14]. Species or environmental-driven differences in ϕ , therefore, will cause differences in $\Delta^{13}C_{leaf}$ independent of changes in C_i/C_a . Much of the literature that details the response of ϕ to growth conditions was initially tested with spot measurements of C_i/C_a and $\delta^{13}C_{leaf}$ using Equations 6 and 7. However, on-line measurements of $\Delta^{13}C_{leaf}$ coupled with leaf gas exchange have raised questions about the relationship between $\Delta^{13}C_{leaf}$, $\delta^{13}C_{leaf}$ and ϕ [See Box 3;11,14].

For example, $\Delta^{13}C_{leaf}$ calculated from $\delta^{13}C_{leaf}$ was higher in drought- and salt-stressed, and nitrogen-limited plants compared to plants grown under optimal conditions [22-28]. Additionally, $\Delta^{13}C_{leaf}$ calculated from dry matter $\delta^{13}C_{leaf}$ increased

when plants were grown under low light [27,29]. These studies suggest from comparisons of spot measurements of C_i/C_a and $\Delta^{13}C_{\text{leaf}}$ estimated from $\delta^{13}C_{\text{leaf}}$ that ϕ is affected by growth under low light, limited nitrogen availability, and water and salt stress [22-27,27-29]. However, because $\delta^{13}C_{\text{leaf}}$ integrates over the life of the leaf, instantaneous measurements of $\Delta^{13}C_{\text{leaf}}$ and $\delta^{13}C_{\text{leaf}}$ do not always correlate, raising questions to how well $\delta^{13}C_{\text{leaf}}$ relates to changes in ϕ . Several studies comparing direct measurements of $\Delta^{13}C_{\text{leaf}}$ and $\Delta^{13}C_{\text{leaf}}$ calculated from $\delta^{13}C_{\text{leaf}}$ have shown significant differences that are not always related to ϕ [30-33]. In general, $\delta^{13}C_{\text{leaf}}$ measurements suggest ϕ values are more variable and higher than those calculated from direct $\Delta^{13}C_{\text{leaf}}$ measurements [30-32,34,35, see Figure 1]. As a result, dry leaf matter-derived $\Delta^{13}C_{\text{leaf}}$ produce ϕ values typically between 0.3 and 0.5, while ϕ values from direct $\Delta^{13}C_{\text{leaf}}$ fall between 0.21 and 0.3 [30,32,36]. The disparity between these estimates of ϕ is likely due to differences in time integration of the measurements. For example, $\delta^{13}C_{\text{leaf}}$ will be influenced by changes in C_i/C_a that occur over the lifetime of the leaf and post-photosynthetic fractionations, which will be discussed below.

Direct measurements of $\Delta^{13}C_{\text{leaf}}$ generally find that ϕ is relatively constant under various environmental conditions [11,12,14-18,31-34]. An exception is that ϕ has been shown to increase under very low light intensities [29,32,36-40], but much of the increase in ϕ was attributed to oversimplification of $\Delta^{13}C_{\text{leaf}}$ model [14,15,17]. In fact, using the full formulation of the $\Delta^{13}C_{\text{leaf}}$ model developed by Farquhar (1984) typically indicates little to no change in ϕ under low light [17,36,37,41]. Additionally, ϕ was generally found to acclimate to rapid changes in light quality [42]. Therefore, direct measurements of $\Delta^{13}C_{\text{leaf}}$ generally indicate that C_4 plants have a remarkable ability to coordinate the metabolic flux through the C_4 and C_3 cycles, and that ϕ is relatively constant between species and across different growth conditions. The C_4 and C_3 cycles have extensive metabolic and redox regulation between the mesophyll and bundle sheath cells, so that the two cycles are really two parts of one pathway [43].

Potential post-photosynthetic fractionations

Fractionation that occurs downstream from photosynthesis is less well characterized but will also influence $\delta^{13}C_{\text{leaf}}$ [11,44]. Post-photosynthetic fractionations are associated with generating carbon pools for leaf construction, export and import into and out of leaves, and respiration [11,44]. However, there is insufficient research in

143 this area and a better understanding of the fractionations associated with these
144 processes is needed to disentangle the relationship between $\delta^{13}\text{C}_{\text{leaf}}$ and direct
145 measurements of $\Delta^{13}\text{C}_{\text{leaf}}$. To further complicate matters, it is not clear how growth
146 conditions influence post-photosynthetic fractionations.

147 The preferential use of isotopically distinct carbon pools for respiration or
148 movement between leaves will also affect $\delta^{13}\text{C}_{\text{leaf}}$ [11]. For example, in both C_3 and C_4
149 plants carbohydrate pools such as sucrose and starch tend to be enriched in ^{13}C relative
150 to bulk leaf tissue, while lipids, organic acids, and proteins are depleted in ^{13}C [11,45].
151 Additionally, sucrose produced from triose-phosphates in the cytosol during the day is
152 depleted in ^{13}C relative to the leaf, but sucrose produced at night from starch
153 remobilization is enriched in ^{13}C relative to the leaf [45]. In C_3 *Ricinus communis*, the
154 isotopic composition of exported sucrose (or imported into sink leaves) depends on the
155 ratio of day/night sucrose synthesis [46]. Unfortunately, the effect that these
156 fractionations have on $\delta^{13}\text{C}_{\text{leaf}}$ is not well known and further research is needed on
157 understanding the fractionations associated with carbon movement between C_4 leaves,
158 especially under drought.

159 Respiration may also influence $\delta^{13}\text{C}_{\text{leaf}}$ by shifting between isotopically different
160 pools of carbon in response to stresses or other physiological changes. For example, in
161 C_3 plants consuming triose-phosphates evolves CO_2 depleted in ^{13}C , but the magnitude
162 of this depletion depends on the decarboxylating enzymes involved and the $\delta^{13}\text{C}$ of
163 carbon atoms in the triose-phosphates [44, 47]. When the Krebs cycle is coupled with β -
164 oxidation of fatty acids, evolved CO_2 is more depleted than when pyruvate
165 dehydrogenation predominates [44]. This likely explains the larger than expected
166 variation in $\delta^{13}\text{C}$ of nocturnal leaf respiration measured in the C_4 grass *Sporobolus*
167 *wrightii* [48]. Furthermore, under stressful growth conditions other carbon sources such
168 as lipids could be used for respiration [44,47]. However, the fractionation associated
169 with the shift in respiratory carbon source would have to be quite large to be
170 responsible for the $\sim 1\text{‰}$ change in $\delta^{13}\text{C}_{\text{leaf}}$ that have been measured between well-
171 watered and drought treatments (Ellsworth et al. unpublished and in review). In C_3
172 plants there were no dramatic shifts in respiratory carbon under drought stress [47,49];
173 however, this has not been characterized in C_4 plants. Although respiration is not likely
174 responsible of the entire change in $\delta^{13}\text{C}_{\text{leaf}}$ measured across water availability it may
175 have some effect and more research is also needed in this area, particularly in C_4 plants.

Drought and $\delta^{13}\text{C}_{\text{leaf}}$

Measurements of $\delta^{13}\text{C}_{\text{leaf}}$ as a proxy for $\text{TE}_{\text{intrinsic}}$ in plant breeding programs has long been suggested for both C_3 and C_4 crop species [10], but recently has seen more interest because of the need for a high throughput proxy for $\text{TE}_{\text{intrinsic}}$ [1,2,50,51]. To date, $\delta^{13}\text{C}_{\text{leaf}}$ has only been successfully used to screen for $\text{TE}_{\text{intrinsic}}$ in wheat breeding programs in Australia [52]. However, the response of $\delta^{13}\text{C}$ to drought has been tested in several C_4 species such as maize [53-57], sugar cane [22], sorghum [58], green foxtail millet and foxtail millet (Ellsworth et al., in review), pearl millet [55,57], and numerous other grass species covering all three C_4 biochemical subtypes [26,39,59,60]. Consistently, across these experiments and C_4 subtypes, the $\delta^{13}\text{C}$ values are lower in water-limited plants compared to the corresponding well-watered plants, independent of the tissue sampled (leaf, kernel, shoot, silk, or cob). This shift in $\delta^{13}\text{C}_{\text{leaf}}$ between treatments is generally dependent on the severity of the drought and interactions with other treatments such as nitrogen, phosphorus, or light limitation and elevated CO_2 . However, plants under drought consistently have lower $\delta^{13}\text{C}_{\text{leaf}}$ values than the well-watered plants suggesting a robust response of $\delta^{13}\text{C}_{\text{leaf}}$ to drought in C_4 plants. Furthermore, studies comparing $\delta^{13}\text{C}$ to g_s and, in some cases, $\text{TE}_{\text{intrinsic}}$ showed C_4 plants under drought decreased g_s , increased $\text{TE}_{\text{intrinsic}}$, and had lower $\delta^{13}\text{C}_{\text{leaf}}$ [22,26,53,56,58, Ellsworth et al., in review].

These studies demonstrate that decreases in C_i/C_a under drought conditions consistently influence $\delta^{13}\text{C}_{\text{leaf}}$, and with further testing may be an effective proxy for $\text{TE}_{\text{intrinsic}}$ in C_4 plants (See example in Figure 1). However, the sensitivity to which $\delta^{13}\text{C}_{\text{leaf}}$ can distinguish variation in WUE still needs to be tested. For example, $\delta^{13}\text{C}_{\text{leaf}}$ may be sufficient as a first pass to separate genotypes with high and low $\text{TE}_{\text{intrinsic}}$ but not suitable for fine scale screening to separate genotypes with only slight differences in $\text{TE}_{\text{intrinsic}}$ [53,54]. Unfortunately, there are few drought experiments where ϕ has been determined using direct measurements of $\Delta^{13}\text{C}_{\text{leaf}}$ [59]. Therefore, the effect of drought on ϕ and its influence on $\delta^{13}\text{C}_{\text{leaf}}$ requires further study. However, considering ϕ appears to be relatively constant, the primary driver of $\delta^{13}\text{C}_{\text{leaf}}$ under drought is likely changes in C_i/C_a . Of course, the influence of post-photosynthetic fractionations associated with respiration and mobilization of carbon pools within the leaf will potentially limit the sensitivity of using $\delta^{13}\text{C}_{\text{leaf}}$.

Conclusion and future directions

With the current focus on breeding more water use efficient crops and the predicted increase in drought and growing season temperatures, C₄ crops will continue to play an important role in agriculture. In the modern age of marker-assisted plant breeding, techniques to screen for physiological traits such TE_{intrinsic} are needed to assess phenotypic variation across large numbers of genotypes. Therefore, the development of $\delta^{13}\text{C}$ as a tool for estimating TE_{intrinsic} in large-scale plant breeding programs will be beneficial. However, more research is needed to solidify the relationship of TE_{intrinsic}, $\delta^{13}\text{C}_{\text{leaf}}$, and C_i/C_a for more C₄ species under various growth conditions. This will include the elucidation of post-photosynthetic fractionations and their effects on $\delta^{13}\text{C}_{\text{leaf}}$ to further refine these relationships. Additionally, more experiments are needed to assess $\delta^{13}\text{C}_{\text{leaf}}$ variation in agriculturally important breeding populations to help identify quantitative trait loci and potential gene candidates influencing $\delta^{13}\text{C}_{\text{leaf}}$ and TE_{intrinsic}.

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226 Figure 1. Relationship between $\Delta^{13}\text{C}_{\text{leaf}}$, $\delta^{13}\text{C}_{\text{leaf}}$ and C_i/C_a in *Setaria italica* and *S. viridis*
227 grown under well-watered or drought conditions (Ellsworth *et al.*, unpublished data).
228 The y-axes show $\Delta^{13}\text{C}_{\text{leaf}}$ calculated from $\delta^{13}\text{C}_{\text{leaf}}$ (Equation 6) and the corresponding
229 $\delta^{13}\text{C}_{\text{leaf}}$, and the x-axis shows the measured C_i/C_a . The $\delta^{13}\text{C}$ of the CO_2 in the growth
230 chambers was -10 ‰. The line represents the theoretical relationship between $\Delta^{13}\text{C}_{\text{leaf}}$,
231 $\delta^{13}\text{C}_{\text{leaf}}$ and C_i/C_a (based on Equation 7; where ϕ was assumed to be 0.21). The $\Delta^{13}\text{C}_{\text{leaf}}$
232 estimated from $\delta^{13}\text{C}_{\text{leaf}}$ are generally above the theoretical line, consistent with post-
233 photosynthetic fractionation that is not taken into account in Equation 7 [30-32,34,35].

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Box 2. Definitions of terms

g_{CO_2} = stomatal conductance to CO_2

$g_{\text{H}_2\text{O}}$ = stomatal conductance to H_2O *

C_i = leaf intercellular CO_2 partial pressure

C_a = ambient CO_2 partial pressure

e_i = water vapor pressures inside the leaf

e_a = water vapor pressures surrounding the leaf

a = fractionation during diffusion of CO_2 in air through stomata (4.4 ‰)

b_3 = fractionation by Rubisco (30 ‰)

b_4 = combined fractionation of PEP carboxylation & preceding isotopic equilibrium during dissolution and hydration of CO_2 (-5.2 ‰ at a leaf temperature of 30 °C)

ϕ = leakiness; fraction of CO_2 released from bundle sheath cells by the C_4 cycle and not fixed by Rubisco

s = fractionation during the leakage of CO_2 out of the bundle sheath cells (1.8 ‰)

*The ratio of diffusivity of H_2O vapor to CO_2 in air is 1.6.

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Box 3. On-line (direct) $\Delta^{13}\text{C}_{\text{leaf}}$ measurements

On-line or direct measurements of leaf carbon isotopic discrimination ($\Delta^{13}\text{C}_{\text{leaf}}$) combine measurements of $\delta^{13}\text{C}$ of CO_2 entering and leaving a leaf gas exchange chamber. Under these conditions $\Delta^{13}\text{C}_{\text{leaf}}$ represents the instantaneous fractionation associated with CO_2 diffusion, photosynthesis, photorespiration, and respiration. Alternatively, the dry matter leaf carbon isotopic composition ($\delta^{13}\text{C}_{\text{leaf}}$) can be used as an indirect measure of $\Delta^{13}\text{C}_{\text{leaf}}$ that integrates variation in CO_2 availability (C_i/C_a), photosynthetic and post-photosynthetic fractionations over the life of the leaf. Tunable diode laser absorption spectroscopy (TDL) measures concentrations of $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$ and allows for continuous measurements of $\Delta^{13}\text{C}_{\text{leaf}}$ in response to changes in measurement conditions [11]. However, the TDL measurements provide a snapshot of leaf level CO_2 isotope exchange and do not necessarily capture all of the integrated fractionation factors that drive variation in $\delta^{13}\text{C}_{\text{leaf}}$.

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Fig. 1