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DEVIATION BETWEEN $\delta^{13}C$ AND LEAF INTERCELLULAR CO_2 IN *SALIX INTERIOR* CUTTINGS DEVELOPING UNDER LOW LIGHT

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Leaf $\delta^{13}C$ values of perennial species are sometimes less negative than model-predicted $\delta^{13}C$ values derived from instantaneous measures of p_i/p_a. It has been hypothesized that the less negative $\delta^{13}C$ values could be caused by ^{13}C-enriched stored carbon imported during the early stages of leaf growth. The $\delta^{13}C$ values of newly emerging leaves could thus represent $\delta^{13}C$ values of stem-stored carbohydrates and may also provide integral measures of p_i/p_a at the end of the past growing season. We tested these hypotheses by sprouting cuttings of *Salix interior* under wet and dry soil-moisture conditions in a controlled environmental chamber. Plants were defoliated after 56 d, and watering treatments were then reversed for half of the plants in each treatment. The $\delta^{13}C$ values of newly emerging leaves did not correlate with p_i/p_a ratios of newly emerging leaves or of mature leaves prior to defoliation, thereby indicative that $\delta^{13}C$ values of newly emerging leaves are not a simple reflection of prior p_i/p_a. Also, the $\delta^{13}C$ values of newly emerging leaves were more enriched in ^{13}C relative to the $\delta^{13}C$ values of stem carbohydrates in the treatments where water regimes were reversed. Newly emerging leaves after defoliation had higher $\delta^{13}C$ values despite the lower instantaneous water-use efficiency and similar values of p_i/p_a to older photosynthetic leaves. Large differences between observed and model-predicted p_i/p_a values also occurred in older, more mature leaves, and this may be because large proportions of their total mass were derived from carbon import.

Keywords: $\delta^{13}C$, carbon import, *Salix*.

Introduction

The carbon isotopic composition ($\delta^{13}C$) of C_3 plants has been an invaluable source of information about ratios of leaf internal-to-atmospheric CO_2 concentrations (p_i/p_a) and intrinsic water-use efficiency (Ehleringer et al. 1993). This is due to the fact that p_i/p_a influences the extent to which ^{13}C is discriminated against during carboxylation and diffusion and because p_i/p_a is inversely related to intrinsic water-use efficiency (Farquhar et al. 1982, 1989; Ehleringer et al. 1991, 1993). As a result, carbon isotopic ratios of plant tissues are commonly used as integrated measures of intrinsic water-use efficiency in studies of plant responses to environment (Ehleringer et al. 1993). Nonetheless, $\delta^{13}C$ values are sometimes less negative than would be expected from model-predicted $\delta^{13}C$ values derived from instantaneous measurements of p_i/p_a (Vitousek et al. 1990; Sauer et al. 1995). One cause for the less negative $\delta^{13}C$ values is import of stored organic carbon. There is now considerable empirical evidence showing that newly emerging leaves may be enriched in ^{13}C by 2‰–5‰ relative to mature leaves of trees (Lowden and Dyke 1974; Leavitt and Long 1985; Sobrado and Ehleringer 1997; Terwilliger 1997). Based on the current models (Farquhar et al. 1982, 1989), the newly emerging leaves should have lower average p_i/p_a and higher water-use efficiency than older leaves. In contrast, newly emerging leaves commonly exhibit lower water-use efficiencies than older, fully expanded leaves (Sobrado 1996; Terwilliger 1997). Two alternative interpretations of the ^{13}C-enriched isotopic signatures of newly emerging leaf material have been offered (Leavitt and Long 1985; Smedley et al. 1991). First, the $\delta^{13}C$ signatures of newly emerging leaves may reflect the $\delta^{13}C$ of carbohydrates imported from other leaves before the newly emerging leaves are photosynthetically competent. These values may simply represent the accumulation of carbon under drier conditions by the mature leaves at the end of the previous growing season (Smedley et al. 1991). If so, then the $\delta^{13}C$ of older leaves may provide an integrated measure of p_i/p_a, water-use efficiency, and environmental conditions over two successive growing seasons. The second viewpoint is that the more enriched $\delta^{13}C$ values of newly emerging leaves may simply represent the carbon isotopic ratios of a more ^{13}C-enriched storage pool of carbon in the stems that are caused by fractionation during secondary metabolism (Gleixner et al. 1998; Pate and Arthur 1998). The implication of this hypothesis is that newly emerging leaves may represent the isotopic signatures of imported carbon (heterotrophic carbon) that carries an additional postcarboxylation fractionation event that is independent of p_i/p_a. If leaf growth incorporates a relatively large proportion of carbon that is derived from fractionated carbon derived from carbon import relative to carbon derived from its own photosynthetic products (autotrophic carbon), then the isotopic signatures present in the heterotrophic, newly emerging leaves may influence the $\delta^{13}C$ values of the older autotrophic leaves. In such instances, $\delta^{13}C$ values of leaf tissue may not solely represent the p_i/p_a and water-use efficiency dur-

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ing the growing season. This influence may be particularly obvious in deciduous tree species where stored organic compounds are the dominant carbon sources for leaf growth in the early spring.

We performed this study to test whether the carbon isotopic ratios of young leaves reflected the carbon isotopic ratios and p_i/p_a of mature leaves in the previous growing season or whether they were influenced by isotopic ratios such as those found in the stem storage pool. Our second objective was to determine whether the initial $\delta^{13}C$ values of newly emerging heterotrophic leaves influence the $\delta^{13}C$ values of the leaves once they have matured. We used cuttings of *Salix interior* because leaf development from the axillary buds could be stimulated easily through the removal of older leaves. This allowed us to initiate development of new leaves under contrasting environmental conditions to determine the potential influence of previous environmental growth conditions on the carbon isotopic ratio of young leaf tissue.

Material and Methods

Experimental Design

Genetically similar individuals were obtained by detaching 100 distal stem sections from a single tree of *Salix interior* (Rowlee) growing near Clinton Lake in northeastern Kansas (38°56′N, 95°20′W). Stem sections were planted with Rootex hormone number 3 (Brooker Chemical, Chatsworth, Calif.) in potting mix in 0.25-L cups in January 1997. Cuttings were placed in a growth chamber with a 14 h light/10 h dark regime. Day and night temperatures were maintained at 25° and 20°C, respectively. Day and night relative humidity ranged between 50% and 60%, and the mean PPFD at plant height was 245 μmol m^{-2} s^{-1}. Half of the plants were watered daily with 50 mL of deionized water, that is, 55 plants (wet), and the other half were watered every third day, that is, 55 plants (dry). Nutrients were applied once every 2 wk in the form of 0.1% Schultz Instant Plant Food (Schultz, St. Louis), which consisted of 20 : 30 : 20 NPK and less than 0.1% Zn, Fe, and Mn. After 3 mo, plants were defoliated and transplanted into 2-L pots (fig. 1). The watering treatments were maintained for 56 d after defoliation. At 56 d, predawn water potentials, gas exchange, and carbon isotopic ratios were measured using five plants in the wet and dry treatments to establish whether the watering regimes had a significant effect on the stomatal conductance. The plants were defoliated again, and watering treatments were reversed for half of the plants, whereas the watering treatments were unchanged in the remaining plants such that four watering treatments were implemented after the second defoliation (fig. 1). Each group received one of the following treatments: daily watering for 56 d followed by 50 d of the same treatment after defoliation (wet-wet), daily watering for 56 d followed by watering every third day for 50 d after defoliation (wet-dry), watering every third day for 56 d followed by daily watering for 50 d after defoliation (dry-wet), and watering every third day for 56 d followed by 50 d of the same treatment after defoliation (dry-dry).

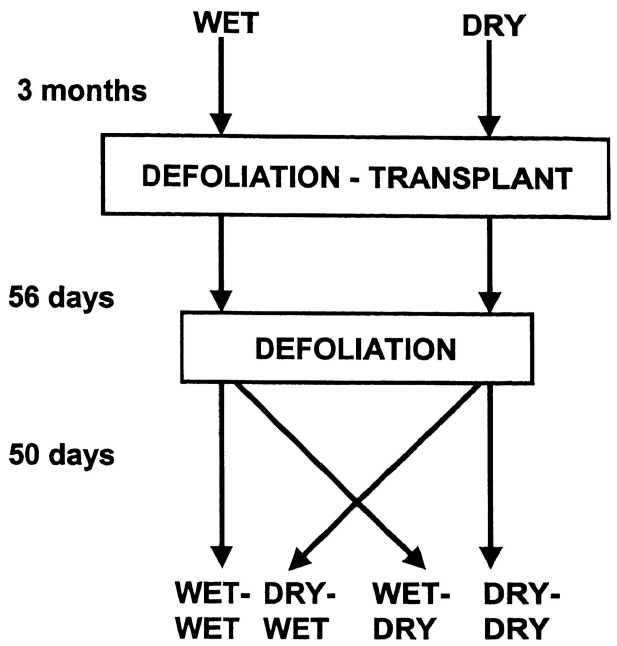

Fig. 1 Experimental design

Instantaneous Physiological Measurements

Leaf water potentials were measured between 0600 and 0700 hours, before the growth chamber lights were switched on, using a Scholander pressure chamber (PMS Instrument, Corvallis, Oreg.). Two leaves from each of five individuals in the wet and dry treatments were measured. Net photosynthesis, stomatal conductance, transpiration, and p_i/p_a ratios were determined in the growth chamber using a CID PS301 open-system IRGA and a CID leaf chamber (CID, Vancouver, Wash.) with a window area of 2.54 cm^2. Two measurements were taken on each of three leaves per plant. Three to five plants were measured depending on leaf availability. Measurements were made between 1000 and 1600 hours in the growth chamber at 7, 20, 30, and 50 d after leaf emergence. When leaves did not completely cover the window of the chamber, the areas of the portions of leaves inside the chamber were measured using a LI-COR LI-3000 area meter (LI-COR, Lincoln, Nebr.). Because leaves were amphistomatous, leaf areas were doubled to calculate the gas-exchange parameters (von Caemmerer and Farquhar 1981). After gas exchange and area measurements, leaves were dried at 70°C to determine their dry masses at different ages.

Tissue Preparation and Organic Extractions

Whole-leaf tissue samples and stem fractions containing soluble sugars, amino acids, organic acids, and starch were prepared for carbon-isotopic-ratio analysis to allow comparison between the $\delta^{13}C$ of young leaf tissue and the various stem organic fractions. Leaves used for gas-exchange measurements were dried at 70°C for 3 d. The three leaves from each plant were pooled and ground into a fine powder using a mortar and pestle. Stems harvested from plants at 7 d after leaf emergence were dried and ground with a coffee mill. Three 100-

mg subsamples of dried stem tissue were used to extract a mixed pool of organic carbon, pure sugars, and starch, respectively, according to the methods of Brugnoli et al. (1988) and Ehleringer (1991).

For the mixed organic fraction, 100 mg of dry-tissue powder was boiled in 10 mL of 80% ethanol for 20 min and then centrifuged at 2000 *g* for 10 min. The supernatant was collected, and the tissue was again boiled in a second 10-mL aliquot of 80% ethanol for 10 min. After centrifugation, the supernatants were combined, and the ethanol was evaporated at 40°C. The residue was dried further in an oven at 70°C for 48 h.

Sugars were extracted by boiling 100 mg of dry-tissue powder in 35 mL water for 30 min. After centrifugation at 2000 *g* for 15 min, the supernatant was collected and mixed with Dowex-50 (pretreated with 1 N HCl) for 20 min to remove all the soluble amino acids from the solution. Samples were filtered, and the filtrates were mixed with Dowex-1 (pretreated with 1 N HCl) for 20 min to remove organic acids from the solution. After several filtrations, the filtrate was evaporated at 40°C, and the residues were dried in an oven for 48 h.

Starch was extracted by adding 10 mL 20% HCl (w/w) to 100 mg of dry-tissue powder and shaking intermittently for 30 min. Samples were then centrifuged at 2000 *g*, and the supernatant was collected and stored. A second 10-mL aliquot of 20% HCl was added to the tissue for 30 min. Samples were centrifuged, and the supernatants were combined. An aliquot of 25 mL 80% ethanol was added to the supernatants and allowed to stand for 24 h, after which starch was observed as a white precipitate. The samples were centrifuged at 2000 *g*, and the white pellet of starch was oven-dried at 70°C for 48 h. The dried organic extracts and ground whole-leaf tissue were used for carbon isotopic analyses.

Carbon Isotopic Analyses

Dried and ground leaf tissue (1–2 mg) powders were processed through an on-line elemental analyzer (Heraeus, CHN-O RAPID) interfaced with a Finnigan Delta-S isotope ratio mass spectrometer through a trapping box system at the Laboratory of Isotope Biodynamics, Department of Biochemistry, University of Nebraska, Lincoln. Air samples were collected in preevacuated 2-L glass flasks from the growth chamber on three occasions at the beginning, middle, and end of the experiment. Carbon dioxide was purified cryogenically on a gas-extraction vacuum line. The yields of ca. 30 mmol CO_2 were sealed under vacuum in Pyrex ampules and analyzed on the dual inlet of the mass spectrometer.

All carbon isotopic ratios are in δ notation, where

$$\delta^{13}C = [(R_{C\,sample}/R_{C\,standard}) - 1]1000, \quad (1a)$$

$$R_C = {}^{13}C/{}^{12}C. \quad (1b)$$

For the samples measured via continuous flow, $R_{C\,sample}$ is compared to an acetanilide (−29.9‰) reference. Samples measured on the dual inlet were compared to a reference gas (Oztech, Dallas, Tex.). Both references had been calibrated against a Pee Dee Belemnite (PDB) standard. The precision of all continuous-flow and dual-inlet measurements was 0.2‰ and 0.1‰, respectively.

Modeled Relationship between $\delta^{13}C$ and p_i/p_a

A model proposed by Farquhar et al. (1982, 1989) was used to calculate predicted $\delta^{13}C$ values and integrated p_i/p_a ratios by substituting measurements of these parameters into the following equation:

$$\delta^{13}C_{plant} = \delta^{13}C_{air} - a - (b - a)(p_i/p_a) + d, \quad (2)$$

where a = discrimination during the diffusion of CO_2 through the stomata (4.4‰) (Craig 1953), b = discrimination during carboxylation, p_i/p_a = the ratio of leaf internal-to-external CO_2 partial pressure, and d = other discrimination (e.g., caused by presence of resistance between the intercellular spaces and sites of carboxylation, respiratory losses, and translocation).

It is generally assumed that $d = 0$ (O'Leary 1993). The carboxylation term b is 27‰ if it constitutes the dual isotopic influences of carboxylation by Rubisco and PEP carboxylase, according to the equation (Farquhar and Richards 1984)

$$b = b_3 - \beta(b_3 - b_4), \quad (3)$$

where b = the molar proportion of carbon fixed by PEP carboxylation, b_3 = the discrimination by Rubisco (29‰) (Roeske and O'Leary 1984), and b_4 = the discrimination by PEP carboxylase (−5.7‰) (Farquhar 1983).

The estimated 2‰ reduction in b caused by PEP carboxylase discrimination assumes that β is 0.06. This estimate appears

Table 1

Gas-Exchange Characteristics and Predawn Ψ of 56-d Mature Leaves before Defoliation and 7-d Young Leaves after Defoliation

	56-d leaves		7-d leaves			
Leaves	Wet	Dry	Wet-wet	Dry-dry	Wet-dry	Dry-wet
Photosynthesis (A)	$2.86^{C} \pm 0.3$	$2.73^{C} \pm 0.2$	$0.44^{A} \pm 0.1$	$0.33^{A} \pm 0.2$	$-0.42^{AB} \pm 0.2$	$-0.03^{AB} \pm 0.2$
Conductance (g)	$181^{D} \pm 45$	$61^{AC} \pm 5$	$116^{A} \pm 15.4$	$63^{AB} \pm 15.5$	$38^{BC} \pm 3.4$	$79^{A} \pm 21.5$
Transpiration (*E*)	$0.95^{A} \pm 0.3$	$0.45^{A} \pm 0.1$	$0.33^{A} \pm 0.1$	$0.37^{A} \pm 0.1$	$0.41^{A} \pm 0.2$	$0.56^{A} \pm 0.1$
p_i/p_a	$0.90^{B} \pm 0.02$	$0.82^{C} \pm 0.01$	$0.98^{AB} \pm 0.01$	$0.97^{AB} \pm 0.02$	$1.06^{A} \pm 0.03$	$1.03^{A} \pm 0.03$
A/*E*	$3.6^{C} \pm 0.6$	$6.3^{D} \pm 0.6$	$1.8^{A} \pm 0.5$	$1.4^{A} \pm 0.5$	$-0.1^{AB} \pm 0.2$	$-0.7^{B} \pm 0.3$
Predawn Ψ (MPa)	$-0.16^{A} \pm 0.03$	$-1.07^{B} \pm 0.06$	...	...	...	...

Note. Values are mean ± SE; $n = 5$. Different letters indicate that means were significantly different among treatments, following Fisher's LSD test. Photosynthesis is represented in micromoles per square meter per second; conductance and transpiration are represented in millimoles per square meter per second.

Table 2

$\delta^{13}C$ Values of Mature Leaves (56 d before Defoliation), Young Leaves, and Stem Organic Fractions (7 d after Defoliation)

Plant part	Wet	Dry	Wet-wet	Wet-dry	Dry-wet	Dry-dry
Young leaf	...	...	-23.6 ± 0.6^{B1}	-24.3 ± 0.6^{B1}	-22.6 ± 0.6^{B1}	-23.5 ± 0.6^{B1}
Mature leaf	-27.9 ± 0.3^{C1}	-25.5 ± 0.4^{C2}	-27.9 ± 0.3^{C1}	-27.9 ± 0.4^{C1}	-25.5 ± 0.4^{C2}	-25.5 ± 0.4^{C2}
Stem mixed organics	...	...	-26.4 ± 0.5^{AC1}	-27.6 ± 0.3^{CD1}	-27.2 ± 0.1^{A1}	-26.4 ± 0.2^{A1}
Stem sugar	...	...	-25.6 ± 0.3^{A1}	-25.8 ± 0.4^{A1}	-26.1 ± 0.4^{AC1}	-24.9 ± 0.7^{B1}
Stem starch	...	...	-25.2 ± 0.9^{AB1}	-26.5 ± 0.3^{AD1}	-26.6 ± 0.4^{AC1}	-25.8 ± 0.5^{C1}

Note. Values are mean ± SE; n = 3–5 plants. Different letters indicate significant differences among means within each treatment, following Fisher's LSD test. Different numerical values indicate significant differences among means among the treatments, following Fisher's LSD test.

realistic for wheat (Holbrook et al. 1984), and b = 27‰ is the commonly accepted carboxylation term in the theoretical model. We used b = 27‰ in equation (2) to derive integrated measures of p_i/p_a.

Estimation of the Influence of Imported Carbon on $\delta^{13}C$ Values

We estimated how much of the leaf carbon was imported to account for the deviations between observed and predicted $\delta^{13}C$ values using the following equation:

$$\delta^{13}C_{day\,n} = m_i(\delta^{13}C_{import}) + (1 - m_i)(\delta^{13}C_{predicted}), \quad (4a)$$

where $\delta^{13}C_{day\,n}$ = observed $\delta^{13}C$ values at the respective leaf ages, $\delta^{13}C_{import}$ = carbon isotopic ratio of imported carbon (assumed to equal the isotopic ratio of leaves at 7 d in each treatment), $\delta^{13}C_{predicted}$ = carbon isotopic ratio of autotrophic carbon (calculated from p_i/p_a), and m_i = proportion of total leaf carbon that is imported.

We estimated m_i by rearrangement as follows:

$$m_i = \frac{\delta^{13}C_{day\,n} - \delta^{13}C_{predicted}}{\delta^{13}C_{import} - \delta^{13}C_{predicted}}. \quad (4b)$$

Data Analyses

Data were tested for normality using n scores (Sokal and Rohlf 1995). Homogeneity of variances was tested using the F_{max}-test. If the assumptions of normality and homogeneity were met, t-tests and one-way ANOVAs were used to test differences among treatment means. When data did not conform to the assumptions of normality and homogeneity of variances, t-tests and one-way ANOVAs were used on rank-transformed data (Conover and Iman 1981). Predicted and adjusted $\delta^{13}C$ values were compared with observed leaf $\delta^{13}C$ values using t-tests. All statistical tests were performed with MINITAB (version 9.0, State College, Pa.).

Results

Physiological Traits of 56-d Old Leaves Measured before Defoliation

Predawn water potentials and stomatal conductances of 56-d leaves that developed under dry conditions were lower than those that developed under moist conditions (table 1). Rates of net photosynthesis were not significantly different between plants in the two water regimes. As a result, instantaneous leaf p_i/p_a was lower in the plants from the dry treatment than in the wet treatment (table 1). The $\delta^{13}C$ values of mature leaf tissue in the dry treatment were significantly less negative than those of leaves in the wet treatment (table 2). This would be expected given the lower instantaneous p_i/p_a and the higher water-use efficiency of leaves on the dry treatment plants (table 1).

Physiological Traits of Leaves 7 d after Defoliation

Seven days after defoliation, newly emerging leaves of wet-wet and dry-dry treatment plants showed a positive net carbon gain, whereas plants of all other treatments still exhibited net CO_2 release (table 1). Both instantaneous p_i/p_a and $\delta^{13}C$ values of the newly developing leaves did not differ among the four treatments (tables 1, 2). Instantaneous p_i/p_a were, in all cases, high (>0.97) due to the low photosynthetic rates. Young leaves following defoliation were significantly more enriched in ^{13}C than were the mature leaves before defoliation in each of the four treatments (table 2). The integrated p_i/p_a ratios derived from the $\delta^{13}C$ values at 7 d (eq. [2]) were much lower than the ratios obtained from instantaneous gas-exchange mea-

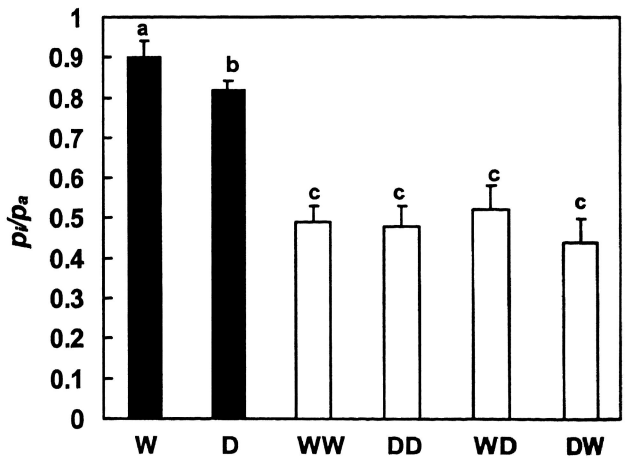

Fig. 2 Mean (±SE) instantaneous p_i/p_a of 56-d leaves (filled bars) before defoliation in wet (*W*) and dry (*D*) treatments and integrated p_i/p_a at 7 d (open bars) after defoliation in wet-wet (*WW*), dry-dry (*DD*), wet-dry (*WD*), and dry-wet (*DW*) treatments. Different letters indicate that means are significantly different, following Fisher's LSD test ($F_{5,20}$ = 76.5, $P < 0.05$).

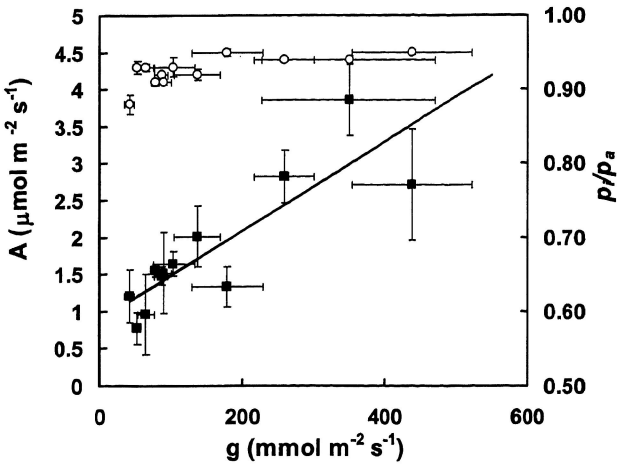

Fig. 3 Relationships between stomatal conductance and photosynthesis (A) ($r = 0.85$, $n = 11$, $P < 0.05$; filled squares) and p_i/p_a and A ($r = 0.14$, $P > 0.05$; open circles) in leaves of *Salix interior* ranging between 20- and 50-d leaf age in all four watering treatments. Each data point represents the mean (±SE) for each treatment at each sampling date.

surements on wet and dry leaves before defoliation at 56 d (fig. 2).

Comparison of $\delta^{13}C$ of Young Leaves and Stem Carbon Sources

No general relationships emerged between the $\delta^{13}C$ values of 7-d-old newly emerging leaves and the $\delta^{13}C$ values of individual stem organic fractions in the four treatments (table 2). In the wet-wet plants, the $\delta^{13}C$ values of the 7-d-old leaves were significantly less negative than the $\delta^{13}C$ values of the mixed organic compounds and sugars but did not differ from the $\delta^{13}C$ values of starch extracted from the stems. In the dry-dry plants, however, $\delta^{13}C$ values of the newly emerging leaves were less negative than $\delta^{13}C$ of stem starch and the mixed organic fraction but were not significantly different from the stem sugars. In both the wet-dry and dry-wet plants, $\delta^{13}C$ of the newly emerging leaves were significantly less negative than $\delta^{13}C$ of mature leaves and the stem organic fractions.

Comparison of Leaf Growth, Gas-Exchange Characteristics, and $\delta^{13}C$ Values during Leaf Development after Defoliation

Rates of net photosynthesis (A) were positively correlated ($r = 0.85$, $P < 0.05$) with stomatal conductance (g) in leaves measured on 20-, 30-, and 50-d leaves after defoliation in all treatments. In contrast, p_i/p_a remained constant throughout leaf development (fig. 3). Photosynthetic rates generally increased to a maximum between 20 and 30 d and declined at 50 d (fig. 4). In addition, leaf mass increased over time in all four treatments (fig. 4). In the dry-dry plants, however, very little leaf mass accumulated over time, which would be expected given the very low rates of photosynthesis observed throughout leaf development. Increases in leaf mass were positively correlated ($r = 0.72$, $P < 0.05$) with rates of photosynthesis in all four treatments. The $\delta^{13}C$ values indicate that the carbon was assimilated at much lower integrated p_i/p_a ratios than those measured instantaneously throughout leaf development (fig. 5).

Deviations in $\delta^{13}C$ Values of Older Leaves

The additional fractionation term d ranged between 4.3‰ and 13.9‰ across all leaf developmental stages and treatments (table 3). The magnitude of d correlated negatively with the instantaneous photosynthetic rates of the plants (fig. 6). It was estimated that the range in d could be accounted for if leaf carbon import ranged between 39% and 100% across all developmental stages and treatments (table 3).

Discussion

$\delta^{13}C$ Values of Newly Emerging Leaves

In this study, $\delta^{13}C$ values of young leaves were significantly less negative by 2‰–4‰ than values for mature leaves in *Salix interior*. This is in agreement with previous findings for many temperate and tropical tree species (Lowden and Dyke 1974; Leavitt and Long 1985; Sobrado and Ehleringer 1997; Terwilliger 1997). The observation that the $\delta^{13}C$ values of the young leaves were the same among the four experimental moisture regimes indicates that the $\delta^{13}C$ values of the tissue at this stage of development were not influenced by the environment under which the leaves were developing. Also, the $\delta^{13}C$ values implied that p_i/p_a was much lower and water-use efficiency much higher than was actually measured in the young leaves. This suggests that the $\delta^{13}C$ values of the young leaves might reflect the isotope ratios of the imported carbon used to produce this tissue, that is, the carbon stored in the stems or accumulated in the previous growing season.

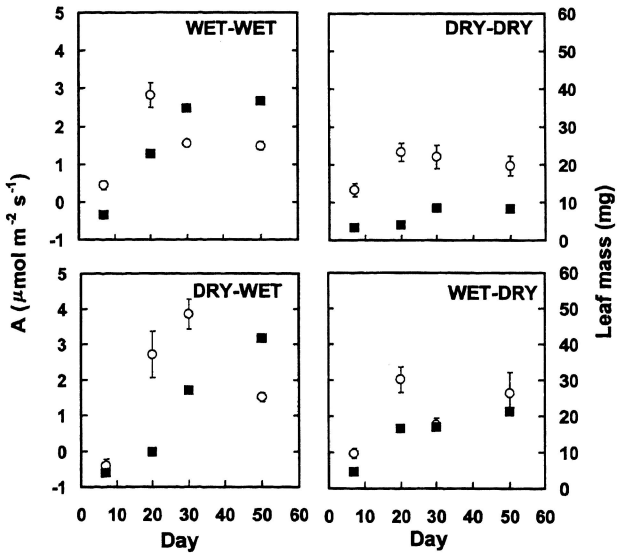

Fig. 4 Relationships between mean A (±SE; open circles) and cumulative leaf mass (filled squares) with leaf age in wet-wet, dry-dry, dry-wet, and wet-dry plants.

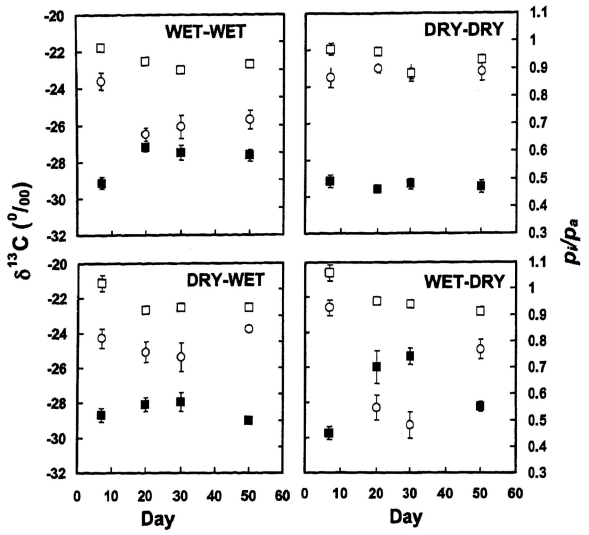

Fig. 5 Relationships between mean (±SE) observed $\delta^{13}C$ (open circles), instantaneous p_i/p_a (open squares), and integrated p_i/p_a (filled squares) measured at 7, 20, 30, and 50 d in wet-wet, dry-dry, wet-dry, and dry-wet plants.

The less negative $\delta^{13}C$ values observed in the young leaves, however, were not representative of $\delta^{13}C$ signatures or measured p_i/p_a values of mature leaves at the end of the previous growing season. Furthermore, there was no clear relationship between $\delta^{13}C$ values of metabolites in the stem and those in newly emerging leaves. Some similarities emerged between $\delta^{13}C$ values of young leaves and $\delta^{13}C$ values of the mobile and storage components of stem carbon, but they were inconsistent among treatments. The $\delta^{13}C$ values of newly emerging leaf tissues were similar to the $\delta^{13}C$ values of stem starches or sugars only in the wet-wet and dry-dry treatments, whereas the $\delta^{13}C$ values of newly emerging leaf tissue were more enriched in ^{13}C relative to the stem starches and sugars in the wet-dry and dry-wet treatments. A few studies have shown that $\delta^{13}C$ of starch is less negative than whole-leaf material (Gleixner et al. 1993; Le Roux 1993). Because carbon stores in the stem are largely composed of starch, it can be expected that sugars originating from this carbon pool during mobilization of reserves at bud break would exhibit less negative $\delta^{13}C$ values than mature leaf tissue. This may explain the less negative $\delta^{13}C$ values of newly emerging leaf tissue in the wet-wet and dry-dry plants. On the other hand, it cannot explain why the young leaf $\delta^{13}C$ of the wet-dry and dry-wet plants were more enriched in ^{13}C than both the stem carbon pools and the mature leaves of the previous growing season. It is possible that changing water availability may have triggered a change in metabolism during carbon import. This may have led to carbon isotopic fractionation during translocation from the stem carbon pool and its subsequent biochemical conversion to form new leaf tissue in those two treatments (Gleixner et al. 1998).

Instantaneous versus Integrated Measures of p_i/p_a

Even after the leaves gained photosynthetic competency in this study, instantaneous measures of p_i/p_a were much higher than the integrated measures derived from $\delta^{13}C$ values throughout leaf development (fig. 5). The leaf gas-exchange data indicate that photosynthesis was limited to a large degree by biochemical factors throughout leaf development in all treatments. This is illustrated by the constancy of p_i/p_a while A and g changed linearly over time (fig. 3). Such relationships are commonly found in plants grown at low light intensities (Farquhar and Sharkey 1982; Wong et al. 1985*a*, 1985*b*). There are several possible causes for the deviations between measured p_i/p_a and integrated p_i/p_a. One possible explanation is that the two measurements represent different time scales; thus, the instantaneous measurements may not be a true reflection of the gas-exchange properties on a diurnal basis throughout leaf development. Although the instantaneous gas-exchange measurements reflect p_i/p_a for only a relatively short time interval in this experiment, p_i/p_a did not vary much at the different leaf ages even though gas-exchange measurements were not made at similar times of day on the separate days. It suggests that the time-scale differences between instantaneous and integrated measurements of p_i/p_a do not appear to be a likely reason for the consistently higher-than-expected $\delta^{13}C$ values observed throughout leaf development. Instead, the strong negative correlation between d and A suggests that, in addition to the model parameters of equation (2), an additional biochemical fractionation factor was influencing the $\delta^{13}C$ values of leaves when A was low.

Table 3

Observed and Predicted $\delta^{13}C$ at 7–50 d after Defoliation in Four Water Treatments in *Salix interior*

Treatment/leaf age	$\delta^{13}C_{leaf}$	Predicted $\delta^{13}C_{predicted}$	d	% import
Wet-wet:				
7	$-23.6^{A} \pm 0.5$	-34.6 ± 0.2	11	...
20	$-26.5^{B} \pm 0.4$	-33.7 ± 0.1	7.2	71
30	$-26.1^{BC} \pm 0.3$	-32.4 ± 0.2	6.3	73
50	$-25.7^{C} \pm 0.5$	-33.2 ± 0.1	7.5	78
Dry-dry:				
7	$-23.5^{A} \pm 0.5$	-34.4 ± 0.5	10.9	...
20	$-23.0^{A} \pm 0.3$	-34.1 ± 0.2	11.1	100
30	$-23.3^{A} \pm 0.4$	-32.4 ± 0.6	9.1	100
50	$-23.2^{A} \pm 0.5$	-33.6 ± 0.2	10.4	100
Wet-dry:				
7	$-24.3^{A} \pm 0.6$	-35.6 ± 0.7	11.3	...
20	$-25.1^{A} \pm 0.6$	-33.3 ± 0.3	8.2	91
30	$-25.4^{A} \pm 0.8$	-33.5 ± 0.3	8.1	88
50	$-23.8^{A} \pm 0.2$	-33.5 ± 0.3	9.7	100
Dry-wet:				
7	$-22.6^{A} \pm 0.6$	-36.5 ± 0.6	13.9	...
20	$-28.3^{B} \pm 0.2$	-34.0 ± 0.2	5.7	50
30	$-29.3^{B} \pm 0.4$	-33.6 ± 0.2	4.3	39
50	$-25.0^{D} \pm 0.4$	-33.1 ± 0.1	8.1	77

Note. Values are mean ± SE. Different letters indicate that means were significantly different at the different leaf ages within each treatment, following Fisher's LSD test ($P < 0.05$). Predicted $\delta^{13}C$ was calculated using equation (2). The additional fractionation term d equals the difference between observed and predicted $\delta^{13}C$ values. Predicted carbon import between leaf developmental stages were estimated using equations (4a) and (4b).

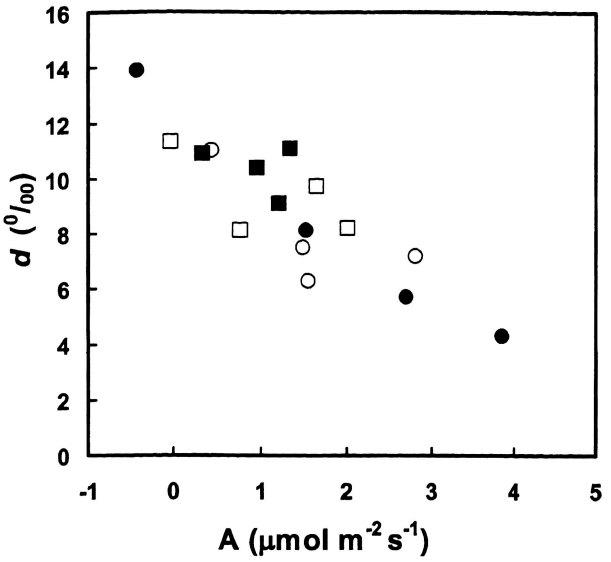

Fig. 6 Relationship between d and A ($r = 0.60$, $P < 0.05$); d = observed $\delta^{13}C$ – predicted $\delta^{13}C$ (eq. [2]) in wet-wet plants (open circles), dry-dry plants (filled squares), wet-dry plants (open squares), and dry-wet plants (filled circles).

Influence of Carbon Import

It is more likely that the initial ^{13}C-enriched signature of leaf tissue at 7 d may have influenced the carbon isotopic signature of the older autotrophic leaves. It is possible that carbon import did not end as soon as the leaves became photosynthetically competent (Turgeon 1989). A relationship between incremental leaf-mass accumulation and photosynthesis for all plants in the four treatments (data not shown) indicated that 52% of the variation in leaf mass was explained by variation in photosynthetic rates. It is possible that the other 48% of unexplained variability may have been caused by variation in levels of leaf import among the developmental stages in the four water treatments. By assuming that the large values of d were caused by continuous import of organic compounds into the leaves after 7 d, the observed deviations in $\delta^{13}C$ values can be explained if the dry-dry plants derived all of their leaf masses from imported carbon throughout leaf development and the wet-wet, wet-dry, and dry-wet plants derived 71%–73%, 88%–91%, and 39%–50% of their leaf masses from imported carbon at 20 and 30 d, respectively. Given the low photosynthetic rates exhibited by the leaves of the experimental plants throughout leaf development, it is feasible that the large values of the additional fractionation term d may have been caused by continuous import of an enriched carbohydrate source from the stems to facilitate leaf developments under such low light conditions when photosynthetic rates remained limited by biochemical factors.

Other Factors

It is important to note, however, that $\delta^{13}C$ values of stem carbohydrates did not always reflect the $\delta^{13}C$ values of the heterotrophic leaf stage (7 d). This suggests that another biochemical factor may have influenced the isotopic signature of the leaves, in part, during carbon import. There is recent evidence showing that PEP carboxylase activities are higher in the presence of sugars in C_3 species (Golombek et al. 1999; Le Roux-Swarthout et al. 2000). It is possible that the contribution of carbon by PEP carboxylase (eq. [3]) may have varied above 6% and caused $\delta^{13}C$ enrichment when photosynthetic rates were low in the leaves developing under low light conditions. The photosythetic rates exhibited under these experimental conditions were ca. 33% of what is commonly measured under full sunlight in other species of *Salix* (Cienciala and Lindroth 1994). The deviations found between $\delta^{13}C$ values and leaf intercellular carbon dioxide concentrations may be typical of leaves that are photosynthetically constrained due to biochemical deficiencies.

The implications of the deviations found in this study are that it should not be commonly assumed that the initial ^{13}C enrichment observed in deciduous trees simply represents the isotopic signature of the carbon that was accumulated by the mature leaves in the previous growing season. It appears that there may be an additional biochemical fractionation occurring during changes in metabolism in response to variation in soil-moisture availability. Labeling studies with ^{13}C are needed to reveal where the fractionation event occurs. Also, the deviations between $\delta^{13}C$ and p_i/p_a throughout leaf development may simulate intraplant physiological processes in the field when photosynthesis is limited biochemically by frequent cloud cover, internal canopy shading, low foliage nutrient status, or an inherently large heterotrophic stage of leaf development. Studies should be carried out to investigate the influence of environmental factors on carbon import and PEP carboyxlase carbon fixation in deciduous tree species.

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