

# Diurnal and seasonal variation in the carbon isotope composition of leaf dark-respired CO<sub>2</sub> in velvet mesquite (*Prosopis velutina*)

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## ABSTRACT

We evaluated diurnal and seasonal patterns of carbon isotope composition of leaf dark-respired CO<sub>2</sub> ( $\delta^{13}\text{C}_l$ ) in the C<sub>3</sub> perennial shrub velvet mesquite (*Prosopis velutina*) across flood plain and upland savanna ecosystems in the south-western USA.  $\delta^{13}\text{C}_l$  of darkened leaves increased to maximum values late during daytime periods and declined gradually over night-time periods to minimum values at pre-dawn. The magnitude of the diurnal shift in  $\delta^{13}\text{C}_l$  was strongly influenced by seasonal and habitat-related differences in soil water availability and leaf surface vapour pressure deficit.  $\delta^{13}\text{C}_l$  and the cumulative flux-weighted  $\delta^{13}\text{C}$  value of photosynthates were positively correlated, suggesting that progressive <sup>13</sup>C enrichment of the CO<sub>2</sub> evolved by darkened leaves during the daytime mainly resulted from short-term changes in photosynthetic <sup>13</sup>C discrimination and associated shifts in the  $\delta^{13}\text{C}$  signature of primary respiratory substrates. The <sup>13</sup>C enrichment of dark-respired CO<sub>2</sub> relative to photosynthates across habitats and seasons was 4 to 6‰ at the end of the daytime period (1800 h), but progressively declined to 0‰ by pre-dawn (0300 h). The origin of night-time and daytime variations in  $\delta^{13}\text{C}_l$  is discussed in terms of the carbon source(s) feeding respiration and the drought-induced changes in carbon metabolism.

**Key-words:** dark respiration; photosynthetic <sup>13</sup>C discrimination; respiratory apparent <sup>13</sup>C/<sup>12</sup>C fractionation; water stress.

## INTRODUCTION

Carbon isotope ratio ( $\delta^{13}\text{C}$ ) measurements have emerged as an important tool for probing metabolic processes and tracing carbon fluxes at ecosystem and larger scales. The  $\delta^{13}\text{C}$  value of CO<sub>2</sub> exchanged between the land surface and atmosphere is useful for partitioning net CO<sub>2</sub> exchange into autotrophic and heterotrophic component fluxes and investigating terrestrial carbon cycle responses to environmental change (Lloyd *et al.* 1996; Bowling, Tans & Monson

2001; Scartazza *et al.* 2004; Knohl & Buchmann 2005). Autotrophic respiration is one of the major components of biosphere–atmosphere CO<sub>2</sub> exchange; approximately 50% of plant-assimilated carbon is quickly released back to the atmosphere through this pathway (Ryan 1991). Estimation of autotrophic respiration fluxes and sources based on  $\delta^{13}\text{C}$  measurements requires that isotopic signatures of these fluxes be modelled or measured at the appropriate spatial and temporal scales. This is challenging over short-time scales because leaf dark-respired CO<sub>2</sub> is enriched in <sup>13</sup>C relative to primary respiratory substrates (Duranceau *et al.* 1999; Ghashghaie, Duranceau & Badeck 2001) and  $\delta^{13}\text{C}$  values of released CO<sub>2</sub> can potentially shift by up to 8‰ over a diurnal period (Hymus *et al.* 2005; Prater, Behzad & Jeffery 2006; Werner *et al.* 2007; Priault, Wegener & Werner 2009).

Highly <sup>13</sup>C-enriched values of leaf dark-respired CO<sub>2</sub> compared with primary respiratory substrates have been observed in a number of species and across a range of environmental conditions (Duranceau *et al.* 1999; Ghashghaie *et al.* 2001). Changes in <sup>13</sup>C enrichment occur over very short (minute) (Barbour *et al.* 2007; Werner *et al.* 2007) and diurnal time scales (Duranceau, Ghashghaie & Brugnoli 2001; Ghashghaie *et al.* 2001; Tcherkez *et al.* 2003; Hymus *et al.* 2005; Prater *et al.* 2006; Werner *et al.* 2007; Gessler *et al.* 2009; Priault *et al.* 2009). At the leaf scale, the apparent <sup>13</sup>C/<sup>12</sup>C fractionation between respiratory substrates and CO<sub>2</sub> is partly attributed to the non-statistical <sup>13</sup>C distribution within hexose molecules (Rossmann, Butzenlechner & Schmidt 1991; Duranceau *et al.* 1999; Ghashghaie *et al.* 2001; Tcherkez *et al.* 2003) resulting from isotope effects of aldolase involved in the formation of fructose-1,6-bisphosphate from triose phosphates (Gleixner & Schmidt 1997; Schmidt 2003). Incomplete oxidation of hexoses associated with C allocation to biosynthesis can cause the  $\delta^{13}\text{C}$  value of leaf dark-respired CO<sub>2</sub> ( $\delta^{13}\text{C}_l$ ) to be enriched by up to 6‰ (Ghashghaie, Badeck & Lanigan 2003; Hobbie & Werner 2004). This respiratory apparent <sup>13</sup>C/<sup>12</sup>C fractionation is believed to be partly responsible for diurnal variation in  $\delta^{13}\text{C}_l$ , which varies among plant functional groups and is highly influenced by plant resource availability (Hymus *et al.* 2005; Prater *et al.* 2006; Werner *et al.* 2007; Priault *et al.* 2009). Decarboxylation of <sup>13</sup>C-enriched phosphoenolpyruvate carboxylase-derived malate associated

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with light-enhanced dark respiration (LEDR) (Azcon-Bieto 1986; Atkin, Evans & Siebke 1998) is also partly responsible for the observed  $^{13}\text{C}$  enrichment in  $\text{CO}_2$  evolved from darkened light-acclimated leaves and the daytime increase in  $\delta^{13}\text{C}_i$  (Barbour *et al.* 2007; Gessler *et al.* 2009).

Diurnal variation of  $\delta^{13}\text{C}_i$  is likely to be affected also by changes in the  $\delta^{13}\text{C}$  signature of primary respiratory substrates associated with short-term changes in photosynthetic  $^{13}\text{C}$  discrimination ( $\Delta_P$ ). Leaf dark respiration is fuelled by two distinct pools: a fast pool that is exchanged within hours and a slow pool with a mean residence time of a few days (Schnyder *et al.* 2003). Recently assimilated photosynthates account for approximately 50% of the carbon lost by leaf dark respiration (Nogués *et al.* 2004). The isotopic signatures of the slow pools (soluble sugars, starch, lipids and cellulose) are expected to be relatively constant over diurnal time periods (Hymus *et al.* 2005; Göttlicher *et al.* 2006), whereas the  $\delta^{13}\text{C}$  signature of the fast pool is likely to shift quickly with changes in environmental and physiological conditions that alter  $\Delta_P$ . The impact of short-term environmental and physiological changes on the cumulative flux-weighted  $\delta^{13}\text{C}$  value of photosynthates ( $\delta^{13}\text{C}_{\text{pw}}$ ) can be modelled from gas exchange measurements (Farquhar, O'Leary & Berry 1982).  $\delta^{13}\text{C}_{\text{pw}}$  integrates effects of current and previous environmental conditions on  $\Delta_P$  over the day, and is likely to provide a reasonable estimation of the  $\delta^{13}\text{C}$  value of respiratory substrates.

Environmental changes over diurnal and seasonal time periods can alter the degree of  $^{13}\text{C}$  enrichment in leaf dark-respired  $\text{CO}_2$  relative to respiratory substrates (Ghashghaie *et al.* 2003; Hymus *et al.* 2005). Drought, for example, can affect  $\delta^{13}\text{C}_i$  through its impact on  $\Delta_P$  and associated changes in the  $\delta^{13}\text{C}$  value of photosynthates, and by altering the size and limitation of metabolite pools and the activities of key biosynthetic pathways affecting respiratory apparent  $^{13}\text{C}/^{12}\text{C}$  fractionation. In general, drought stress is expected to reduce respiratory apparent fractionation as key substrates would be limiting. Soil drying alters  $\delta^{13}\text{C}_i$  under controlled conditions (Duranceau *et al.* 1999; Ghashghaie *et al.* 2001), but the effects of drought on diurnal and seasonal variation in  $\delta^{13}\text{C}_i$  under field conditions have not been explored. Drought-stressed plants are predicted to show less diurnal variation in  $\Delta_P$  because water limitation reduces the diurnal range of stomatal conductance and  $C_i/C_a$  (Valladares & Pearcy 1997). Understanding relationships between drought and diurnal patterns of  $\delta^{13}\text{C}_i$  is required to properly interpret the isotope signature of  $\text{CO}_2$  exchange in terrestrial ecosystems where water plays a key role in constraining plant physiological processes.

We measured  $\delta^{13}\text{C}_i$  in velvet mesquite (*Prosopis velutina* Woot.), a widespread and invasive leguminous shrub in the semiarid south-western USA, in xeric upland and mesic riparian habitats to evaluate how drought influences diurnal and seasonal patterns of this important isotopic signal. This work was motivated by our interest in how woody plant encroachment into grasslands alters coupling of carbon and water cycles in different landscape positions (Williams *et al.* 2006). This deep-rooted shrub can access ground water in

riparian settings, reducing its reliance on precipitation, whereas it is completely dependent on limited rainfall supplies in upland habitats (Cable 1980; Snyder & Williams 2000; Fravolini *et al.* 2005). Our research objectives were to characterize diurnal variation in  $\delta^{13}\text{C}_i$  in *P. velutina* and to examine the effects of soil and atmospheric drought on the diurnal pattern and amplitude of  $\delta^{13}\text{C}_i$ . We expected to observe large diurnal variation in  $\delta^{13}\text{C}_i$  in both riparian and upland sites and through the dry pre-monsoon and wet monsoon seasons, but the magnitude of apparent respiratory  $^{13}\text{C}/^{12}\text{C}$  fractionation would be lowest in the xeric upland setting and during the dry pre-monsoon period. We also expected to observe less diurnal shift in  $\delta^{13}\text{C}_i$  in *P. velutina* during the pre-monsoon dry period compared to the wetter part of the growing season, as severe soil and atmospheric drought would reduce the magnitude of diurnal variation in the  $\delta^{13}\text{C}$  value of recently fixed photosynthates.

## MATERIALS AND METHODS

### Study sites

Two velvet mesquite (*P. velutina*) populations, one in an upland xeric habitat and another in a riparian mesic habitat, were selected for this study. The riparian population was located on a remnant flood plain terrace of the San Pedro River, approximately 12 km east of Sierra Vista, Arizona, USA (31°40'N, 110°10'W, 1200 m elevation). Long-term (1982–2007) mean maximum and minimum temperatures are 25.1 and 9.5 °C, respectively, and mean annual precipitation is 368 mm in Sierra Vista (1982–2007; Western Regional Climate Center; <http://www.wrcc.dri.edu>). Approximately 60% of the total annual precipitation occurs during the summer monsoon period from July through September. The plant community at this site was dominated by 3 to 4 m tall *P. velutina*. Sacaton grass (*Sporobolus wrightii* Munro ex Scribn.) and smaller shrubs were abundant in scattered patches in the tree interspaces. Depth to groundwater at this site is about 6.5 m (Scott *et al.* 2006). Deep-rooted *P. velutina* plants on alluvial terraces along the San Pedro River obtain a large fraction of their water from the near-surface water table and experience only mild water stress over the growing season compared with plants in upland areas (Snyder & Williams 2000; Fravolini *et al.* 2005). Soils at this site consist mainly of gravelly sandy loam layers interspersed with clay and gravel lenses (Scott *et al.* 2006).

The upland *P. velutina* population was located in the Santa Rita Experimental Range 35 km south of Tucson, AZ, USA (31°47'N, 110°50'W, 1190 m elevation). The mean maximum and minimum temperatures are 24.7 and 11.1 °C, respectively, and mean annual rainfall is 563 mm (1950–2007; Western Regional Climate Center; <http://www.wrcc.dri.edu>). More than 51% of the total annual precipitation occurs during the summer monsoon. The plant community at this upland site was dominated by *P. velutina*, perennial grasses and sub-shrubs. Although

*P. velutina* roots extend to great depths, roots do not access stable groundwater in these upland sites and winter and summer precipitation rarely penetrate below 2 m (Cable 1980; Frasier & Cox 1994; Fravolini *et al.* 2005). The Holocene-aged alluvial soils at this site have a sandy loam texture (Fravolini *et al.* 2005).

### Field sampling of leaf dark-respired $\text{CO}_2$

Sampling of leaf dark-respired  $\text{CO}_2$  was conducted during the dry pre-monsoon (June 5 and 7 at upland and riparian sites, respectively) and rainy monsoon (August 25 and 27 at riparian and upland sites, respectively) seasons to capture differences in water stress conditions. Large-bore (60 ml) syringes were used to collect dark-respired  $\text{CO}_2$  samples from *P. velutina* leaves using a method modified from Werner *et al.* (2007). Several young, fully expanded leaves were collected from upper parts of *P. velutina* canopies that were fully illuminated during daytime periods. For each plant ( $n = 5$ ), approximately 10 leaves were composited into a single sample, placed inside the syringe barrel and immediately flushed completely with  $\text{CO}_2$ -free air five times by actuating the syringe plunger. Syringe barrels were completely opaque to prevent photosynthesis.  $\text{CO}_2$  from leaf respiration in the barrel was allowed to build for 15 min and then 5 ml of air in the barrel was injected into 12 ml helium-flushed vials fitted with septum caps. This process was repeated nine times for each of the five plants over a 24-h period (0600, 0900, 1200, 1500, 1800, 2100, 0000, 0300 and 0600 h). Vials were stored in the field in dry, insulated coolers and shipped overnight to the University of Wyoming Stable Isotope Facility for isotopic analysis.

### Water potential and leaf gas exchange measurements

Leaf gas exchange rates were measured concurrently with the collection of leaf dark-respired  $\text{CO}_2$  using a LI-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA) on three replicate leaves on each of the five *P. velutina* trees used for respiration sampling. Young, fully expanded leaves from near the top of the canopy in fully illuminated locations were used for these measurements. For each measurement, environmental conditions inside of the leaf chamber (i.e. photosynthetically active radiation, chamber block temperature, relative humidity and  $\text{CO}_2$  concentration) were set to match ambient conditions. Vapour pressure deficit at the leaf surface ( $D_l$ ) was calculated as the difference between the saturation vapour pressure in the sub-stomatal cavity and the vapour pressure in the leaf chamber, which closely matched that of ambient conditions. Dark respiration rate ( $R$ ) during the daytime was measured by setting photosynthetically active radiation to zero. Leaf area was determined by scanning the leaflets used for gas exchange measurements. Pre-dawn leaf water potential ( $\Psi_{pd}$ ) was measured on three *P. velutina* twigs for

each of the five trees at each site between 0300 and 0500 h using a pressure chamber (PMS Instruments, Corvallis, OR, USA).

### Starch and soluble carbohydrate extractions

Leaves comparable with those used for  $\text{CO}_2$  sampling and gas exchange measurements (same canopy position) were collected at each sampling interval over the 24 h measurement periods. Leaves were immediately flash frozen in liquid nitrogen, stored on dry ice and then freeze-dried using a Labconco freeze dryer (Labconco, Kansas City, MO, USA). Freeze-dried *P. velutina* leaves were ground into fine powder using a ball mill. Starch and soluble carbohydrates were then extracted following the procedures described by Göttlicher *et al.* (2006). Briefly, 100 mg powdered dry leaf material was extracted with 1.5 mL methanol/chloroform/water (MCW, 12:5:3, v/v/v) for 30 min at 70 °C. After centrifuging at 10 000  $g$  for 2 min, 800  $\mu\text{L}$  of the supernatant was transferred into a new vial for the extraction of soluble carbohydrates. The pellet was washed with deionized water and oven-dried after re-extraction with MCW three times. Chloroform was added to the supernatant to induce phase separation. The upper phase containing soluble carbohydrates was transferred to a self-made ion exchange column for the separation of the neutral fraction from the total low molecular weight compounds. The column was filled with a mixture of anion-exchange resin (Dowex 1  $\times$  8, Sigma-Aldrich, St. Louis, MO, USA) and cation-exchange resin (Dowex 50W  $\times$  8, Sigma-Aldrich) with deionized water as mobile phase. The eluted neutral fraction was collected, oven-dried and re-dissolved in 1 mL water. Fifty microlitres of this sample was placed into a tin capsule, oven-dried at 65 °C, and then analysed on an isotope ratio mass spectrometer (described below).

Starch in the oven-dried pellet was gelatinized at 100 °C in a water bath for 15 min. Gelatinized starch was hydrolysed by heat stable  $\alpha$ -amylase (Sigma-Aldrich) at 85 °C for 120 min. The aqueous phase, containing sugars derived from starch, was separated from the pellet through centrifugation at 10 000  $g$ . The aqueous phase was purified in a centrifugal filter device (Microcon YM-10, Millipore, Billerica, MA, USA). Fifty microlitres of the filtrate was transferred to a tin capsule and oven-dried at 65 °C for stable isotope analysis. We extracted starch and sucrose standards with the methods described above and measured their stable carbon isotope composition. No significant isotopic fractionation was detected during both starch and soluble carbohydrate extractions.

### Isotope ratio mass spectrometry

All isotope analyses were performed at the University of Wyoming Stable Isotope Facility. Leaf dark-respired  $\text{CO}_2$  was analysed within 5 d of field collection using a GasBench II attached to a Finnigan Delta + XP continuous flow inlet isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany). Repeated measurements with

laboratory CO<sub>2</sub>-in-air working standards had a precision of <0.1‰. The δ<sup>13</sup>C of CO<sub>2</sub> in respiration samples were corrected to the international Vienna Pee Dee Belemnite (VPDB) standard using CO<sub>2</sub>-in-air working standards calibrated with CO<sub>2</sub>-in-air standards obtained from National Oceanic and Atmospheric Administration, Climate Monitoring and Diagnostic Laboratory. The δ<sup>13</sup>C of leaf biomass, starch and soluble carbohydrates were determined by a Micromass IsoPrime continuous flow isotope ratio mass spectrometer (Micromass, Manchester, UK). Precision of repeated measurements of laboratory standards was <0.1‰. Carbon isotope ratios are reported in parts per thousand relative to VPDB as: δ<sup>13</sup>C (‰) = (R<sub>sample</sub>/R<sub>standard</sub> - 1) × 1000.

### δ<sup>13</sup>C value estimates of the leaf photosynthate pool

δ<sup>13</sup>C of the recently fixed photosynthate pool was estimated as:

$$\delta^{13}C_{pwi} = \frac{\sum_{i=1}^n A_i \times \delta^{13}C_{pi}}{\sum_{i=1}^n A_i} \quad (1)$$

where δ<sup>13</sup>C<sub>pwi</sub> is the assimilation-weighted, cumulative carbon isotopic value of the recently fixed photosynthates at time *i* (0600, 0900, 1200, 1500 and 1800 h); A<sub>*i*</sub> and δ<sup>13</sup>C<sub>pi</sub> are the instantaneous net assimilation rate and the δ<sup>13</sup>C value of photosynthates at time *i*, respectively. δ<sup>13</sup>C<sub>pi</sub> was solved from:

$$\Delta_p = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p} \quad (2)$$

where δ<sup>13</sup>C<sub>a</sub> represents the δ<sup>13</sup>C value of atmospheric CO<sub>2</sub> (assumed to be -8‰).

Δ<sub>p</sub>, the discrimination against <sup>13</sup>C during photosynthesis, was estimated from the simplified linear model described by Farquhar *et al.* (1982), which assumes that mesophyll conductance is constant and relatively large.

$$\Delta_p = a + (b - a)C_i/C_a \quad (3)$$

The constant *a* represents the fractionation occurring during diffusion through air (4.4‰); *b* is the net fractionation associated with carboxylation (27‰); C<sub>*i*</sub> and C<sub>*a*</sub> are the concentrations of CO<sub>2</sub> in the sub-stomatal cavity and the atmosphere, respectively. Although *b* could vary slightly with temperature (O'Leary, Madhavan & Paneth 1992), the range of daytime leaf temperatures expected across sites and from pre-monsoon to monsoon seasons in this study would not be expected to alter this term significantly.

### Statistical analysis

We conducted repeated measures analysis of variance to test the effects of site, season and sampling time as well as

their interactions on the δ<sup>13</sup>C of leaf dark-respired CO<sub>2</sub>, leaf starch, leaf carbohydrates and bulk leaf biomass. Since we sampled leaves repeatedly from the same trees over 24 h field campaigns during both pre-monsoon and monsoon seasons within each site, time and season were treated as within subjects factors and site (riparian and upland) was treated as a between subjects factor in the analysis. Simple linear regression analysis was conducted to evaluate the dependence of δ<sup>13</sup>C<sub>*i*</sub> on δ<sup>13</sup>C of photosynthates, leaf starch, leaf-soluble carbohydrates and bulk leaf biomass. All statistical analyses were carried out using SAS version 9.0 (SAS Institute Inc., Cary, NC, USA). Average values are reported as the arithmetic mean ± 1 standard deviation.

## RESULTS

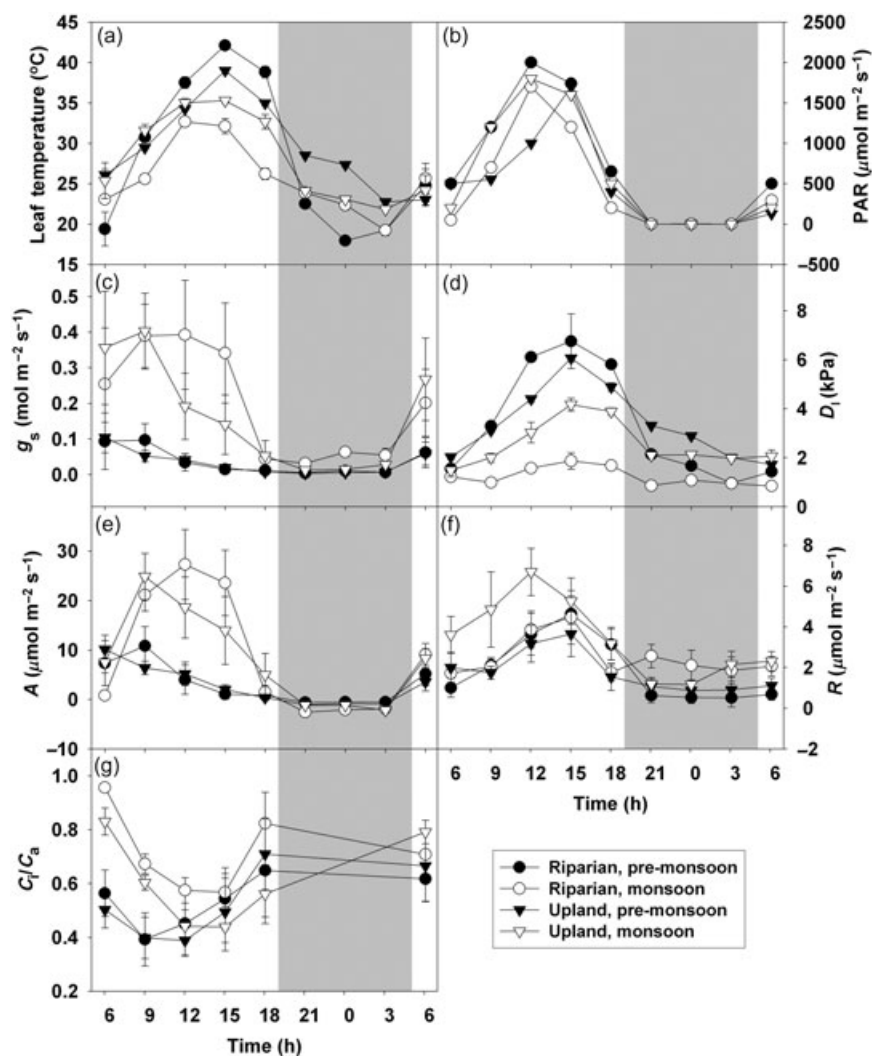
### Water potential and leaf gas exchange

*Prosopis velutina* twigs at the riparian site during the pre-dawn period equilibrated to higher water potentials (Ψ<sub>pd</sub>) than did those at the upland site during both pre-monsoon and monsoon sampling periods. Ψ<sub>pd</sub> increased from -3.7 ± 0.2 to -1.3 ± 0.1 MPa from the pre-monsoon to monsoon sampling periods in *P. velutina* at the upland site, whereas only a slight increase in Ψ<sub>pd</sub>, from -1.1 ± 0.2 to -0.5 ± 0.1 MPa, was observed at the riparian site over this same period.

Net carbon assimilation rate (*A*) of *P. velutina* trees growing at both riparian and upland sites reached a maximum between 0600 and 0900 h, and between 0900 and 1200 h, respectively, for pre-monsoon and monsoon sampling periods, and then gradually declined thereafter over the remaining daylight period (Fig. 1e). Surprisingly, no differences were observed in the mean *A* values for plants at the riparian and upland sites during either the pre-monsoon or monsoon, although those at the riparian site had higher pre-dawn water potential values. Stomatal conductance (*g*<sub>s</sub>), vapour pressure deficit at the leaf surface (*D*<sub>1</sub>) and C<sub>*i*</sub>/C<sub>*a*</sub> also did not differ among plants at the two sites during the pre-monsoon period (Fig. 1c, d & g), suggesting that soil water availability was not the only factor constraining plant carbon assimilation. High *D*<sub>1</sub> (Fig. 1d) during the pre-monsoon season at both sites likely had important impacts on *A*, *g*<sub>s</sub> and C<sub>*i*</sub>/C<sub>*a*</sub>.

*A* increased after the onset of monsoonal rainfall at both riparian and upland sites. Increased *A* during the monsoon season was likely due to increased soil water supply and reduced *D*<sub>1</sub> and leaf temperature, as photosynthetically active radiation was not appreciably different between the pre-monsoon and monsoon sampling dates (Fig. 1a, b & d).

Leaf respiration rate (*R*) gradually increased over the daytime period, reaching maximum values at 1500 h (Fig. 1f). *R* rapidly decreased thereafter to minimum values at the beginning of the night-time period and fluctuated near minimum values for the remainder of the night-time (Fig. 1f). *R* at the upland site, in general, was higher than that at the riparian site, but only during the monsoon period.



**Figure 1.** Diurnal patterns of environmental and physiological parameters for *Prosopis velutina* trees growing at riparian (circle) and upland (triangle) sites during pre-monsoon (solid) and monsoon (open) sampling periods. Diurnal trends in (a) leaf temperature ( $^{\circ}\text{C}$ ), (b) photosynthetically active radiation (PAR,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), (c) stomatal conductance ( $g_s$ ,  $\text{mol m}^{-2} \text{s}^{-1}$ ), (d) leaf vapour pressure deficit ( $D_l$ , kPa), (e) net assimilation rate ( $A$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), (f) dark respiration rate ( $R$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and (g)  $C_i/C_a$  ratio. Data are the means of five replicate trees (standard deviations are shown when larger than symbols). The shaded area denotes the dark period.

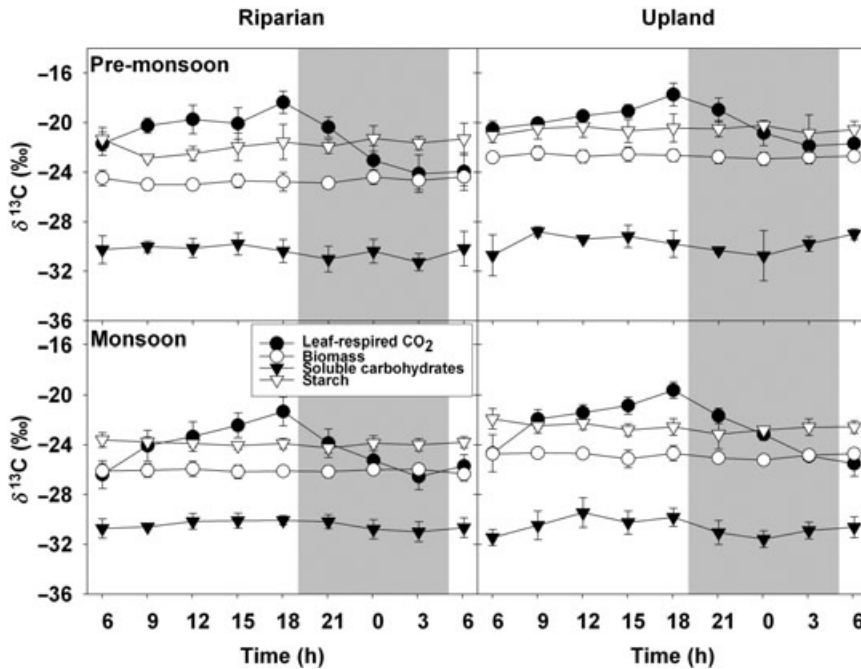
### $\delta^{13}\text{C}$ of biomass, soluble carbohydrates, starch and leaf dark-respired $\text{CO}_2$

No diurnal variation was detected for carbon isotope composition of leaf biomass ( $\delta^{13}\text{C}_b$ ), but  $\delta^{13}\text{C}_b$  was lower in leaves collected during the monsoon compared with those collected during the pre-monsoon period at both upland and riparian sites (Fig. 2; Table 1). Significant site differences ( $P < 0.001$ ) in  $\delta^{13}\text{C}_b$  were also detected;  $\delta^{13}\text{C}_b$  values for the upland *P. velutina* plants were  $2.0 \pm 0.3$  and  $1.2 \pm 0.3\text{‰}$  more positive than for plants at the riparian site during pre-monsoon and monsoon sampling periods, respectively (Table 1). The carbon isotope composition of starch ( $\delta^{13}\text{C}_s$ ) and total soluble carbohydrates ( $\delta^{13}\text{C}_{sc}$ ) showed no clear diurnal shift (Fig. 2). Compared with bulk leaf biomass, soluble carbohydrates were enriched in  $^{13}\text{C}$  by 2–3‰. No diurnal shifts or site differences were observed in  $\delta^{13}\text{C}_{sc}$ , but  $\delta^{13}\text{C}_{sc}$  values did decrease slightly from pre-monsoon to the monsoon period (Table 1). Soluble carbohydrates were depleted in  $^{13}\text{C}$  by  $7.0 \pm 0.6$  and  $5.8 \pm 0.6\text{‰}$  relative to bulk leaf biomass during

pre-monsoon and monsoon periods, respectively, at the upland site and by  $5.7 \pm 0.5$  and  $4.4 \pm 0.4\text{‰}$  at the riparian site.

The carbon isotope ratio of leaf dark-respired  $\text{CO}_2$  ( $\delta^{13}\text{C}_i$ ) demonstrated systematic diurnal variation (Fig. 2). Similar diurnal patterns in  $\delta^{13}\text{C}_i$  were observed between *P. velutina* plants at riparian and upland sites;  $\delta^{13}\text{C}_i$  values became progressively more positive during the daytime period reaching maximum values at 1800 h, and gradually declined thereafter at the end of the photoperiod.  $\delta^{13}\text{C}_i$  values were significantly different between pre-monsoon and monsoon sampling periods within each site (Table 1).  $\delta^{13}\text{C}_i$  values during the monsoon sampling period were  $3.0 \pm 0.9$  and  $2.6 \pm 0.9\text{‰}$  more negative than those observed during the pre-monsoon period at riparian and upland sites, respectively.  $\delta^{13}\text{C}_i$  values during the pre-monsoon and monsoon periods were  $1.3 \pm 0.8$  and  $1.7 \pm 0.6\text{‰}$  more positive, respectively, for plants at the upland compared with those at the riparian site.

Leaf dark-respired  $\text{CO}_2$  in *P. velutina* was enriched in  $^{13}\text{C}$  relative to the soluble carbohydrate pool at riparian and



**Figure 2.** Diurnal patterns of carbon isotope signatures (‰) of leaf dark-respired CO<sub>2</sub> (solid circle), bulk leaf biomass (open circle), soluble carbohydrates (solid triangle) and starch (open triangle) from pre-monsoon and monsoon sampling periods for *Prosopis velutina* trees growing at upland and riparian sites. Carbon isotope ratios of leaf dark-respired CO<sub>2</sub> are the means of five replications. Carbon isotope ratios of bulk leaf biomass, soluble carbohydrates and starch are the means of three replications (standard deviations are shown when larger than symbols). The shaded area denotes the dark period.

upland sites and during pre-monsoon and monsoon periods. The magnitude of this enrichment varied with the time of the day. Compared with starch, leaf dark-respired CO<sub>2</sub> was <sup>13</sup>C-enriched for most of the daytime during the pre-monsoon sampling period at the riparian site. <sup>13</sup>C enrichment in leaf dark-respired CO<sub>2</sub> relative to starch was observed only between 1500 and 2100 h at the upland site during both pre-monsoon and monsoon periods and at the riparian site during the monsoon period (Fig. 2). Leaf dark-respired CO<sub>2</sub> was either <sup>13</sup>C-depleted relative to starch or had similar <sup>13</sup>C values compared with the starch pool during the night-time period. Leaf dark-respired CO<sub>2</sub> was <sup>13</sup>C-enriched relative to the bulk leaf material, except late in the night-time through the early morning hours.

Significant positive correlations were detected between  $\delta^{13}\text{C}_1$  and  $\delta^{13}\text{C}_b$  (Fig. 3a) and  $\delta^{13}\text{C}_{sc}$  (Fig. 3b) and  $\delta^{13}\text{C}_s$  (Fig. 3c) across all sampling periods and sites, but the coefficients of determination were low and the distribution of data was highly clustered by season and site. The slopes of the relationship between  $\delta^{13}\text{C}_1$  and  $\delta^{13}\text{C}_b$  (1.17) and  $\delta^{13}\text{C}_s$  (1.10) were lower than that of  $\delta^{13}\text{C}_1$  and  $\delta^{13}\text{C}_{sc}$  (1.95).

Isotopic differences between  $\delta^{13}\text{C}_1$  and  $\delta^{13}\text{C}_b$  and  $\delta^{13}\text{C}_{sc}$  and  $\delta^{13}\text{C}_s$  were highly influenced by diurnal and seasonal variation in  $\delta^{13}\text{C}_1$ .

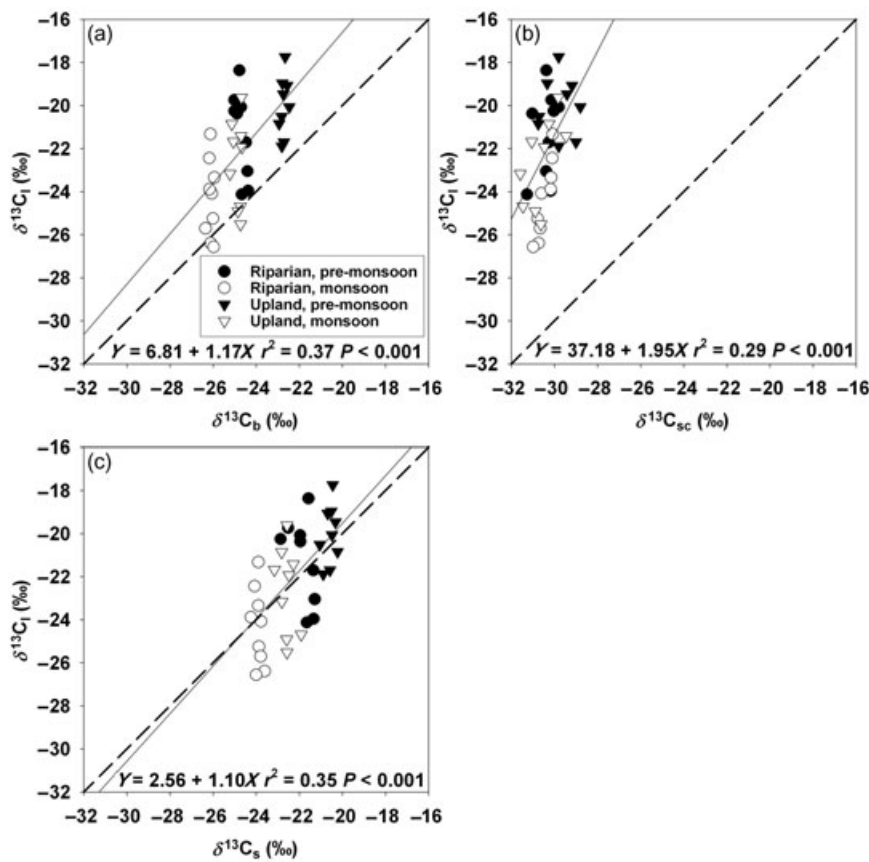
### $\delta^{13}\text{C}$ of the leaf photosynthate pool

The carbon isotope values of photosynthates ( $\delta^{13}\text{C}_p$ ) in *P. velutina* at both riparian and upland sites modelled from gas exchange parameters varied widely during the light period (Table 2). During the pre-monsoon period,  $\delta^{13}\text{C}_p$  varied by  $5.5 \pm 0.5$  and  $7.4 \pm 0.4$ ‰ for riparian and upland sites, respectively. The diurnal range in  $\delta^{13}\text{C}_p$  increased during the monsoon period, with  $8.8 \pm 0.7$  and  $8.9 \pm 0.5$ ‰ for riparian and upland sites, respectively. We compared the modelled  $\delta^{13}\text{C}_p$  with the observed  $\delta^{13}\text{C}_1$  at five time points (0600, 0900, 1200, 1500 and 1800 h) over daytime periods. Significant correlation was detected between  $\delta^{13}\text{C}_1$  and instantaneous values of  $\delta^{13}\text{C}_p$  (Fig. 4a). However,  $\delta^{13}\text{C}_1$  was more strongly correlated with the  $\delta^{13}\text{C}$  values of the cumulative, assimilation-weighted photosynthates ( $\delta^{13}\text{C}_{pw}$ ; Fig. 4b).

**Table 1.** Degrees of freedom (d.f.) and *P* values from the repeated measures ANOVA of carbon isotope composition of leaf dark-respired CO<sub>2</sub> ( $\delta^{13}\text{C}_1$ ), soluble carbohydrates ( $\delta^{13}\text{C}_{sc}$ ), starch ( $\delta^{13}\text{C}_s$ ) and bulk leaf biomass ( $\delta^{13}\text{C}_b$ )

Factors		Site	Season	Time	Site × season	Site × time	Season × time	Site × season × time
d.f.		1	1	8	1	8	8	8
$\delta^{13}\text{C}_1$	<i>P</i>	<0.01	<0.01	<0.01	0.12	0.52	0.02	0.06
$\delta^{13}\text{C}_{sc}$	<i>P</i>	0.57	0.02	0.67	0.44	0.41	0.61	0.35
$\delta^{13}\text{C}_s$	<i>P</i>	<0.01	<0.01	0.70	0.78	0.71	0.52	0.65
$\delta^{13}\text{C}_b$	<i>P</i>	<0.01	<0.01	0.99	<0.01	0.72	0.90	0.89

Site is the between subjects factor and season and time are within subjects factors. ANOVA, analysis of variance.



**Figure 3.** Relationships between carbon isotope signatures (‰) of leaf dark-respired  $\text{CO}_2$  ( $\delta^{13}\text{C}_1$ ) and carbon isotope values of (a) bulk leaf biomass ( $\delta^{13}\text{C}_b$ ), (b) soluble carbohydrates ( $\delta^{13}\text{C}_{sc}$ ) and (c) starch ( $\delta^{13}\text{C}_s$ ) for riparian (circle) and upland (triangle) habitats and pre-monsoon (solid) and monsoon (open) seasons. Dashed lines represent the 1:1 line. Linear regression functions,  $r^2$  and  $P$  values are provided.

## DISCUSSION

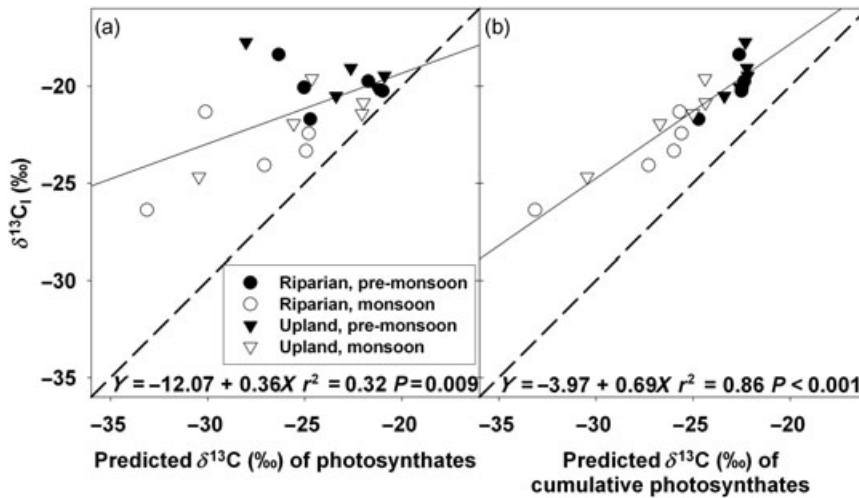
Large diurnal changes in the carbon isotope ratio value of leaf dark-respired  $\text{CO}_2$  ( $\delta^{13}\text{C}_1$ ) were observed in *P. velutina*, with  $\delta^{13}\text{C}_1$  increasing to maximum values late during daytime periods and declining gradually over night-time periods to minimum values at pre-dawn. These findings are similar to those for other drought-tolerant trees (Hymus *et al.* 2005; Prater *et al.* 2006; Priault *et al.* 2009). This general pattern of diurnal variation in  $\delta^{13}\text{C}_1$  in *P. velutina* held across upland and riparian habitats and from the dry pre-monsoon to the wet monsoon seasons, but the mean value and magnitude of diurnal variation in  $\delta^{13}\text{C}_1$  were strongly impacted by soil and atmospheric drought. Diurnal changes in  $\delta^{13}\text{C}_1$

have important implications for using  $\delta^{13}\text{C}$  measurements to constrain estimates of  $\text{CO}_2$  fluxes between ecosystems and the atmosphere (Bowling *et al.* 2001). The pronounced night-time shift in  $\delta^{13}\text{C}_1$  observed in *P. velutina* is especially relevant for studies employing the Keeling-plot approach (Keeling 1958) to partition night-time ecosystem respiration into its component fluxes. This approach assumes that the  $\delta^{13}\text{C}$  signatures of respiration sources within the ecosystem are constant over short time periods and can be accurately estimated or measured directly (Pataki *et al.* 2003). Accounting for diurnal and seasonal shifts in  $\delta^{13}\text{C}_1$  within this context requires better understanding of the effects of environmental conditions such as drought on  $\delta^{13}\text{C}_1$  and the mechanisms underlying these patterns.

**Table 2.** Diurnal variation in the  $\delta^{13}\text{C}$  value (‰) of photosynthates for *P. velutina* at riparian and upland sites during pre-monsoon and monsoon sampling periods

Site	Season	0600 h	0900 h	1200 h	1500 h	1800 h
Riparian	Pre-monsoon	-24.7 ± 1.8	-21.0 ± 2.3	-21.7 ± 2.8	-25.0 ± 1.8	-26.3 ± 2.3
	Monsoon	-33.1 ± 0.1	-27.1 ± 0.7	-24.9 ± 0.8	-24.8 ± 0.9	-30.1 ± 2.1
Upland	Pre-monsoon	-23.4 ± 1.1	-21.3 ± 1.6	-20.9 ± 0.7	-22.6 ± 1.5	-28.0 ± 2.1
	Monsoon	-30.5 ± 1.2	-25.6 ± 0.2	-22.1 ± 1.2	-22.0 ± 0.6	-24.6 ± 1.7

Values are modelled from gas exchange parameters. See text for details. Data are the means ( $\pm 1$  standard deviation) of five trees at each site sampled multiple times through the daytime period.



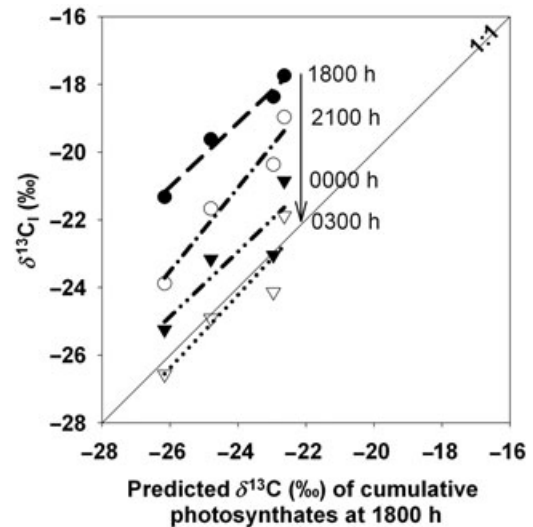
**Figure 4.** Relationships between carbon isotope signatures (‰) of leaf dark-respired CO<sub>2</sub> ( $\delta^{13}\text{C}_1$ ) and predicted carbon isotope ratios of (a) photosynthates ( $\delta^{13}\text{C}_p$ ) and (b) predicted carbon isotope ratios of cumulative photosynthates ( $\delta^{13}\text{C}_{pw}$ ) for riparian (circle) and upland (triangle) habitats and pre-monsoon (solid) and monsoon (open) seasons at 0600, 0900, 1200, 1500 and 1800 h. Dashed lines represent the 1:1 line. Linear regression functions,  $r^2$  and  $P$  values are provided.

### Photosynthetic $^{13}\text{C}/^{12}\text{C}$ discrimination and primary respiratory substrates

An important starting point for understanding the  $\delta^{13}\text{C}$  signature of leaf dark-respired CO<sub>2</sub> is to examine the  $\delta^{13}\text{C}$  signature of putative respiratory substrates. However, in *P. velutina*, diurnal variation in  $\delta^{13}\text{C}_1$  was not explained by changes in the carbon isotope ratio of leaf starch ( $\delta^{13}\text{C}_s$ ) or total soluble carbohydrates ( $\delta^{13}\text{C}_{sc}$ ) (Fig. 3). Hymus *et al.* (2005) also did not observe co-variation over short time periods between  $\delta^{13}\text{C}_1$  and either  $\delta^{13}\text{C}_s$  or  $\delta^{13}\text{C}_{sc}$ . Rather, isotopic analysis of sugars directly associated with leaf respiratory metabolism, such as glucose and recently fixed photosynthates, are likely to better reflect the controls on  $\delta^{13}\text{C}_1$  (Ghashghaie *et al.* 2001). Indeed, recently fixed photosynthates can account for about 50% of leaf dark respiration (Nogués *et al.* 2004). We estimated the carbon isotope composition of recently fixed photosynthates from gas exchange measurements and the simplified version of the photosynthetic discrimination model of Farquhar *et al.* (1982). During the daytime,  $\delta^{13}\text{C}_1$  in *P. velutina* was strongly correlated with the carbon isotope ratio value of the cumulative photosynthate pool ( $\delta^{13}\text{C}_{pw}$ ) associated with short-term changes in photosynthetic carbon isotope discrimination ( $\Delta_p$ ) (Fig. 4b). Increases in daytime  $\delta^{13}\text{C}_1$  values observed in other studies were correlated with cumulative photosynthesis, but not the instantaneous  $\delta^{13}\text{C}$  value of photosynthates estimated from gas exchange parameters (Hymus *et al.* 2005; Priault *et al.* 2009). However, these studies did not report values for  $\delta^{13}\text{C}_{pw}$ . Our results suggest that short-term changes in the  $\delta^{13}\text{C}$  value of photosynthates play an important role in determining daytime increases of  $\delta^{13}\text{C}_1$  in *P. velutina* growing under field conditions.

We observed progressive decreases of  $\delta^{13}\text{C}_1$  in *P. velutina* at the end of the photoperiod and through the early nighttime period under natural field conditions, similar to patterns observed in leaves of other tree species (Hymus *et al.* 2005; Prater *et al.* 2006). Other studies have noted similar decreases in  $\delta^{13}\text{C}_1$  during extended dark periods in controlled settings (Nogués *et al.* 2004; Barbour *et al.* 2007;

Werner *et al.* 2007; Priault *et al.* 2009). We plotted  $\delta^{13}\text{C}_1$  at 1800, 2100, 0000 and 0300 h against  $\delta^{13}\text{C}_{pw}$  recorded at the end of the daytime (1800 h) (Fig. 5) to investigate the role of recently fixed photosynthates in controlling night-time  $\delta^{13}\text{C}_1$ .  $\delta^{13}\text{C}_{pw}$  was strongly correlated with night-time  $\delta^{13}\text{C}_1$  across upland and riparian sites and the wet and dry seasons. But important offsets were observed between  $\delta^{13}\text{C}_1$  and  $\delta^{13}\text{C}_{pw}$  and the magnitude of the offset shifted over the night-time period. If we assume that the flux-weighted photosynthates were the primary respiratory substrates at night, then  $^{13}\text{C}$  enrichment of respired CO<sub>2</sub> relative to these substrates decreased from about 6‰ at 1800 h to approximately 0‰ at 0300 h. The convergence of  $\delta^{13}\text{C}_1$  on  $\delta^{13}\text{C}_{pw}$  at



**Figure 5.** Relationships between predicted carbon isotope ratios of the cumulative photosynthate pool ( $\delta^{13}\text{C}_{pw}$ ) at 1800 h with carbon isotope signatures of leaf dark-respired CO<sub>2</sub> ( $\delta^{13}\text{C}_1$ ) at 1800 h (solid circle and dash line), 2100 h (open circle and dash-dot line), 0000 h (solid triangle and dash-dot-dot line) and 0300 h (open triangle and dot line), respectively, for both sites and both seasons. The solid line represents the 1:1 line.

0300 h suggests that the night-time  $\delta^{13}\text{C}$  value of  $\text{CO}_2$  respired by the plant canopy in ecosystem scale studies can be accurately estimated from daytime gas exchange characteristics.

### Drought effects on $\delta^{13}\text{C}_i$

Leaf dark-respired  $\text{CO}_2$  in *P. velutina* during the dry pre-monsoon season was significantly  $^{13}\text{C}$  enriched compared to that during the wet monsoon season (Table 1). Water limitation-induced  $^{13}\text{C}$  enrichment in leaf dark-respired  $\text{CO}_2$  has also been observed in *Nicotiana sylvestris* and *Helianthus annuus* growing in controlled environment settings (Duranceau *et al.* 1999; Ghashghaie *et al.* 2001). Seasonal differences in  $\delta^{13}\text{C}_p$  values in *P. velutina* (Table 2) illustrate how soil water limitation and vapour pressure deficit at the leaf surface ( $D_i$ ) influenced  $\delta^{13}\text{C}_i$  values through impacts on photosynthetic carbon isotope discrimination ( $\Delta_P$ ). Drought-induced shifts in  $\delta^{13}\text{C}_i$ , therefore, can be an important component of seasonal variation in the carbon isotope composition of ecosystem respiration ( $\delta^{13}\text{C}_R$ ) in savanna ecosystems where physiological processes are strongly controlled by water availability.

The amplitude of the diurnal variation in  $\delta^{13}\text{C}_i$  in *P. velutina* was significantly affected by differences in soil water availability and  $D_i$  across riparian and upland sites and between the pre-monsoon and monsoon seasons (Fig. 2; Table 1). The diurnal amplitude in  $\delta^{13}\text{C}_i$  (calculated as  $\delta^{13}\text{C}_i$  at 1800 h minus  $\delta^{13}\text{C}_i$  at 0600 h) was greater during the monsoon period at riparian ( $5.1 \pm 1.1\text{‰}$ ) and upland ( $5.1 \pm 0.9\text{‰}$ ) sites than during the pre-monsoon period ( $3.3 \pm 0.8\text{‰}$  and  $2.8 \pm 0.7\text{‰}$ , respectively, at riparian and upland sites). Changes in the magnitude of diurnal variation in  $\delta^{13}\text{C}_i$  could potentially influence patterns of  $\delta^{13}\text{C}_R$ , if foliar respiration fluxes are large, relative to other component fluxes. Indeed, Bowling *et al.* (2003) observed that  $\delta^{13}\text{C}_R$  varied significantly over a single night in a grassland ecosystem, which may have been due to night-time shifts in  $\delta^{13}\text{C}_i$ .

A positive correlation between  $\delta^{13}\text{C}_i$  and the  $\delta^{13}\text{C}$  value of photosynthates modelled from gas exchange measurements suggests that the magnitude of daytime increases in  $\delta^{13}\text{C}_i$  in *P. velutina* were controlled primarily by daytime changes in  $\Delta_P$  (Fig. 4b). Progressive changes in daytime  $\Delta_P$  can also affect the pattern of night-time decreases in  $\delta^{13}\text{C}_i$ . Transitory starch synthesis during the daytime and remobilization at night may induce progressive night-time shifts in the  $\delta^{13}\text{C}$  value of primary respiratory substrates. The remobilization of transitory starch follows the 'first in, last out' rule, which means that the first glucose to be polymerized into starch will be the last one to be broken down into maltose and exported out of the chloroplast (Zeeman, Smith & Smith 2007). Hence, if photosynthates that were used to synthesize starch were gradually  $^{13}\text{C}$  enriched during the daytime, then the  $\delta^{13}\text{C}$  value of respiratory substrates should become progressively reduced over the night-time period. This hypothesis is consistent with observations of the gradual increase in  $\delta^{13}\text{C}$  of photosynthates

from 0600 to 1500 h modelled from gas exchange and the gradual decrease in  $\delta^{13}\text{C}_i$  over the night-time period. Although the instantaneous modelled  $\delta^{13}\text{C}$  value of photosynthates declined at 1800 h (Table 2), its effect on the  $\delta^{13}\text{C}$  value of starch is likely to be negligible considering the low assimilation rates at that time point (Fig. 1e).

Although we demonstrate that drought can alter the diurnal amplitude of  $\delta^{13}\text{C}_i$  potentially through its impact on  $\Delta_P$  and resultant  $\delta^{13}\text{C}$  values of respiratory substrates and transitory starch pools, other processes impacted by drought stress may also affect short-term variation in  $\delta^{13}\text{C}_i$ . For example, the pool size of malate fuelling respiration in light-acclimated darkened leaves (i.e. LEDR), and the partitioning of intermediate respiratory metabolites between biosynthesis and adenosine triphosphate (ATP) production in mitochondria are also likely impacted by drought stress. Positional  $^{13}\text{C}$  labelling experiments suggest that variation in the pyruvate dehydrogenase (PDH) to acetyl coenzyme A (acetyl-CoA) oxidation ratio governs the magnitude of the daytime increase in  $\delta^{13}\text{C}_i$  (Priault *et al.* 2009). We hypothesize that reductions in the ratio of PDH:acetyl-CoA oxidation leading to decreases in  $\delta^{13}\text{C}_i$  are also likely to occur over the night-time period. The ratio of PDH:acetyl-CoA oxidation is sensitive to substrate availability (Priault *et al.* 2009), which is likely to be affected by drought. Reduced substrate availability during drought is therefore likely to dampen the diurnal variation in  $\delta^{13}\text{C}_i$  by decreasing the ratio of PDH:acetyl-CoA oxidation.

A gradual increase in the malate pool size during the day could partially contribute to the observed daytime increases in  $\delta^{13}\text{C}_i$  (Gessler *et al.* 2009). We do not know the degree to which the decarboxylation of malate associated with LEDR influenced the daytime increase of  $\delta^{13}\text{C}_i$  in *P. velutina*. Drought-stressed plants are likely to have a reduced and less variable pool size of malate compared to non-stressed plants, which would limit daytime increases in  $\delta^{13}\text{C}_i$ . However, the night-time decrease of  $\delta^{13}\text{C}_i$  observed in *P. velutina* was unlikely a result of changes in malate decarboxylation associated with LEDR.

Finally, drought may affect diurnal variation in  $\delta^{13}\text{C}_i$  through its impact on  $^{13}\text{C}/^{12}\text{C}$  fractionations associated with respiratory enzymatic reactions (Deniro & Epstein 1977; Tcherkez & Farquhar 2005). In general, the range of diurnal variation in isotope effects associated with respiratory enzymatic reactions is determined by substrate availability and the magnitude of diurnal shifts in leaf temperature. Large diurnal shifts in leaf temperature during the dry pre-monsoon season (Fig. 1a) are expected to produce greater diurnal changes in the isotope effects of respiratory enzymes. However, we observed less diurnal variation in  $\delta^{13}\text{C}_i$  during the dry pre-monsoon season than during the wet monsoon season, suggesting that temperature-dependent isotope effects associated with respiratory enzymes did not play a dominant role in controlling variation in  $\delta^{13}\text{C}_i$ .

Diurnal and seasonal changes in the  $\delta^{13}\text{C}$  value of respired  $\text{CO}_2$  from autotrophic and heterotrophic tissues can provide unique insight into metabolic activities in plants and ecosystems. The observed large diurnal

amplitude of  $\delta^{13}\text{C}_1$  in *P. velutina* and in other trees and shrubs (Hymus *et al.* 2005; Prater *et al.* 2006; Priault *et al.* 2009) is apparently common for woody  $\text{C}_3$  species. Resolving mechanisms responsible for short-term changes in  $\delta^{13}\text{C}_1$  would assist in the interpretation of carbon isotope fluxes between ecosystems and atmosphere and provide further insight into plant metabolic responses to environmental change.

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