Diurnal variation of $\Delta^{13}\text{CO}_2$, $\Delta^{18}\text{O}^{16}\text{O}$ and evaporative site enrichment of $\delta^{18}\text{H}_2\text{O}$ in *Piper aduncum* under field conditions in Trinidad

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ABSTRACT

Concurrent measurements of gas exchange, instantaneous isotope discrimination ($\Delta$) against $^{13}\text{CO}_2$ and $^{18}\text{O}^{16}\text{O}$, and extent of $^{18}\text{O}$ enrichment in $\text{H}_2\text{O}$ at the evaporative sites, were followed in a tropical forest pioneer, *Piper aduncum*, on two different days in Trinidad during February 1995. $\Delta^{13}\text{CO}_2$ differed from that predicted from measurements of internal:external $\text{CO}_2$ concentration ($C_i/C_a$) and showed a wide range of values which decreased throughout the course of the day. Derivation of $C_i$ (the $\text{CO}_2$ concentration at the carboxylation site) was not possible using carbon isotope discrimination under field conditions in situ and was derived assuming a constant value of internal transfer conductance ($g_w$). Under low rates of assimilation the derived $C_i/C_a$, like $C_i/C_a$, remained relatively stable over the course of both days and $\Delta^{18}\text{O}^{16}\text{O}$ followed evaporative demand. Lower values of $\Delta^{18}\text{O}^{16}\text{O}$ on day 2 occurred in response to the indirect effect of increased leaf-to-air vapour pressure deficits (VPD) and reduced stomatal conductance. For the first time, direct determination of the $\Delta^{18}\text{O}$ of transpired water vapour ($\delta_i$) allowed derivation of evaporative site enrichment without the prerequisite of isotopic steady state (ISS) defined in the Craig and Gordon model. Generally, $\delta_i$ was less enriched than the source water ($\delta_s$) in the morning and more enriched in the afternoon, which would be predicted from an increase and decrease in ambient VPD, respectively. On both days, leaves of *P. aduncum* approached ISS (indicated where $\delta_i = \delta_s$) between 1300 and 1500 h. Evaporative site enrichment was maintained into the late afternoon, despite a decrease in ambient VPD. The data presented provide a greater insight into the natural variation in isotopic discrimination under field conditions, which may help to refine models of terrestrial biome discrimination.

Key-words: *Piper aduncum*; $^{13}\text{C}$; instantaneous isotope discrimination; leaf gas exchange; $^{18}\text{O}$; Trinidad; water vapour.

INTRODUCTION

Plant processes serve a unique role as integrators and indicators of global environmental change (Yakir et al. 1993). Isotopic analysis of atmospheric $^{13}\text{CO}_2$ (Mook et al. 1983; Broadmeadow et al. 1992; Ciais et al. 1995), $^{18}\text{O}^{16}\text{O}$ (Francey & Tans 1987; Farquhar et al. 1993; Ciais & Meijer 1997; Ciais et al. 1997) and $\text{H}_2^{18}\text{O}$ (Salati et al. 1979; Jacob & Sonntag 1991) has suggested that the terrestrial biosphere exerts a substantial influence on global cycles of $\text{CO}_2$ and water. Attention has, therefore, focused on the mechanisms controlling fractionation in biological systems. Intuitively, terrestrial photosynthetic gas exchange will provide a large biospheric contribution to global biogeochemical cycles, along with soil respiration and aquatic photosynthesis. In order to assess any contribution by terrestrial photosynthesis, we must first understand the processes governing fractionation and the natural variation in biological discrimination.

During photosynthetic gas exchange, a combination of diffusive and enzymatic discrimination against the heavier $^{13}\text{C}$ leaves plant material depleted in the heavier isotope relative to atmospheric $\text{CO}_2$ (Farquhar, Ehleringer & Hubick 1989). Additionally, through rapid exchange between $\text{CO}_2$ and water in the chloroplast, the $^{18}\text{O}$ leaf water isotope signal is transferred to atmospheric $\text{CO}_2$ (Francey & Tans 1987; Farquhar & Lloyd 1993; Yakir et al. 1994) and provides a non-destructive probe to investigate terrestrial-atmospheric exchanges of $\text{CO}_2$ and water vapour (Farquhar et al. 1993; Yakir & Wang 1996). The magnitude of discrimination expressed by the leaf is a result of both leaf water enrichment and the diffusional resistance of $\text{CO}_2$ from ambient air to the chloroplast (Farquhar & Lloyd 1993; Flanagan et al. 1994). The latter is represented by the expression $C_i/C_a$, the concentration of $\text{CO}_2$ within the chloroplast of a leaf relative to that in ambient air, and is a key intracellular parameter which represents leaf photosynthetic gas exchange and is important for interpreting oxygen and carbon isotope discrimination of $\text{CO}_2$ during photosynthesis. Estimation of $C_i$ is possible by analysing the difference between observed instantaneous carbon isotope discrimination and that predicted from concurrent measurements of gas exchange [$\Delta_i - \Delta_{\text{obs}}$; Evans et al. (1986); von Caemmerer & Evans (1991)].
Measurements of photosynthetic response and isotope discrimination under steady-state conditions are necessary to gain an understanding of the interaction between physiological and biochemical processes that occur during photosynthetic gas exchange. Because response times of gas exchange vary with light and water stress, the natural variation in environmental conditions under some field situations may mean that photosynthetic steady state is rarely achieved. Stomatal conductances can take 20 min to achieve steady state following changes in irradiance (Barradas & Jones 1996) and have been reported to take between 20 and 40 min for *Piper auritum* and *P. aequale* (Tinoco-Ojanguren & Pearcy 1992). However, field data have shown that despite large shifts in A and gs, C/Ca can remain relatively constant throughout the day [see Tenhunen *et al*. (1984); Wise *et al*. (1991) and references therein] from which simple discrimination models would predict a constant Δ13CO2 (Farquhar *et al*. 1989). This suggests that steady-state discrimination may occur without the prerequisite of steady-state photosynthesis.

However, initial observations of diurnal changes in instantaneous discrimination for *P. aduncum* (a tropical pioneering tree) under field conditions in Trinidad revealed large variability in the measured discrimination signal compared with that predicted (M. S. J. Broadmeadow, unpublished field observations; Gillon *et al*. 1997). Recent laboratory work has suggested that under low rates of assimilation, changes in (photo)respiratory CO2 evolution can affect the on-line signal (Gillon & Griffiths 1997). Models predicting discrimination are essential for understanding both net and gross exchanges of CO2 between the biosphere and the atmosphere. However, whether the observed oxygen and carbon discrimination in CO2 follows that predicted, despite large changes in A and gs, under field conditions, has not been extensively investigated for 13CO2 and only under laboratory conditions for C18O16O.

Additionally, estimation of 18O leaf water enrichment at the sites of evaporation (δ) is important in analysing isotopic leaf water budgets. Using the Craig & Gordon (1965) model of evaporative enrichment for hydrological systems, the enrichment of water at the evaporating surfaces of a leaf can be expressed as the proportion of H218O evaporated relative to H216O (White 1988; Flanagan, Comstock & Ehleringer 1991a; see Appendix A).

Whilst the model is useful for estimating the degree of enrichment at the evaporation sites (δ), it is, by derivation, dependent on the leaf being at isotopic steady state (ISS), the point at which transpired water vapour has the same oxygen isotope composition as the stem water. The time for leaf water to attain ISS is proportional to leaf water turnover rate [defined as the ratio of leaf water volume to transpiration rate, V/E; Farris & Strain (1978); Flanagan, Bain & Ehleringer (1991b); Wang & Yakir (1995)] and the magnitude of the change in environmental conditions that the leaf is exposed to (Flanagan *et al*. 1991b; Yakir *et al*. 1994). Under transient environmental conditions, we cannot always assume that a leaf is at ISS (Wang & Yakir 1995). Direct measurement of the δ18O of transpired water vapour during photosynthetic gas exchange would, therefore, allow: (i) the identification of proximity to ISS; (ii) an estimation of evaporative site enrichment irrespective of ISS; and (iii) provide a greater insight into the range of δ18O water vapour signal transferred to the surrounding environment, which may have important implications for scaling up of water transfer from leaf to canopy to atmosphere.

The objectives of this investigation were to follow photosynthetic gas exchange, discrimination against 13CO2 and C18O16O along with the extent of evaporative site enrichment in H218O, under field conditions for a deciduous rainforest in Trinidad. In particular, the natural fluctuations throughout the day were examined, irrespective of whether steady-state photosynthetic gas exchange or isotope discrimination were attained. From this we hoped to ascertain whether steady-state photosynthesis was a prerequisite for steady-state isotopic discrimination, as well as evaluating whether discrimination models reliably predict discrimination, and therefore Cc, under natural conditions in the field.

**MATERIALS AND METHODS**

Experiments were conducted in the field, at the Asa Wright, Simla Research Station, Trinidad [see Borland *et al*. (1993) for a full site description] on 2 d in February 1995. An elevated bamboo platform was constructed so that the upper leaves of the *P. aduncum* canopy (those exposed to full sunlight) could be reached. Leaves selected were fully expanded and located at the same height and position within the canopy. To gain a representative sample of gas exchange and discrimination characteristics, random leaves were used on day 1 (8 February 1995). On day 2 (16 February 1995), the same three leaves were measured through the day to account for any effects of leaf-to-leaf variation.

Additionally, three leaves were collected for δ18O leaf water determination, at 0930 and 1230 h on the first day and placed into Exetainers (Eurosta Scientific, Crewe, UK). Spring water was collected from a ground water spring within 0.5 km of the field site. Rain water was collected in a rain gauge, positioned in a clearing, fitted with a funnel to minimize evaporation and the oxygen isotope composition was analysed for each major precipitation event, throughout the field campaign. Volumes of 1 cm3 of collected rain or spring water were injected into pre-evacuated Exetainers (Eurosta Scientific, Crewe, UK).

**Photon flux and temperature**

Instantaneous photon flux density (PFD) was followed throughout the day using the sensor attached to the portable infra red gas analysis system (IRGA) used for gas exchange measurements (see below). Measurements of total daily PFD were also recorded using an integrating light meter (Delta T, Cambridge, UK). Shade air temperature and relative humidity measurements were taken with
the dew point humidity sensor (Protimeter, Marlow, UK) throughout the course of both days.

**Leaf water potential**

Xylem sap pressure was measured using a pressure chamber to indicate leaf water potential (Soil Moisture Equipment Corporation, Santa Barbara, CA, USA) for day 1 only (because of the limited nitrogen supply in the remote field location). Leaves were taken from the same height and position in the canopy over the day ($n=3$).

**Gas exchange**

Gas exchange measurements were conducted by enclosing 10–20 cm$^2$ sections of leaf within a leaf cuvette attached to a CIRAS-1 portable IRGA (PP Systems, Hitchin, UK) on leaves throughout the day, under ambient light, CO$_2$ concentration and temperature. The leaf temperature was calculated using an energy balance calculation (Parkinson 1983) and gas exchange parameters were recalculated for the respective leaf areas. On day 1, a conifer PLC 3 head (ADC, Hoddeston, U.K.) was used, whilst in the hotter conditions on day 2 this was replaced with a broad-leaf PLC (PP Systems, Hitchin, UK) attached with an external cooling fan. The air supply was taken from a 5 dm$^3$ buffering volume $>15$ m upwind of the platform to provide a reference supply with a stable CO$_2$ concentration and isotopic signature. The flow of air passed through the cuvette ranged between 350 and 430 cm$^3$ min$^{-1}$. Gas exchange measurements were taken immediately before and after the 15 min on-line collection periods, and were averaged for the collection time. The $P$. aduncum leaves were hypostomatous with an average total leaf area of 85 cm$^2$. Mean stomatal density taken from five stomatal epidermal impressions of three leaves was 193 mm$^{-2}$. Leaf conductance to water vapour was corrected for hypostomy (CIRAS manual, PP systems 1994). Differences between the two types of cuvettes used were accounted for by correcting for boundary layer resistances of 0.45 and 0.28 m$^2$ s$^{-1}$ mol$^{-1}$ for days 1 and 2, respectively.

**Collection of CO$_2$ and water vapour for isotope analysis**

Atmospheric CO$_2$ and water vapour were collected using a modified glass collection line (Griffiths et al. 1990; Broadmeadow et al. 1992) attached to a rotary vacuum pump. The standard collection procedure was modified to enable separation of CO$_2$ from water vapour for C$^{18}$O$^{16}$O and H$_2$$^{18}$O analysis. Air from either upstream (reference) or downstream (analysis) of the leaf cuvette was passed at positive pressure to a metering valve (Swagelock, Ohio, USA). The atmospheric CO$_2$ and water vapour were trapped in a 6 mm internal diameter double spiral cold trap, immersed in liquid nitrogen, under a partial vacuum. Reference air was typically collected twice at the beginning and end of the day and on average after every third analysis collection, to account for diurnal variations in the ambient isotopic composition of canopy air, $\delta_a$ [Fig. 1, see also Borland et al. (1993); Harwood (1997)]. Cryogenically trapped CO$_2$ and water vapour were isolated and evacuated down to $10^{-2}$ mbar ($10^{-3}$ kPa).

The CO$_2$ was then liberated using acetone cooled to $-70$ °C (by addition of liquid nitrogen), for at least 2 min and condensed into a Pyrex side arm and sealed using a butane blow torch. As a precautionary measure, the sealed Pyrex tube was immediately stored in a liquid nitrogen dry shipper (Jencons, Leighton Buzzard, UK). Should complete separation of the CO$_2$ and H$_2$O not have been

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** Diurnal variation in (a) $\delta_a^{13}$CO$_2$, (b) $\delta_a^{18}$O$^{16}$O and (c) $\delta_a^{18}$O$_2$ of cuvette air supply on day 1 (○) and day 2 (●). Best fit polynomial regressions were applied for both days from which the isotopic composition at the time of the analysis ($\delta_{in}$) was derived. The fitted trend lines are represented by fine and thick lines for day 1 and day 2, respectively. The CO$_2$ reference at 1014 h on day 1 (arrowed) was excluded from the regression as the internal mass spectrometer precision of the sample was four times greater than that observed for all other samples.
achieved during sampling, cold storage prevented equilibration (Harwood 1997) allowing the C18O16O signal to be preserved.

A new Pyrex side arm was then fitted to the collection line and evacuated. The water was liberated by heating the trap using a butane blow torch and cryodistilled into the freshly evacuated side arm until no more water was observed to condense in the side arm (this was usually in the order of 2–3 min). An aliquot of CO2 was then introduced into the collection line, so that the δ18O2 could be measured through equilibration with CO2. This was achieved by backfilling a gas tight syringe with CO2, of known isotopic signature from a modified commercial drink carbonation cylinder and injecting 1 cm3, via a septum fitting, into the collection line. The CO2 was then passed into the side arm, frozen above the water sample and sealed in the vial using the blow torch.

**Protocol for determination of C18O16O**

Samples of CO2 were stored in liquid nitrogen until purification. Each sample was then taken individually, warmed to room temperature, wiped to remove any external moisture and placed in a bottle breaker attached to a sample purification line. The transfer time from liquid nitrogen to breaking the sample tube under vacuum was typically less than 2 min, so as not to allow equilibration with any residual water.

Technique development showed that small volumes of CO2 inferred incomplete separation, or collection, of the CO2 and water vapour which lead to large outliers. Such samples were, therefore, rejected. This was validated in laboratory trials leading to precision for separated CO2 of ±0.08‰ for δ13CO2 and ±0.07‰ for δ18O16O (standard deviations, n = 8) where isotope compositions are expressed as δ using the per mille (‰) notation given by δ (‰) = [(Rsample/Rstandard)−1]×1000. For 13CO2, R = 13C/12C and the standard is Pee Dee Belemnite (PDB) and for C18O16O, R = 18O/16O and the standard is CO2 derived from PDB (PDB–CO2).

**Protocol for determination of δH18O of water vapour**

Samples containing water and equilibration CO2 were left for a minimum of 3 d to equilibrate, after which the sample was purified in accordance with the standard procedure. Corrections were made for equilibration temperature and respective volume of gas and liquid according to Scrimgeour (1995).

The volume of water vapour present in the side arm (involved in equilibration) was calculated from the flow rate of air passing through the collection line (the rate set by the needle valve), the duration of the collection, and the water vapour concentration of the air supply as measured by the CIRAS water vapour IRGAs attached to the collection line. The average volume of water collected over 15 min generally varied between 0.05 and 0.07 cm3, depending on the ambient vapour pressure. Reproducibility of water vapour samples equilibrated with CO2 were typically ±0.09‰ (n = 8) and were expressed in the δ notation relative to the water standard, standard mean ocean water (SMOW).

**Purification and mass spectrometric analysis of on-line samples**

All atmospheric CO2 and water equilibrated CO2 samples were purified by cryodistillation under vacuum on a purification line by passing the gas through two acetone traps at dry ice temperature (−70 °C, as described in Griffiths et al. (1990) to remove any water and non-condensable gases. Samples were analysed using a dual inlet mass spectrometer modified to a VG 903 triple collector specification by Provac Services (Crewe, UK) and run using the Europa IRMS (Crewe, UK) data collection software, against a working standard of 99.995% CO2 (BDH High Purity Gases, Poole, UK), with a δ3CO2 of −43.18‰ and a δ18O of −28.72‰ versus PDB–CO2.

Samples of CO2 were corrected for the presence of N2O (as N2O has the same molecular mass, 44, as CO2). Mass spectrometer sensitivity to N2O was determined using the method described in Freidli & Siegenthaler (1988), whereby pure CO2 and N2O are mixed using mass flow controllers to produce different CO2–N2O gas mixtures (where ρ represents the ratio of the volume of N2O to CO2) and admitted into the mass spectrometer. The corrections derived for the ratio of N2O:CO2 representing ambient air (ρ = 0.00088, 310 p.p.b.:350 p.p.m., respectively) of +0.24‰ for δ13C and +0.31‰ for δ18O were in agreement with those observed in other investigations (Freidli & Siegenthaler 1988; Flanagan & Varney 1995). Diurnal variation in ambient N2O was considered as negligible and, thus, corrections to CO2 were made using an N2O concentration of 310 p.p.b. and the respective CO2 concentration using the equations of Freidli & Siegenthaler (1988).

**Source and rain water**

Rain and source water were analysed using a direct equilibration method (Scrimgeour 1995), whereby the water is equilibrated with CO2 of known volume and isotopic composition. Samples were left for at least 3 d to equilibrate, after which they were run on the ANCA CF-IRMS (Europa Scientific, Crewe, UK). Working standards also underwent the same equilibration process as the samples, to account for any variation in sample preparation. After mass spectrometer analysis, samples were corrected for their respective volumes, δ18O of the equilibration CO2 and equilibration temperature. Reproducibility of repeated runs of the same sample generally yielded a standard deviation of ±0.07‰ (n = 8). The oxygen isotope composition of all source, rain and leaf water (see below) samples were expressed in the δ notation relative to SMOW.

$\Delta H_2^{18}O$ Leaf water

The $\Delta H_2^{18}O$ of the leaf water samples were kindly analysed by Prof. D. Yakir and colleagues at the Weizmann Institute, Rehovot, Israel. Leaf water was extracted via vacuum distillation for analysis as described in Wang & Yakir (1995). External precision for the water samples was approximately $\pm 0.2\%$.

On-line discrimination of $^{13}CO_2$ and $^{18}O^{16}O$

Measured (observed) discrimination of $^{13}CO_2$ ($\Delta_{obs}$) was calculated as described by Evans et al. (1986) below, whereby:

$$\Delta_{obs} = \frac{\xi (\delta_{out} - \delta_{in}) \times 1000}{1000 + \delta_{c} - \xi (\delta_{out} - \delta_{in})},$$

(1)

where $\xi = c_r(c_c - c_b)$ and $\delta_{in}$, $c_c$ and $\delta_{out}$, $c_b$ are the isotopic composition and $CO_2$ concentration entering and leaving the cuvette, respectively. Values of $c_b$ were corrected to the vapour pressure of $c_c$ to account for any dilution of $CO_2$ leaving the cuvette from increased water vapour concentration. Background ambient $CO_2$ typically varied between $-9.5$ and $-8.5\%$ $\delta^{13}CO_2$ (Fig. 1a) whilst $\delta^{18}O^{16}O$ ranged from $-0.5$ to $0.5\%$ (Fig. 1b). To ensure consistency in the selection of $\delta_{obs}$ for measurements of concurrent discrimination of $^{13}CO_2$, $^{18}O^{16}O$ and $H_2^{18}O$, values were interpolated from temporal polynomial regressions of the reference air isotope composition ($\delta_{obs}$) for both days at the time of any given analysis (Fig. 1). The more conventional method, expressing the isotope composition of ambient air as a function of the inverse of the concentration, could not be applied as no relationship held with either $\delta^{13}CO_2$ and $1/\delta^{18}O^{16}O$ or $\delta^{18}O$ and $1/\delta^{18}O^{16}O$, consistent with other observations within this canopy (Harwood 1997).

Instantaneous ‘on-line’ discrimination of $^{18}O^{16}O$ was derived using the same equation as for $\Delta^{13}CO_2$ (Farquhar & Lloyd 1993) by simply substituting the oxygen isotope composition of $CO_2$ for $\delta_{in}$ and $\delta_{out}$ in Eqn 1.

**Modelled discrimination of $\Delta^{13}CO_2$ and $\Delta^{18}O^{16}O$**

Modelled discrimination against $^{13}CO_2$ ($\Delta_t$) was derived from the following equation (Evans et al. 1986):

$$\Delta_t = a + \frac{(C_a - C_i)}{C_a} + \frac{b'(C_i)}{C_a} - \frac{f^*}{C_a},$$

(2)

where $C_a$ and $C_i$ refer to the concentration of $CO_2$ in the air and intercellular leaf spaces, respectively (where $C_i$ represents the $CO_2$ concentration exposed to the leaf and is represented by the $CO_2$ concentration leaving the cuvette), $b'$ is an assumed value of net discrimination of Rubisco (here taken as $29\%$), $a$ is the fractionation due to diffusion through the stomata (4-4\%); $f^*$ is the $CO_2$ compensation point in the absence of dark respiration (calculated from the regression equations listed by Brooks & Farquhar (1985) and $f$ represents the fractionations associated with photorespiration [here taken as 7\%; Rooney (1988); Gillon & Griffiths (1997)].

The offset between the modelled $^{13}CO_2$ discrimination, $\Delta_t$ (Eqn 2) and that measured, $\Delta_{obs}$ (Eqn 1), expressed as $\Delta_t - \Delta_{obs}$ has traditionally been attributed to additional drawdown imposed by the internal resistance to $CO_2$ diffusion from the intercellular air space $CO_2$ concentration ($C_i$) to that at the sites of carboxylation in the chloroplast ($C_c$; Evans et al. 1986; von Caemmerer & Evans 1991).

The difference between measured and modelled discrimination ($\Delta_t - \Delta_{obs}$) has, therefore, been used to estimate $C_c$ by rearranging the following equation (von Caemmerer & Evans 1991):

$$\Delta_t - \Delta_{obs} = (b' - a)(C_i - C_c)/C_a + (f^*)/C_a.$$  

(3)

Additionally, if values of $g_w$ are known then from Fick’s law of diffusion, estimates of $C_c$ can also be derived using the following expression (von Caemmerer & Evans 1991; Harley et al. 1992):

$$A = g_w(C_i - C_c)/P,$$  

(4)

where $A$ is assimilation in $\mu mol m^{-2} s^{-1}$, $P$ is atmospheric pressure and $g_w$ is the internal transfer conductance for $CO_2$ (in $mol m^{-2} s^{-1} bar^{-1}$). Whilst conductance for $CO_2$ ($g_w$) between $C_a$ and $C_i$ can vary, the internal transfer conductance ($g_w$) from $C_i$ to $C_c$ within the same leaf can be considered as constant.

Assuming there is complete equilibration between $CO_2$ and water within the chloroplast, the on-line discrimination of $^{18}O^{16}O$ can be predicted using the following equation (Farquhar & Lloyd 1993):

$$\Delta C^{18}O^{16}O = \bar{a} + \frac{C_c}{C_a - C_c} (\delta_{in} - \delta_{out}).$$  

(5)

where $\delta$ is the oxygen isotope composition of $CO_2$, $C$ is the concentration of $CO_2$ and subscripts $c$ and $a$ refer to the chloroplast and ambient air, respectively, and $\bar{a}$ is the weighted mean of discrimination occurring during the diffusion from ambient air to the sites of carboxylation within the chloroplast, here taken as 7.4\% (Farquhar et al. 1993).

**Determination of the $\Delta H_2^{18}O$ of transpired water vapour ($\delta$)**

The $\Delta H_2^{18}O$ of transpired water vapour was derived by mass balance from measuring the water vapour concentration and isotope composition before and after a leaf cuvette, as follows (see Appendix A):

$$\delta_i = \xi_i (\delta_{out} - \delta_{in}) + \delta_{in},$$  

(6)

where $\xi_i$ = $e_{out}(e_{out} - e_{in})$ and $\delta_{in}$, $e_{in}$ and $\delta_{out}$, $e_{out}$ are the oxygen isotope composition and vapour pressure (in mbar) of the water vapour entering and leaving the cuvette, respectively. This was substituted for $R_1$ in Eqn A3 and solved for $R_c$ to provide an estimate for enrichment at the evaporative sites of the $P. aduncum$ leaves, without the prerequisite of ISS. The oxygen isotope composition of the
reference water vapour, \( \delta_{2}^{18} \text{O} \), varied between –7·5 and –10·0‰ on day 1 and between –9·0 and –10·9‰ on day 2 (Fig. 1c). Variation in the flux and isotope composition of transpired water vapour under the high and variable leaf-to-air VPDs may have accounted for the differences in \( \delta_{2}^{18} \text{O} \) between days 1 and 2. As with \( \text{CO}_2 \), the value of \( \text{H}_2\text{O} \delta_n \) at the time of the analysis was derived from a temporal polynomial regression of the \( \delta^{18} \text{O} \) of water vapour entering the cuvette (Fig. 1c).

RESULTS

Photon flux, temperature and leaf water status

The average PFD on day 1 was lower and more intermittent than on day 2, due to greater cloud cover (Fig. 2a & b). The accumulated PFD recorded reached 26·7 and 37·2 mol m\(^{-2}\) d\(^{-1}\), for days 1 and 2, respectively. In general, day 1 experienced substantially lower temperatures and higher relative humidities throughout the photoperiod than day 2 (data not shown). These brighter, drier conditions on day 2 resulted in higher average leaf temperatures of 37·3 °C between 1000 and 1400 h on day 2, compared with a mean of 31·6 °C for the same period on day 1 (Fig. 2c).

The leaf water potential (\( \Psi \), measured on day 1 only) decreased from early morning, reaching a minimum of –1·1 MPa at 1300 h and then increased in the afternoon (Fig. 2d). The leaf water potential had fully recovered to the predawn value of –0·21 MPa, by 1800 h. In general, the diurnal transition in leaf water potential was gradual, reflecting the overall diel progression in evaporative demand, and contrasted with the variability seen in leaf gas exchange which was influenced by individual leaf stomatal responses (Fig. 3a & b).

Gas exchange

On day 1, gas exchange measurements were made continuously throughout the day on a range of leaves. Assimilation was greatest in the morning around 0800 h and declined as the day progressed (open symbols, Fig. 3a), whilst the general diurnal trend in stomatal conductance tended to follow leaf-to-air VPD (Fig. 3b & d). Under cloud cover at midday (Fig. 2a), a decrease in leaf-to-air VPD (Fig. 3d) gave rise to a transient increase in average \( A \) and \( g_s \) from 4·2 to 5·8 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Fig. 3a) and 120–185 \( \text{mmol m}^{-2} \text{s}^{-1} \) (Fig. 3b), respectively. The concentration of substomatal \( \text{CO}_2 \), expressed as \( C_i/C_a \), remained relatively stable throughout the day (Fig. 3c), with a mean value of 0·78 (standard error = 0·02, \( n = 12 \)), with the exception of the early morning, between 0800 and 0900 h, where high rates of assimilation increased the drawdown from \( C_a \) to \( C_i \) reducing \( C_i/C_a \) to 0·65.

For day 2, the same three leaves were followed throughout the day (closed symbols, Fig. 3). Higher PFD and temperatures (Fig. 2) induced greater leaf-to-air VPDs (Fig. 3d) reaching a maximum of 42 mbar (4·2 kPa) at midday. Assimilation and stomatal conductance were greatest in the morning before 1000 h and declined over the course of the day. Both \( A \) and \( g_s \) on day 2 were slightly reduced in the drier conditions, compared with day 1. For all leaves,
On day 1, measured $\Delta^{13}\text{CO}_2$ declined gradually over the day, from above 30‰ in the morning to around 10‰ in the late afternoon (open symbols, Fig. 4a). Whilst the sampling procedure showed that individual leaves can have distinct responses, as demonstrated by the regular decline over the course of day 2 in all three leaves (closed symbols, Fig. 4a), variation in measured discrimination was also systematically related to changing environmental conditions. The first measurements of both days, taken as soon as rates of photosynthesis for isotopic discrimination were measurable (the time at which solar elevation exceeded the surrounding canopy) produced unusually high discrimination signals (circled points, Fig. 4a) compared with that modelled (Fig. 4b). This ‘postdawn’ response was observed not only on both days in 1995, but also on the same $P.\ aduncum$ canopy 3 years earlier (M. S. J. Broadmeadow, unpublished field observations 1992; Gillon et al. 1997).

With $C_i/C_a$ relatively constant over both days (Fig. 3c), modelled $\Delta^{13}\text{CO}_2$ ($\Delta_i$) also remained constant (Fig. 4b), so that the observed discrimination ($\Delta_{obs}$) could be either greater or lower than that modelled ($\Delta_i$) from $C_i/C_a$ (Fig. 4c). The offset between observed and modelled discrimination ($\Delta_i - \Delta_{obs}$), when compared with the quotient of assimilation and CO$_2$ concentration, $A/C_a$, can usually be used to derive the internal resistance to CO$_2$, from ambient air to the sites of carboxylation in the chloroplast, $C_c$ (Evans et al. 1986; von Caemmerer & Evans 1991; Evans & von Caemmerer 1996). However, a general decrease in $\Delta_i - \Delta_{obs}$ with $A/C_a$ was observed (Fig. 4c) and contrasted with the increase expected in order to calculate $g_w$.

The systematic shift in $\Delta^{13}\text{CO}_2$ was not a function of the gas exchange system: calculation of $\Delta_{obs}$ is partly dependent on $\xi$, the fractional uptake of CO$_2$ (Eqn 1), where large values of $\xi$ (low CO$_2$ drawdown) may reduce the accuracy of $\Delta_{obs}$ (von Caemmerer & Evans 1991). However, the relationship between $\xi$ and $\Delta_i - \Delta_{obs}$ for $P.\ aduncum$ revealed that scatter occurred over the full range of $\xi$ (8–45), not just at large values (Fig. 4d). In addition, the majority of the data points fell outside the range of $\Delta_i - \Delta_{obs}$ associated with a mass spectrometric precision of ±0.1‰ (shaded triangle, Fig. 4d).

On day 1, measured $\Delta^{18}\text{O}^{16}\text{O}$ generally increased from 10‰ in the morning, to 37‰ at 1330 h, and declined again in the afternoon (open symbols, Fig. 5a). As with $\Delta^{13}\text{CO}_2$, variations from this trend were related to both distinct leaf responses (demonstrated by the three individually measured leaves on day 2, closed symbols, Fig. 5a) and changes in environmental conditions induced by intermittent cloud cover (Fig. 2a & b). For day 2, leaves 2 and 3 followed a similar diurnal pattern, but had maximum midday enrichment of 21 and 23‰, respectively, somewhat lower than that measured on day 1. In contrast, $\Delta^{18}\text{O}^{16}\text{O}$ of leaf 1 remained fairly constant between 8 and 12‰, over the three time intervals measured (also Fig. 5a).

Because of the large variation in $\Delta_{obs}$ relative to $\Delta_i$ for $\Delta^{13}\text{CO}_2$ discrimination (Fig. 4c), the concentration of CO$_2$ in the chloroplast ($C_c$) for $P.\ aduncum$ was estimated using Eqn 4. Internal CO$_2$ transfer conductance ($g_w$) was derived from taking the assimilation of 16 $\mu$mol m$^{-2}$ s$^{-1}$ attained under 1000 $\mu$mol m$^{-2}$ s$^{-1}$ PFD for the pioneering species $P.\ auritum$ (Tinoco-Ojanguren & Pearcy 1992) and the relationship between assimilation under high light and $g_w$ in the literature (von Caemmerer & Evans 1991; Lloyd et al.

Figure 3. Diurnal variation in (a) assimilation ($A$), (b) stomatal conductance ($g_s$), (c) $C_i/C_a$ and (d) leaf-to-air vapour pressure deficits (VPD), for different $P.\ aduncum$ leaves on day 1 (□) and three separate leaves on day 2 (▲ leaf 1, ● leaf 2, ■ leaf 3). Points are the average of two readings.

$C_i/C_a$ slightly increased over the day (Fig. 3c), due to the drop in assimilation, but on average was significantly lower ($P = 0.00026$, students $t$-test) than day 1 at 0.68 (standard error = 0.02, $n = 11$).

Instantaneous isotope discrimination

On day 1, measured $\Delta^{13}\text{CO}_2$ declined gradually over the day, from above 30‰ in the morning to around 10‰ in the late afternoon (open symbols, Fig. 4a). Whilst the sampling procedure showed that individual leaves can have distinct responses, as demonstrated by the regular decline over the course of day 2 in all three leaves (closed symbols,
Assuming constant $g_w$ and under low rates of assimilation, by definition, the offset between $C_i$ and $C_c$, and, hence, $C_i/C_a$ in *P. aduncum* will have remained relatively stable over the course of the day. A reduced chloroplast CO$_2$ concentration on day 2 (closed symbols, Fig. 5a) is, therefore, suggested to partially account for the lower observed values of $\Delta^{13}$CO$_2$. Furthermore, the low C$^{18}$O$^{16}$O discrimination observed for leaf 1 on the second day corresponded with lower values of $C_i/C_a$ (closed triangles Fig. 5a & b). Measured values of $\Delta^{13}$C$^{18}$O$^{16}$O generally lay between that predicted (Eqn 5, dashed lines, Fig. 5b) for chloroplast water in equilibration with that at the evaporative sites of between 2 and 10‰. This agreed well with the evaporative enrichment derived from the $\delta^{18}$O of transpired water vapour (see Fig. 6b) for day 1, but did not reflect the high evaporative enrichment (20‰) in the late afternoon of day 2.

On both days, the $\delta^{18}$O signal of transpired water was generally more depleted than stem water in the morning and more enriched in the afternoon (Fig. 6a). The sole exception to this trend was at 0740 h on day 1, which approximated that of source water and may have occurred due to low and stable VPD conditions early in the morning. An increase in the $\delta^{18}$H$_2$O of transpired water vapour over the day, would be expected with an approach to steady state, under increasing and decreasing VPD, respectively. The transpired water vapour had, on average, approximately the same signal as the stem water ($-3.2‰$) between 1300 and 1500 h on both days indicating that the leaves were probably close to or at ISS.

Leaf water enrichment at the evaporative sites ($\delta_e$) derived from the measurements of transpired water vapour, was tightly related for all leaves over both sampling days, but consistently higher on day 2 (Fig. 6b). Again, there was no systematic relationship with the fractional proportion of water lost ($\xi_l$), as for both days 17 of the 23 data points ranged between 3 and 7 (data not shown). The largest remaining values of $\xi_l$ recorded occurred shortly after dawn and approaching dusk and ranged from 8 to 15.

Evaporative site enrichment ($\delta_e$) generally increased in the morning, peaked between 1300 and 1400 h, and then...
became slightly reduced in the afternoon, on both days. On day 1, $\delta_e$ reached a maximum enrichment of 12.7‰ at 1334 h and remained high in the afternoon (Fig. 6b). For day 2, the maximum enrichment was higher, reaching 20.9‰ at 1423 h, for both leaves 1 and 2. The transient decrease in $\delta_e$ at 1300 h on day 1 was likely to have been due to a decrease in air temperature and leaf-to-air VPD, as a result of cloud cover. The dashed lines in Fig. 6(b) represent values of $\delta_e$ assuming that ISS held throughout the entire day. Such an assumption would have over-estimated evaporative site enrichment under periods of increasing leaf-to-air VPD and under-estimated $\delta_e$ at times of decreasing leaf-to-air VPD, with an increased effect under high VPDs (up to 4‰, in Fig. 6b).

Bulk $\delta H_{2}^{18}O$ leaf water values for P. aduncum (taken on day 1) were 2.56‰ (± 0.2, n = 3) and 7.01‰ (± 0.3, n = 3) for 0930 and 1230 h, respectively (crosses, Fig. 6b). Both measurements were in close agreement with that calculated for the evaporation sites ($\delta_e$), including the transient decrease in enrichment around 1300 h on day 1 due to cloud cover.

**DISCUSSION**

**Gas exchange and leaf water status**

On both days, maximum assimilation of $P$. aduncum occurred in the morning, around 0800 h and gradually declined over the day as leaf-to-air VPD increased and water potential decreased (Figs 2d & 3a). This is similar to that found for $J$. copaia, in French Guiana (Huc, Fehri & Guehl 1994) and in other field observations of diurnal gas exchange (Wise et al. 1990; Wise et al. 1991; Epron, Dreyer & Breda 1992). The minimum leaf water potential ($\Psi$) at midday of ~1.1 MPa for $P$. aduncum was similar to that of ~1.5 MPa for the pioneering tree species $P$. aduncum.
Higher leaf-to-air VPDs in the brighter conditions of day 2 are likely to have accounted for the reduction in stomatal conductance observed in *P. aduncum*. Reduced stomatal conductance under high VPDs has been reported for another pioneering *Piper* species (*P. auritum*, Tinoco-Ojanguren & Pearcy 1993), maintaining instantaneous water use efficiency, as a decline in *g*s reduced assimilation and transpiration in tandem. The variability in stomatal conductance on day 1 was due to leaf-to-leaf variation and intermittent light resulting from cloud cover. Concurrent variation in assimilation was minimal and may have been due to the more rapid response of assimilation to changes in PFD (Tinoco-Ojanguren & Pearcy 1992; Barradas & Jones 1996).

Despite variation in *A* and *g*s, *C*/*C*a remained relatively stable over the day. Observations of relatively stable *C*/*C*a have also been reported, despite variation in *g*s during light flecking in a related species, *P. auritum* (Tinoco-Ojanguren & Pearcy 1992). Furthermore, due to the low rates of assimilation, the theoretical drawdown between *C*t and *C*a never exceeded 30 µmol mol⁻¹ (corresponding with a maximum assimilation of 7 µmol m⁻² s⁻¹), thus, the diurnal variation in *C*/*C*a approximated that of *C*/*C*a.

**On-line discrimination of ¹³CO₂**

Measured Δ¹³CO₂ (*D*₁₃) in *P. aduncum* was similar to that found for other *C₃* mesophytes (Evans et al. 1986). However, the variation in *D*₁₃ could not be accounted for by *C*/*C*a alone (Fig. 4). The observation of a wide range in *D*₁₃ compared with that modelled from gas exchange was observed on the same *P. aduncum* canopy 3 years earlier (M. S. J. Broadmeadow, unpublished field observations 1992; Gillon et al. 1997).

Differences between modelled and observed on-line Δ¹³C have previously been accounted for by *g*s, the CO₂ transfer conductance from the leaf intercellular spaces to the chloroplast (Evans et al. 1986; von Caemmerer & Evans 1991). The maximum drawdown of 30 µmol mol⁻¹ from *C*t to *C*a likely to be encountered in *P. aduncum*, only reduces modelled Δ¹³C by 2% and does not explain the full extent of the shift between both modelled and measured Δ¹³CO₂ (*Δ* = *D*₁₃). Any effect of drawdown to *C*a on *D*₁₃ should be reflected by a positive correlation between *Δ* = *D*₁₃ and *A*/*C*a (Evans et al. 1986). In contrast, on both days, *P. aduncum* appeared to exhibit a negative correlation, whereby the greatest values of *Δ* = *D*₁₃ coincided with low assimilation rates, when the drawdown from *C*t to *C*a should have been minimal. Thus, the data are not explained by conventional theory, as neither *C*t or *C*a could fully account for the observed range of measured Δ¹³CO₂.

Further analysis also revealed that large variations in *D*₁₃ could not be explained by the influence of mass spectrometric precision at high *ξ* (Fig. 4d) or diurnal variation in δ¹³CO₂ (Fig. 1a). Thus, similarities between data on both days and that recorded 3 years previously on the same *P. aduncum* stand suggest the range of Δ¹³C observed here may be a function of plant physiology rather than any technical problems.

Increased stomatal patchiness has been associated with water stress (see Eckstein et al. (1996) for a recent review) and also with concurrent changes in relative humidity and irradiance (Cardon, Mott & Berry 1994). Any existence of patchy stomatal closure may lead to an over-estimation of *C*t calculated from measurements of gas exchange (Farquhar 1989; Mott 1995). In this case, actual leaf sub-stomatal CO₂ concentration would be lower, and in turn reduce modelled discrimination (*Δ*). Any shift in *Δ* would still leave values of *D*₁₃ both above and below that modelled and, thus, stomatal patchiness seems unlikely to be wholly attributable for the full range of discrimination values observed. However, investigation of asymmetric stomatal closure was beyond the scope of the field work and more experimental work is required to assess any interaction with discrimination.

The range of *A*/*C*a encountered with *P. aduncum* in the field, 0.005–0.02 (Fig. 3c), is generally lower than values found in controlled conditions [e.g. 0.04–0.12; Evans et al. (1986)]. Values of *Δ* = *D*₁₃, increasing at lower assimilation rates, suggest that measured Δ¹³CO₂ is lower than modelled from *C*/*C*a. This phenomenon was also observed in *Phaseolus vulgaris*, where the Δ¹³C of dark respired CO₂ was very negative at c. −50‰ when plants were grown air depleted in ¹³C (Gillon & Griffiths 1997), as well as other gas exchange studies under field conditions [M. S. J. Broadmeadow, unpublished field data 1992; K. G. Harwood, unpublished field data on *Quercus* 1996; J. S. Gillon, unpublished field data on *Prosopis* 1997; see also Gillon et al. (1997)]. At such low assimilation rates, it was suggested that CO₂ from dark respiration in the light can make a significant contribution to net CO₂ exchange, whereby respiratory CO₂ is more depleted in ¹³C than the CO₂ being assimilated via photosynthesis. Any back diffusion or re-fixation of this depleted CO₂ from the leaf reduces the enrichment in ¹³C observed in air passing over the leaf (i.e. a smaller difference between *δ*a and *δ*net, Eqn 1). Thus, the net discrimination expressed by the leaf is lower than predicted (i.e. reduced *D*₁₃). The data would also suggest that respired CO₂ is relatively more depleted in ¹³C than the initial Rubisco product. Although no fractionation during respiration has been reported for cells in *vitro* (Lin & Ehleringer 1997), such a situation may arise from short-term differences in the isotopic composition of the respiratory substrates and CO₂ being assimilated, due to the slow turnover time of carbohydrate pools, c. 4 h (Parnik, Keerberg & Vil 1972). Similarly, a transient enrichment in the ¹³C of respired CO₂ (relative to that fixed by photosynthesis), possibly as a function of a nocturnal metabolic modification of the carbohydrate pools’ isotope composition, may account for the occurrence of very high *D*₁₃ above that predicted, evident in the few hours after dawn on both days.
Any effects of slow carbohydrate pool turnover on $\Delta_{obs}$ due to increased rates of dark respiration or re-fixation occurring at high temperatures, may be exacerbated in the tropics, and are unlikely to be encountered in crop species usually studied, with characteristically higher values of $A/C_a$ under controlled laboratory conditions.

**On-line discrimination in $^{18}$O$^{16}$O**

Discrimination in $^{18}$O$^{16}$O reflects both the enrichment of $^{18}$O in leaf water, with which it equilibrates before diffusing back out of the chloroplast, and also the extent of back diffusion of CO$_2$ which allows the leaf water enrichment to be expressed (Farquhar et al. 1993). With a relatively small variation in $C_i/C_a$ (derived from $C_i/C_a$) over the course of both days, $\Delta^{18}$O$^{16}$O generally reflected leaf water enrichment, increasing in the morning, until just after midday and slightly decreasing in the afternoon (Fig. 5a). Discrimination in $^{18}$O$^{16}$O is strongly influenced by the non-linear relationship with $C_i/C_a$ (Farquhar et al. 1994). This was demonstrated by a general reduction in values of $\Delta^{18}$O$^{16}$O on day 2 compared with day 1, when stomatal conductance and $C_i/C_a$ were reduced under high VPDs (Fig. 5b). Using the estimated $g_w$ of 0·25 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$, the observed $\Delta^{18}$O$^{16}$O generally lay between that predicted for chloroplast enrichment of 2 and 10‰ (assuming complete equilibration between leaf water and the chloroplast). The extent of back diffusion of CO$_2$ from the chloroplast (Evans et al. 1986; Farquhar et al. 1993; Williams & Flanagan 1996) was low, particularly for day 2, when stomatal conductance and $C_i/C_a$ were high (Farquhar et al. 1994). This was demonstrated by a general reduction in values of $\Delta^{13}$CO$_2$ and $\Delta^{18}$O$^{16}$O on the extent of back diffusion of CO$_2$ from the chloroplast (Evans et al. 1986; Farquhar et al. 1993; Williams & Flanagan 1996) the two processes were not necessarily coupled in field conditions (Figs 4a & 5a), largely because $C_i/C_a$ imposes a linear control on $\Delta^{13}$CO$_2$, whereas $\Delta^{18}$O$^{16}$O is dependent on the non-linear relationship with $C_i/C_a$ and the interaction with VPD-induced evaporative enrichment (Williams, Flanagan & Coleman 1996). Additionally, any contribution from dark respiratory CO$_2$ may alter the signal of on-line $\Delta^{13}$CO$_2$, yet not affect the $\Delta^{18}$O$^{16}$O, as the $\delta^{18}$O signal of respiratory CO$_2$ will be lost in the equilibration with leaf water in the chloroplast.

**Enrichment of leaf water at the evaporative sites ($\delta_{H_2^{18}O/\delta_i}$)**

In an approach to ISS, transpired water vapour was generally less enriched in $^{18}$O than source water during periods of increasing VPD (e.g. morning) and more enriched than source water during times of decreasing VPD (e.g. afternoon). A relative stability in VPD combined with a slight lag in leaf water turnover rate, indicated that leaf water enrichment was close to ISS between 1300 and 1500 h (Fig. 6a). This observation of proximity to ISS in the field has important implications in modelling the contribution of transpired water vapour to terrestrial water budgets using the isotopic composition of atmospheric water vapour (Bariac et al. 1989, 1994; Brunel et al. 1992). This supports the assumption of ISS in modelling $\Delta^{18}$O$^{16}$O discrimination (Farquhar et al. 1993), because the time when ISS occurs coincides with the most photosynthetically active time of the day, when the greatest fluxes of CO$_2$ and H$_2$O should take place, for many terrestrial plants. However, both this and other field observations have demonstrated that under some conditions, optimum exchange of CO$_2$ and water vapour can occur in the early part of the day, both at the leaf level (Roberts et al. 1980; Leverenz et al. 1982; Roberts, Wallace & Pitman 1984; Briggs, Jurik & Gates 1986; Roberts, Cobral & Ferreira de Aguiar 1990; Wise et al. 1990; Eprou et al. 1992; Hinckley et al. 1994) and at the whole canopy level (Grace et al. 1995). In these cases, the associated changes in environmental conditions may yield non-steady-state photosynthesis and transpiration, for which steady-state estimations of both $\Delta^{13}$CO$_2$ and $\Delta^{18}$O$^{16}$O, derived largely from laboratory experiments, may be in error.

Analysis of transpired water vapour allowed the direct calculation of $^{18}$O enrichment of water at the evaporation sites ($\delta_i$) over the full diurnal period, without the prerequisite of ISS. For the conditions experienced, a conventional derivation of $\delta_i$, assuming ISS throughout the day would lead to an over-estimation of evaporative site enrichment early in the morning and an under-estimation later in the afternoon. The extent of evaporative site enrichment was largely maintained in the afternoon, despite the decrease in leaf-to-air VPD (Fig. 6b) suggesting that $^{18}$O enrichment at the evaporative sites may also be affected by species-dependent rates of leaf water turnover (Wang & Yakir 1995), in addition to evaporative control imposed by ambient VPD. Maintenance of evaporative enrichment of leaf water late into the afternoon has also been noted for cotton (Yakir, DeNiro & Gat 1990), alfalfa (Bariac et al. 1989) and maize (Bariac et al. 1994) and also to some extent in mature oak and beech leaves (Forstel 1978) and barley (Walker & Lance 1991), but contrasts with the relatively stable leaf water enrichment found in sclerophyllous juniper and mistletoe by Flanagan, Marshall & Ehleringer (1993). Two direct measurements of bulk leaf water $^{18}$O composition were close to that derived for the evaporative sites and suggest that the evaporative enrichment model provided approximate estimates of bulk leaf water $\delta H_2^{18}O$ at the two times investigated (cf. Farris & Strain 1978; Forstel 1978; Walker et al. 1989; Yakir et al. 1990; Flanagan et al. 1991a, b; Flanagan et al. 1993). The diurnal trend in measured $\Delta^{18}$O$^{16}$O agreed qualitatively with the modelled evaporative enrichment ($\delta H_2^{18}O$ at the evaporation sites, or $\delta_i$). However, quantitatively, $\Delta^{18}$O$^{16}$O was greater on day 1 and this chloroplast signal was tightly coupled to changes in $C_i/C_a$, associated with VPD and conductance effects. Meanwhile, there was a much more gradual transition in leaf water enrichment at the evaporation sites ($\delta_i$, Fig. 6b), similar to overall leaf water status (Fig. 2d) and contrasted with the more variable stomatal influence on $\Delta^{18}$O$^{16}$O (Fig. 5a). Values of measured $\Delta^{18}$O$^{16}$O were low, particularly for day 2, when compared with that predicted from the Farquhar and Lloyd
model using measured values of δc (Eqn 5, Fig. 5b).

By rearranging the Farquhar and Lloyd C18O16O discrimination model the measured ΔC18O16O can be used to yield an estimate of isotopic composition of water within the chloroplast (δc), which can then be compared with δH218O at the evaporative sites (δe). Preliminary analysis for P. aduncum reveals that in most cases, δc is greater than derived δe (below the 1:1 line of unity, Fig. 7). The model used to derive δc is partially dependent on the estimated value of gw used for deriving Cc (here taken as 0·25 mol m–2 s–1 bar–1). However, estimation of δc using both double and half of the internal conductance value selected of 0·125 and 0·5 mol m–2 s–1 bar–1, respectively [representing the natural range of gw reported in the literature; Loreto et al. (1992)], only results in an average respective shift of 1·5 and 0·7% (error bars, Fig. 7). Thus, uncertainty in Cc can still not account for the full offset observed between δc and δe for P. aduncum.

Observations of chloroplast water less enriched than that at the evaporative sites agrees with the conventional theory of a continuum of enrichment from the unfractonated source water, to the most fractionated water at the sites of evaporation (Farquhar & Lloyd 1993). Chloroplast water can be depleted by up to 10% in δH218O compared with that at the evaporative sites (Yakir et al. 1994) and could partially account for the low values of δc observed in this study. However, since the bulk leaf water values were close to that derived for the evaporative sites (Fig. 6), the lower values of ΔC18O16O (Fig. 5) and, hence, δc (Fig. 7) may have been a result of incomplete isotopic equilibration between CO2 and water vapour within the chloroplast (Farquhar & Lloyd 1993; Flanagan et al. 1994), although this may also be questionable under such low rates of gas exchange. Understanding variations in δc and δe are important for discerning the physiological controls of leaf water enrichment and provides a greater insight into the relationship between leaf water enrichment and the δ18O signal transferred to the leaf cellulose. These observations suggest that further investigation into the variation in δc with derived δc is required under field conditions and in particular for reliable estimates of Cc.

Under high temperatures in Trinidad, measurement of Δ13CO2 in the field deviated from that modelled from gas exchange measurements of C/Cw. Simple models applied to calculating discrimination and deriving single point values of gw, under tropical field conditions, should, therefore, be used with caution. This study also suggests discrepancies may still exist between C18O16O and H218O estimation of 18O leaf water enrichment under field conditions. Additionally, the assumption of ISS may not hold under changeable VPDs and a more faithful representation of δc can be derived from measuring the oxygen isotope composition of transpired water vapour. Thus, observed isotopic discrimination under natural field conditions can differ from that predicted under steady-state photosynthesis in controlled laboratory experiments. Whilst the models provide us with an insight into the mechanism of discriminatory processes, investigation of discrimination under field conditions is necessary to ensure accurate prediction of biome discrimination for global models (Farquhar et al. 1993; Ciais et al. 1995, 1997) and to provide a greater understanding of the factors controlling discrimination under natural, transient, environmental conditions.

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APPENDIX A: ESTIMATION OF EVAPORATIVE SITE ENRICHMENT IN $^{18}$O

Flanagan et al. (1991a) modified the Craig & Gordon (1965) equation, whereby the evaporative enrichment within a leaf is derived from the proportion of $^{18}$O evaporated relative to $^{16}$O ($E^{18}/E^{16}$). The difference in evaporation of the heavy and light isotope can be seen in the transpired water, and expressed as a function of the isotope composition of water at the evaporation sites, the kinetic and equilibrium isotope effects, the leaf-to-air VPD experienced and the atmospheric water vapor signal, as follows:

$$ E^{18}/E^{16} = R_{t(\text{vapour})} = \frac{1}{a_k} \left( \frac{R_a^{s(\text{liquid})} e_i e_a - R_a^{s} e_a}{e_i - e_a} \right). $$

where $R$ is the ratio of heavy to light isotopes, and subscripts $t$ and $e$ represent transpired water vapor and (liquid) water at the evaporation sites, respectively, $a_k$ is the liquid- vapor equilibrium isotope effect corrected to leaf temperature (Majoube 1971); $k$ is the kinetic isotope diffusion fractionation factor, through the stomatal pore, which is derived from the relative rates of diffusion of the light to heavy isotope, which is 1-0285 (Merlivat 1978). When using a leaf cuvette to measure photosynthetic gas exchange, $e_a$ represents the H$_2$O concentration the leaf is exposed to and is reflected by the H$_2$O concentration leaving the cuvette.

Because at ISS the transpired water vapour has the same signal as source water, $R_t$ can be substituted by $R_e$, the isotopic composition for stem water, and then solved for $R_e$ as follows (White 1988; Flanagan et al. 1991a):

$$ R_e = a_k \left[ \alpha_k R_a \left( \frac{e_i - e_a}{e_i} \right) + R_a \left( \frac{e_a}{e_i} \right) \right]. $$

Since fractionation is different through turbulent and laminar flow, the model was further adapted to take into account leaf boundary layer effects as follows (Flanagan et al. 1991a):

$$ R_e = \alpha_k \left[ \alpha_k R_a \left( \frac{e_i - e_a}{e_i} \right) + \alpha_{kb} R_a \left( \frac{e_a - e_a}{e_i} + R_a \left( \frac{e_a}{e_i} \right) \right) \right]. $$

where $e_i$ is the vapour pressure at the leaf surface and $\alpha_{kb}$ is the ratio of the diffusion of light to heavy isotope molecules in a boundary layer (1-0189). Vapour pressure at the leaf surface is calculated from the evaporation and boundary layer resistance measured during leaf gas exchange by rearranging the following:

$$ E = \frac{1}{r_b} \frac{(e_{in} - e_a)}{P}, $$

where $E$ is the evaporation, $r_b$ the boundary layer resistance and $P$ atmospheric pressure.

$R_e$ can then be expressed in the delta notation with respect to a set standard by $\delta(\%) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$ where $R_{\text{standard}}$ for $^{18}$O/$^{16}$O is SMOW. Correct derivation of $\delta_e$ using the above, therefore requires the leaf to be at ISS.

By consideration of mass balance, the vapour pressure of H$_2$O in air as it passes through a leaf chamber can be described by

$$ e_{in} e_{in} + e_{diff} R_e = e_{out} e_{out}, $$

where $e$ is the vapour pressure, $R$ is the ratio $^{18}$O/$^{16}$O and the subscripts in, out and diff refer to the water vapour entering the cuvette, leaving the cuvette and that added by evaporation from the leaf. $R_e$ is the oxygen isotope ratio of transpired water vapour. The above expression holds assuming there is no change in temperature, no condensation within the cuvette, that all the water vapour is collected and the $e_{diff}$ remains constant over the measurement period (the authors acknowledge X. F. Wang for helpful discussion).

Solving for $R_e$, and expressing isotopic composition in the small delta notation ($\%$) gives

$$ \delta_i = \frac{e_{in}}{e_{diff}} \frac{\delta_{out} - e_{in}}{e_{diff}} \delta_{in}. $$

Using the term $\xi_i$ to represent the proportion of H$_2$O transpired relative to that in the ambient air simplification of the above expression to one analogous to measured $A^{13}$CO$_2$, as follows:

$$ \delta_i = \xi_i (\delta_{out} - \delta_{in}) + \delta_{in}, $$

where $\xi_i = e_{out}/(e_{out} - e_{in})$ and $\delta_{out}$, $e_{out}$ are the oxygen isotope composition and vapour pressure (in mbar) of the water vapour entering and leaving the cuvette, respectively.