Developing Phloem $\delta^{13}\text{C}$ and Sugar Composition as Indicators of Water Deficit in *Lupinus angustifolius*

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Additional index words. legumes, phloem, isotope, pinitol, carbohydrates, water deficit water use, carbon

Abstract. The use of naturally occurring carbon isotope ratios (expressed as $\delta^{13}\text{C}$) obtained from phloem sap offers considerable promise for evaluations of short-term physiological status of plants. We investigate the suitability of $\delta^{13}\text{C}$ among carbon pools isolated from both leaves and phloem sap as surrogate measures of plant gas exchange in *Lupinus angustifolius* L. We also investigate the use of phloem osmotic potential (ψ_s), and metabolite concentrations as surrogate measures of $\delta^{13}\text{C}$. Phloem sap carbon bled from the distal tip of the fruit offers considerably improved correlations between $\delta^{13}\text{C}$ and plant gas exchange compared with $\delta^{13}\text{C}$ of carbon pools obtained from extractions of excised phloem tissue and leaf soluble carbon. The concentration of phloem sugars and sugar alcohols correlates strongly with measures of phloem $\delta^{13}\text{C}$ but not with phloem ψ_s . We discuss these results in the context of developing fast, reliable, in-field tools for the assessment of plant physiological status.

Plant physiological processes change in response to short-term variation in resource availability. Developing non-invasive tools for the rapid and reliable evaluation of short-term variation in resource availability is of critical importance to land managers and agriculturalists across a range of managed and natural systems.

The use of natural abundance carbon isotopes to monitor plant gas exchange is well established at the leaf level. More recently, significant attention has been devoted to the use of $\delta^{13}\text{C}$ obtained from phloem sap ($\delta^{13}\text{C}_{\text{phl}}$) as a surrogate measure of whole canopy leaf $\delta^{13}\text{C}$ (Cernusak et al., 2003; Keitel et al., 2006; Merchant et al., 2010c; Rascher et al., 2010; Salmon et al., 2011). Transport of metabolites through the phloem is the central pathway for long-distance transport of carbon thus has potential for spatially and temporally integrated measures of canopy-level $\delta^{13}\text{C}$. Carbon obtained from the phloem sap is also “filtered” from a background of leaf metabolism potentially offering a clearer reflection of ^{13}C abundance obtained by recent photoassimilation (Merchant et al., 2010b). Improved predictions of canopy-scale gas exchange (Cernusak et al., 2005; Keitel et al., 2003) and growth (Merchant et al., 2010c) across a range of plant species support these hypotheses suggesting that techniques based

on the $\delta^{13}\text{C}$ of phloem sap may be further developed across a range of plant species.

Harvesting of phloem sap remains a major impediment to adaptations of phloem sap-based diagnostic assessments of plant function. A range of phloem bleeding and extraction techniques have been developed including stem bleeding (Pate and Arthur, 1998), phloem tissue removal and extraction (Gessler et al., 2004), and bleeding from the distal tip of the fruit (Pate et al., 1974). Whereas each technique offers advantages, their deployment is reliant on the characteristics of the plant species under investigation. Gessler et al. (2004) investigated the correlation of $\delta^{13}\text{C}$ obtained through different phloem sap collection methods applicable to tree species; however, no study has compared such methods with phloem collected from fruit tips (e.g., Pate et al., 1974) or aligned isotope abundance with that predicted by leaf-level models of isotope discrimination and measured gas exchange.

Development of tools based on measures of isotope abundance for use in the field is also restricted by the expense and time taken to measure $\delta^{13}\text{C}$. Pate et al. (1998) identified that the ψ_s (π) of phloem sap obtained from *Eucalyptus globulus* trees correlates strongly with $\delta^{13}\text{C}$. Using a refractometer, calibrated measures of π can be used as a surrogate measure of $\delta^{13}\text{C}$ (e.g., Tausz et al., 2008) enabling in-field detection and mitigation of water availability. More recently, significant and systematic changes in phloem sugar composition have also been detected (Merchant et al., 2010a, 2010b, 2011) to levels that would have a significant influence on π .

Transport of carbon in the phloem sap of plants requires “packaging” into highly reduced compounds. For *L. angustifolius*, phloem is thought to consist largely of sucrose; however, the contribution of sugar alcohols is yet to be investigated. The sugar alcohol D-pinitol is common to all legumes reaching molar concentrations equivalent to that of sucrose at the leaf level (Kuo et al., 1997; Streeter, 2001; Wanek and Richter, 1997) increasing in concentration according to plant stress (Adams

et al., 1992; Ford, 1982; McManus et al., 2000; Richter and Popp, 1992) and ecological gradients (Streeter et al., 2001). To date, no study has characterized the breakdown and metabolism of D-pinitol in plant tissues. In addition, the contribution of pinitol to phloem ψ_s is largely unknown.

In light of recent developments in the use of phloem sap as a rapid and reliable integrated measure of leaf $\delta^{13}\text{C}$ and the potential for use of phloem π as a cheap and reliable surrogate for $\delta^{13}\text{C}$, we sought to determine the effects of water deficit on the concentration of sugars and $\delta^{13}\text{C}$ in both leaves and phloem sap of *L. angustifolius*. We sought to determine the suitability of phloem sap carbon $\delta^{13}\text{C}$, obtained by different collection techniques, to reflect measures of leaf gas exchange. We also sought to evaluate the use of changes in phloem metabolite composition, in particular changes in the ratio of sugars to sugar alcohols as both a surrogate measure of plant water status and a potential confounding effect in overall measures of phloem sap $\delta^{13}\text{C}$ and ψ_s .

MATERIALS AND METHODS

Experimental design. Seeds of *Lupinus angustifolius* were germinated in a mixture of peat and coarse river sand (50/50 v/v) and raised under greenhouse conditions in Creswick, Australia (lat. 31°57' S, long. 115°52' E). In spring (Nov. 2010), ≈ 2 months before the start of treatments, seedlings were transplanted into 4-L pots and grown under glasshouse conditions with a light supplement to maintain a 14-h photoperiod (0700–2100 hr) of greater than 800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density for a 14-h photoperiod with ambient air temperature between 28 °C (day) and 15 °C (night) ramped within 1 h at the start and conclusion of the photoperiod. Seedlings were again planted into a 50:50 mix of peat and coarse river sand with 4 g·L⁻¹ slow-release fertiliser added (Nutricote-100 13N–13P–13 with oligo elements). On each day, plants were watered to field capacity of the soil by drip irrigation.

Received for publication 7 Oct. 2011. Accepted for publication 8 Nov. 2011.

This paper was part of the colloquium, “Emerging Techniques to Evaluate and Mitigate Crop Environmental Stress in a Changing Climate” held 28 Sept. 2011 at the ASHS Conference, Waikoloa, HI, and sponsored by the Environmental Stress Physiology (STRS) Working Group.

Dr. Merchant is supported by an Australian Research Council Postdoctoral Fellowship (DP0988731). Thank you to Dr. Antanas Spokevicius and Dr. Liubov Volkova for their assistance with the experimental setup. Thank you also to Dr. Claudia Keitel for isotopic analysis.

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Eight plants were allocated to each of a control treatment (watered to field capacity daily), mild water deficit [75% of water used by control plants (determined gravimetrically)], and severe water deficit (25% of water used by control plants). Treatments started 8 weeks after germination and were applied for 6 weeks. By the time of sampling, individual plants were 3 months old, ≈ 20 cm in height, and the stems were ≈ 0.8 cm in diameter.

Leaf gas exchange measurements. After 6 weeks, treatment conditions, measures of leaf net carbon assimilation (A , $\mu\text{mol CO}_2/\text{m}^2\cdot\text{s}^{-1}$) and stomatal conductance (g_s) ($\text{mol H}_2\text{O}/\text{m}^2\cdot\text{s}^{-1}$) were determined on each plant using a LI-COR 6400 infrared gas analyzer (LICOR, Lincoln, NE). Three independent measurements were taken on each plant six times throughout the day before the collection of phloem sap (see subsequently). Gas exchange results were scaled according to leaf area within the chamber. Light conditions within the LICOR chamber were set to tracking mode to approximate the growth chamber conditions. CO_2 mole fraction in the reference air stream was set to $400 \mu\text{mol}\cdot\text{mol}^{-1}$ and temperature and relative humidity in the measuring chamber was maintained within the LICOR chamber to approximate ambient conditions. Net CO_2 A ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) as well as g_s to water vapor (g_s , $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were recorded and used to compute the ratio of intercellular to atmospheric CO_2 concentration c_i/c_a . The daily integral of assimilation-weighted c_i was calculated using the approach of Cernusak et al. (2005).

Leaf tissue and phloem sap collection. At 1400 HR on the day of LICOR measurements, one blade from each of six leaflets was taken, pooled for each plant, and immediately frozen at -86°C . Leaves used for gas exchange measurements were not sampled to avoid collection of carbon diluted by CO_2 from the LICOR reference gas canister.

Phloem sap was collected through two methods, one as an extract of excised phloem tissue and the other using a phloem bleeding technique from the distal tip of the fruit (Pate et al., 1974). Each method was conducted following gas exchange measurements between 11 and 14 h after illumination. Phloem sap collection was made late in the photoperiod to avoid the effects on $\delta^{13}\text{C}$ of transitory starch remobilization during the night identified by Gessler et al. (2007).

Collections of phloem material for phloem sap extraction were made using a single-sided razor blade. A section of phloem material ≈ 0.3 cm in area was excised, weighed, placed in 1.5 mL deionized water, and incubated at 4°C for 90 min. Phloem material was then removed and the remaining solution transferred to -86°C . Phloem tissue was then dried at 60°C for 12 h and reweighed. For the bleeding technique, phloem sap formed a droplet on the surface of the cut that was collected into a glass disposable pipette and bulked for each tree. Samples were transferred to -4°C during the sampling period. Before freezing at -86°C , 3 μL of phloem sap was placed

into a separate microtube for measures of osmolality (see subsequently).

Analysis of phloem sap and leaf extracts for soluble carbohydrates. Leaf samples for metabolite analysis were extracted using a solution of methanol, chloroform, and water (MCW) according to the protocol outlined Merchant et al. (2006). For determination of $\delta^{13}\text{C}$, the MCW extract was substituted with a hot water extract whereby 1 mL of hot (80°C) water was used to extract 20 mg of dried leaf material. Extracts of leaf material, phloem sap, and the bled phloem sap were dried down and resuspended into 1 mL of deionized water.

Samples were then deionized over a mixed bed resin (see Merchant et al., 2006) consisting of one part Dowex 1 $\times$ 8 (anion exchange Cl form) and one part Dowex 50W (cation exchange, formate form; Dow Chemical Company, MI) and frozen awaiting analysis. For bled phloem sap, 50 μL was diluted with deionized water to a total volume of 1 mL, deionized, dried, resuspended into 50 μL of deionized water, and then frozen awaiting analysis.

Sixty microliters of deionized MCW extracts were dried and resuspended in 400 μL anhydrous pyridine to which 50 μL of trimethylchlorosilane/bis-trimethylsilyl-trifluoroacetamide mix (1:10; Sigma Aldrich, St. Louis, MO) was added. Samples were incubated for 1 h at 75°C and analyzed by gas chromatography (GC) within 24 h. Analysis of soluble carbohydrates was performed by gas chromatography as per Merchant et al. (2006) using a Shimadzu 17A series gas chromatograph (Shimadzu Corporation Limited, Columbia, MD) with a DB1 column (0.25 mm i.d., 30 m, 0.25- μm film thickness). Split injection was made at 300°C with an initial oven temperature program of 60°C for 2 min ramping to 300°C at $10^\circ\text{C}\cdot\text{min}^{-1}$ and maintained for 10 min. Column flow rate was maintained at $1.5 \text{ mL}\cdot\text{min}^{-1}$. Peak integration was made using Class VP analysis software (Shimadzu Corporation Limited). $\delta^{13}\text{C}$ was recorded with a continuous-flow isotope ratio mass spectrometer (Delta S; Thermo Electron, Bremen, Germany).

Osmolality of phloem bled from the distal tip of the fruit was determined using a freeze point depression osmometry using an OSMOMAT 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Using solute concentrations obtained from GC analysis, molar concentrations of solutes in phloem sap were converted to ψ_s (π_s) using the van't Hoff equation:

$$\pi_s = -R T c_s$$

where R is the universal gas constant ($8.32 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$), T is the temperature in Kelvin (set at 293), and c_s is the concentration of solutes in phloem sap ($\text{mol}\cdot\text{L}^{-1}$)

Isotope analysis and modeling of $\delta^{13}\text{C}$. For the analysis of carbon isotope composition of the soluble extract in leaves ($\delta^{13}\text{C}_{\text{sol}}$), 500 μL of extract was progressively transferred into tin cups and dried at 45°C for 12 h and then kept over desiccant awaiting analysis. For $\delta^{13}\text{C}_{\text{phloem}}$ and $\delta^{13}\text{C}_{\text{phloem}}$, 500 μL and 5 μL , respectively, were placed into tin cups

and dried at 45°C for 12 h and then kept over desiccant until analyzed.

Isotope ratio mass spectrometry (IRMS) was used to determine the $\delta^{13}\text{C}$ in samples. Samples were analyzed on an Isochrom mass spectrometer (Micromass, Manchester, U.K.) coupled to a Carlo Erba elemental analyser (CE Instruments, Milan, Italy). Samples were dropped from an AS200 autosampler and combusted by Dumas-combustion in a furnace kept at 1060°C . Carbon isotope ratios are expressed in delta-notation, where $\delta^{13}\text{C} = R_{\text{sample}}/R_{\text{standard}} - 1$ and R is the ratio of ^{13}C to ^{12}C in a sample and standard, respectively.

Predicted isotope values were calculated using the following equation originally devised by Farquhar and Richards (1984):

$$\Delta = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (b_s - a_i) \frac{C_i - C_c}{C_a} + b \frac{C_c}{C_a} - f \frac{\Gamma^*}{C_a} - e' \frac{R_d}{A + R_d} \frac{C_c - \Gamma^*}{C_a} \quad (1)$$

where a_b is the fractionation caused by boundary layer (2.9%), a is the fractionation caused by gaseous diffusion (4.4%), and b is the effective fractionation caused by carboxylating enzymes (Rubisco and PEPC, $\approx 29\%$). b_s is the ^{13}C fractionation as CO_2 enters solution (1.1%) and a_i is the ^{13}C fractionation resulting from diffusion of CO_2 in water (0.7%; O'Leary, 1984). f is the fractionation resulting from photorespiration ($\approx 11\%$; Lanigan et al., 2008) and Γ^* is the CO_2 partial pressure at which CO_2 assimilation compensates the production of photorespiratory CO_2 calculated according to Brooks and Farquhar (1985) by:

$$\Gamma^* = 44.7 + 1.88(T - 25) + 0.036(T - 25)^2 \quad (2)$$

where T is the leaf temperature ($^\circ\text{C}$). C_a was set at $400 \mu\text{mol}\cdot\text{mol}^{-1}$ and C_i calculated using the LICOR 6400 software (LICOR, 2008). Mesophyll conductance (g_i) at each growth temperature was estimated using the log normal calculation outlined by Warren (2008). C_c was then calculated as:

$$C_c = C_i - (A/g_i) \quad (3)$$

Fractionation of ^{13}C resulting from day respiration (R_d , e') has been ignored in modeling for our experiment (i.e., $e' = 0$). Similarly, transfer resistance of the boundary layer was set to 0 (i.e., $C_s = C_a$).

For calculations of ^{13}C discrimination by plants, the isotopic composition of the source (i.e., the atmosphere in the growth chamber), was assumed to be -7.8% (see Farquhar et al., 1982). Discrimination (Δ) from air was calculated using the formula:

$$\Delta = (\delta_{\text{air}} - \delta_{\text{plant}})/(1 + \delta_{\text{plant}}) \quad (4)$$

Statistical analysis. Effects of water deficit treatments were analyzed by analysis of variance using Statistica analytical software (Version 6; StatSoft, Tulsa, OK). P values were calculated using Tukey's honestly significant difference post hoc test mean

Fig. 1. Leaf photosynthesis, gas exchange, and c_i/c_a across a daily time course for 3-month-old *Lupinus angustifolius* subjected to 25%, 75%, and 100% of water used by control plants (determined gravimetrically) for a period of 6 weeks. Bars represent the SEM values of eight replicate plants. Gas exchange was measured on a randomly selected fully expanded leaf. Plants were grown under conditions of 25/15 °C (day/night) and between 500 and 1200 $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetic photon flux density (PPFD) for a photoperiod of 14 h per day.

values. Linear regressions were calculated using a general linear model.

RESULTS

The influence of water deficit treatments over the photoperiod was observed on net photosynthetic rate with 100% and 75%

treatments approximately three- and twofold higher than the 25% treatment, respectively (Fig. 1A). Similarly, g_s was higher in both 100% and 75% treatments than those detected in 25% treatment with the 100% treatment sustaining higher g_s rates throughout a longer proportion of the photoperiod (Fig. 1B). Delineation between treatments

was also reflected in c_i/c_a values throughout the photoperiod (Fig. 1C).

Leaf, phloem sap, and phloem extract $\delta^{13}\text{C}$ versus modeled values. $\delta^{13}\text{C}_{\text{sol}}$, $\delta^{13}\text{C}_{\text{phlext}}$, and $\delta^{13}\text{C}_{\text{phlsap}}$ were significantly enriched in ^{13}C relative to $\delta^{13}\text{C}_{\text{modeled}}$ ($P < 0.001$). $\delta^{13}\text{C}_{\text{phlsap}}$ was more closely aligned with $\delta^{13}\text{C}_{\text{modeled}}$ with a consistent offset of $\approx 4\%$ across the range of assimilation weighted c_i/c_a values obtained in this study (Fig. 2; Table 1). In contrast, both the slope and intercepts of $\delta^{13}\text{C}_{\text{phlext}}$ and $\delta^{13}\text{C}_{\text{sol}}$ significantly differed from that of $\delta^{13}\text{C}_{\text{modeled}}$ across the same range of c_i/c_a values (Fig. 2; Table 1).

Phloem sap and leaf sugar contents. Phloem sap sucrose significantly increased by ≈ 80 mM in response to both the 75% and 25% treatments (Table 2). Similarly, pinitol concentrations increased more than twofold for the 75% treatment and threefold for the 25% treatment. Common reducing sugars, glucose, and fructose were both detected at less than 1 mM concentrations and, although mean values increased according to the severity of water deficit, did not significantly differ among treatments.

In contrast, leaf tissues showed a decreasing trend in metabolite concentrations in response to water deficit treatments (Table 2). Leaf sucrose and pinitol concentrations were similar ranging between 40 and 70 $\mu\text{mol} \cdot \text{g}^{-1}$ dry weight.

Variation in phloem osmotic potential. Comparison of $\delta^{13}\text{C}_{\text{phlsap}}$ with that of phloem ψ_s calculated from analyzed phloem contents yielded a positive significant relationship (Fig. 3). Osmotic potential calculated from measured metabolites was consistently less than that measured by freeze point depression osmometry (Fig. 4). This relationship showed an increasing deviation from 1:1 with increasing ψ_s .

DISCUSSION

The present study confirms that $\delta^{13}\text{C}$ of phloem sap carbon obtained through bleeding from the distal tip of the fruit represents the best reflection of plant gas exchange when compared with carbon sources either extracted from phloem or leaf tissues. We also show that D-pinitol is present in phloem sap but only constitutes a small proportion (2% to 3%) of the overall phloem sap carbon content despite representing a significant proportion of the leaf-level soluble sugars. Osmotic potential of phloem sap holds great promise as a surrogate measure of phloem sap $\delta^{13}\text{C}$; however, we highlight here an additional osmotic component(s) of phloem sap that may significantly influence this relationship. The influence of this additional osmotic component may have important implications for the use of refractometry (e.g., Tausz et al., 2008) as a surrogate measure of phloem sap $\delta^{13}\text{C}$.

Patterns in $\delta^{13}\text{C}$. $\delta^{13}\text{C}_{\text{phlext}}$ best reflected those modeled values as predicted by Eq. 1 and offers valid predictions of leaf gas exchange. In contrast, carbon obtained through the phloem extraction technique and purified

Fig. 2. Modeled (predicted) $\delta^{13}\text{C}$ calculated from Eq. 2 and $\delta^{13}\text{C}$ phloem bleed $\delta^{13}\text{C}$ extracted phloem and $\delta^{13}\text{C}$ leaf soluble neutral fraction obtained from 3-month-old *Lupinus angustifolius*. Bars represent the SEM values of eight replicate plants. Gas exchange was measured on a randomly selected fully expanded leaf. Plants were grown under conditions of 25/15 °C (day/night) and between 500 and 1200 $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density (PPFD) for a photoperiod of 14 h per day.

Table 1. Slope, intercept and regression coefficients of the relationships between leaf, phloem, and modeled $\delta^{13}\text{C}$ values plotted against measures of c_i/c_a .^z

Origin	Slope	Intercept	r^2
$\delta^{13}\text{C}_{\text{modeled}}$	-21.27 (<0.0001)	-11.23 (<0.0001)	0.9404
$\delta^{13}\text{C}_{\text{phlsap}}$	-17.81 (<0.0001)	-15.65 (<0.0001)	0.8069
$\delta^{13}\text{C}_{\text{phlext}}$	-2.88 (<0.3379)	-28.62 (<0.0001)	0.0613
$\delta^{13}\text{C}_{\text{sol}}$	-7.77 (<0.0009)	-24.15 (<0.0001)	0.5344

^zPlants of *Lupinus angustifolius* were subjected to 25%, 75%, and 100% of water used by control plants (determined gravimetrically) for a period of 6 weeks ($n = 24$). Gas exchange was measured on a randomly selected fully expanded leaf. Plants were grown under conditions of 25/15 °C (day/night) and between 500 and 1200 $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density for a photoperiod of 14 h per day.

Table 2. Concentrations of sucrose, pinitol, fructose, and glucose in phloem and leaves of *Lupinus angustifolius* plants subjected to 25%, 75%, and 100% of water used by control plants (determined gravimetrically) for a period of 6 weeks ($n = 24$).^z

Tissue	Treatment	Sucrose	Pinitol	Fructose	Glucose
Phloem sap (mm)	100%	375 ^a ± 5.5	5.1 ^a ± 1.36	0.16 ^a ± 0.10	0.19 ^a ± 0.09
	75%	457 ^b ± 13.9	13.7 ^b ± 1.88	0.59 ^a ± 0.12	0.26 ^a ± 0.09
	25%	445 ^b ± 12.9	16.2 ^b ± 1.77	0.84 ^a ± 0.38	0.64 ^a ± 0.37
Leaf ($\mu\text{mol}\cdot\text{g}^{-1}$ dry weight)	100%	66 ^a ± 4.4	55.4 ^a ± 6.87	17.1 ^a ± 1.95	8.94 ^a ± 0.86
	75%	55 ^b ± 4.5	51.7 ^a ± 4.11	19.1 ^a ± 3.41	3.08 ^b ± 1.98
	25%	47 ^b ± 2.6	44.2 ^b ± 2.89	10.8 ^b ± 2.01	1.25 ^b ± 1.43

^zPlants were grown under conditions of 25/15 °C (day/night) and between 500 and 1200 $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density for a photoperiod of 14 h per day. Superscript letters represent between treatment Tukey's honestly significant difference post-hoc test mean values ($P < 0.05$).

metabolites from leaf tissues showed poor relationships with modeled $\delta^{13}\text{C}$ predicted by measured c_i/c_a . For leaf tissues, this is likely a result of a background of leaf metabolism present in the neutral fraction carbon pool. For phloem extracted from excised phloem tissue, poor relationships are likely attributable to the extraction of living material from the stem pieces that are not part of the transported carbon pool. A similar comparison between extraction techniques was made by Gessler et al. (2004) using a chelating reagent (EDTA polyphosphate) in the extraction

solution. For our study, a chelating reagent was not required because phloem pieces yielded enough carbon that, when purified, remained within the detection limits of the IRMS.

Despite a superior fit for the relationship between $\delta^{13}\text{C}_{\text{phlext}}$ and modeled values, a significant offset in $\delta^{13}\text{C}$ from leaf to phloem was observed suggesting a level of heterotrophic enrichment of $\approx 4\%$. This supports the majority of previous studies including substantial reviews detailing enrichment of phloem sap compared with leaf tissues

across a range of plant genera (e.g., Bowling et al., 2008). Despite this observation, the processes underpinning this phenomenon are yet to be fully understood (see Cernusak et al., 2009). Whereas transport fractionation, respiration, and the cycling of carbon through starch biosynthesis are, among many things, likely to play an important role (see Cernusak et al., 2009), changes in phloem composition, combined with differences in fractionation events attributable to synthesis, may account for at least part of this heterotrophic enrichment (Merchant et al., 2011).

Phloem and leaf metabolites. In response to water deficit, phloem metabolite concentrations significantly increased in agreement with previous studies across a range of plant genera (Cernusak et al., 2002; Gessler et al., 2008; Merchant et al., 2010b; Pate et al., 1998). In contrast, leaf metabolites showed significant decreases in concentration indicating reductions in photoassimilate production, increased export of photoassimilates from leaf tissues, increased respiration, increased allocation of carbon to alternative pools (e.g., starch accumulation), or a combination of effects. This pattern suggests that export of metabolites to phloem transport was not a significantly limiting factor to carbon movement during the onset of water deficit. An absence of carbohydrate accumulation in leaf tissues may have a significant influence on sugar-mediated repression of photosynthesis (e.g., Halford et al., 2003; Stitt et al., 2010) and osmotic adjustment hence impedes a plant's responsiveness to short-term changes in resource availability and environmental conditions.

Preservation of phloem structure and function is critical for plant health. Among a range of mechanisms for preserving and protecting the phloem structure, plants "package" carbon into non-reducing forms for long-distance transport. Sucrose is the most common form in which plants transport carbon in the phloem (see Turgeon, 1996), often accompanied by raffinose oligosaccharides and/or polyols such as alditols (e.g., sorbitol, mannitol) or cyclic polyols such as D-pinitol (see Bielecki, 1982; Loescher, 1987; Noiraud et al., 2001b; Reidel et al., 2009). D-Pinitol, a methylated inositol, is of particular interest as a result of its distribution among a number of plant taxa (Angyal and Anderson, 1959; Loewus and Dickinson, 1982; Posternak, 1965), most notably the *Leguminoasae*. D-Pinitol has been shown to accumulate up to 28 $\text{mg}\cdot\text{g}^{-1}$ in seed tissues of leguminous species (Kuo et al., 1997) indicating the importance of phloem-mediated movement of this class of compounds. To date, no study has characterized the metabolism of methylated cyclitols in plant tissues despite their accumulation to significant concentrations. In this study, the substantial accumulation of D-pinitol in leaf tissues supports previous hypotheses for roles in osmotic adjustment (Paul and Cockburn, 1989; Streeter et al., 2001) and the sequestration of excess photochemical energy (Hare et al., 1998).

Fig. 3. $\delta^{13}\text{C}$ of carbon contained in phloem sap plotted against the osmotic potential (ψ_s) of phloem sap calculated through the additive effects of sucrose, pinitol glucose, and fructose concentrations. Phloem sap was obtained from distal fruit tip of 3-month-old *Lupinus angustifolius* subjected to water deficit treatments while grown under conditions of 25/15 °C (day/night) and between 500 and 1200 $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density (PPFD) for a photoperiod of 14 h per day. Water deficit treatments of 100%, 75% and 25% are proportions of daily water use by control plants.

Fig. 4. Osmotic potential in phloem sap calculated from measured metabolites plotted against osmotic potential (ψ_s) in phloem sap calculated from measured osmolality. Phloem sap was obtained from distal fruit tip of 3-month-old *Lupinus angustifolius* subjected to water deficit treatments whilst grown under conditions of 25/15 °C (day/night) and between 500 and 1200 $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density (PPFD) for a photoperiod of 14 h per day. Water deficit treatments of 100%, 75%, and 25% are proportions of daily water use by control plants.

Transport of carbon in the phloem is a major sink for leaf-level carbon balance. In recognition of their important role in carbon transport, several transmembrane proteins for the movement of polyols such as mannitol and sorbitol have been characterized (Gao et al., 2003; Noiraud et al., 2001a). Despite the extensive distribution of cyclitols such as D-pinitol in leaf tissues, less is known regarding their role in the transport of carbon among plant tissues. In this study, significant concentrations of D-pinitol in the leaf, coupled with relatively minor concentrations in

the phloem sap, suggest that movement may be attributable to diffusion with no phloem-loading mechanism in operation. It is important to recognize that the low concentrations of D-pinitol obtained from phloem sap in this study are not directly indicative of flux and highlight the need for further exploration of this carbon transport mechanism across a range of environmental conditions and developmental stages.

The concentration of metabolites in phloem sap increased in response to water deficit; however, no significant change in the ratio of

sucrose to D-pinitol was detected. Changes in the composition of the phloem sap may have important consequences for measures of $\delta^{13}\text{C}$ as has been shown in previous studies in *Eucalyptus* (Merchant et al., 2011). Whereas the significant concentrations of D-pinitol obtained here from the soluble fraction of leaves are likely to have important consequences for leaf-level measures of $\delta^{13}\text{C}$, it is unlikely to have significant implications for phloem sap under conditions experienced in this study.

Osmotic potential as a surrogate measure of $\delta^{13}\text{C}$ and water status. The concentration of phloem sugars and sugar alcohols is a viable predictor of $\delta^{13}\text{C}$ for *L. angustifolius*. Previous studies investigating this relationship have yielded similar results across a range of plant species (Cernusak et al., 2002, 2003; Gessler et al., 2007; Keitel et al., 2003; Merchant et al., 2010c; Pate and Arthur, 1998). Our results also indicate the significant influence of additional solutes in phloem sap on ψ_s . The residual ψ_s unaccounted for in this study is likely to be attributed to a range of solutes, most likely organic acids and inorganic ions. Potassium has been isolated from phloem sap (Pate et al., 1974; Pritchard, 2007) and is likely to have significant influence over osmotic relations attributable to its concentration and low molecular weight. Similarly, free amino acids are often isolated from phloem sap in concentrations large enough to have a discernible influence over osmotic relations (Pritchard, 2007). The use of refractometry as a surrogate measure of phloem carbohydrate concentration requires standardization against measured sugar and sugar alcohol concentrations (e.g., Cernusak et al., 2005; Tausz et al., 2008) and assumes an insignificant or at least constant contribution of additional substances to the refraction index. Substantial differences in phloem composition have been detected across a diversity of plants grown under a broad range of conditions. For our study, a proportionally larger contribution to total phloem sap ψ_s was made by unknown solutes as total ψ_s increased. Our study suggests that the use of ψ_s as a surrogate measure of $\delta^{13}\text{C}$ is valid; however, further understanding of the composition of phloem sap in *L. angustifolius* is required for the use of refractometry methods to be used at least under those conditions outlined in this study.

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