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Original Article

Nitrogen fertilization and $\delta^{18}O$ of CO_2 have no effect on ^{18}O-enrichment of leaf water and cellulose in *Cleistogenes squarrosa* (C_4) – is VPD the sole control?

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ABSTRACT

The oxygen isotope composition of cellulose ($\delta^{18}O_{Cel}$) archives hydrological and physiological information. Here, we assess previously unexplored direct and interactive effects of the $\delta^{18}O$ of CO_2 ($\delta^{18}O_{CO2}$), nitrogen (N) fertilizer supply and vapour pressure deficit (VPD) on $\delta^{18}O_{Cel}$, ^{18}O-enrichment of leaf water ($\Delta^{18}O_{LW}$) and cellulose ($\Delta^{18}O_{Cel}$) relative to source water, and $p_{ex}p_x$, the proportion of oxygen in cellulose that exchanged with unenriched water at the site of cellulose synthesis, in a C_4 grass (*Cleistogenes squarrosa*). $\delta^{18}O_{CO2}$ and N supply, and their interactions with VPD, had no effect on $\delta^{18}O_{Cel}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$. $\Delta^{18}O_{Cel}$ and $\Delta^{18}O_{LW}$ increased with VPD, while $p_{ex}p_x$ decreased. That VPD-effect on $p_{ex}p_x$ was supported by sensitivity tests to variation of $\Delta^{18}O_{LW}$ and the equilibrium fractionation factor between carbonyl oxygen and water. N supply altered growth and morphological features, but not ^{18}O relations; conversely, VPD had no effect on growth or morphology, but controlled ^{18}O relations. The work implies that reconstructions of VPD from $\Delta^{18}O_{Cel}$ would overestimate amplitudes of VPD variation, at least in this species, if the VPD-effect on $p_{ex}p_x$ is ignored. Progress in understanding the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ will require separate investigations of p_{ex} and p_x and of their responses to environmental conditions.

Key-words: growth and morphology; leaf water; nitrogen fertilizer; oxygen isotopes in CO_2 and water; $p_{ex}p_x$; vapour pressure deficit.

INTRODUCTION

The oxygen isotope composition of cellulose ($\delta^{18}O_{Cel}$) is a function of environmental conditions and morpho-physiological plant traits that influence the $\delta^{18}O$ of water in chloroplasts, cellular compartments involved in the synthesis of transport sugars, stores and sink tissues where sugars are metabolized and used in cellulose synthesis (Barbour 2007; Gessler *et al*. 2014). As it contains environmental and physiological information, the $\delta^{18}O$ of cellulose is of great interest to a broad range of scientific disciplines, including palaeoecology, global change science, plant physiology and plant breeding (Roden *et al*. 2000; Barbour 2007; Farquhar *et al*. 2007; Kahmen *et al*. 2011; Xiao *et al*. 2012; Flanagan & Farquhar 2014; Gessler *et al*. 2014). Current knowledge on the relationship between $\delta^{18}O$ of cellulose and $\delta^{18}O$ of source and leaf water has been summarized in a quantitative model by Barbour & Farquhar (2000):

$$\Delta^{18}O_{Cel} = (1 - p_{ex}p_x)\,\Delta^{18}O_{LW} + \varepsilon_o \quad (1)$$

in which $\Delta^{18}O_{Cel}$ is the ^{18}O enrichment of cellulose above source water; $\Delta^{18}O_{LW}$ is the ^{18}O-enrichment of bulk leaf water above source water; $p_{ex}p_x$ is an attenuation factor – reducing the slope of the relationship between $\Delta^{18}O_{Cel}$ and $\Delta^{18}O_{LW}$ – that is determined by p_{ex}, the proportion of oxygen in cellulose that has exchanged with medium water at the site of cellulose synthesis and p_x the proportion of source water (i.e. unenriched by transpiration effects) at that site; and ε_o is the equilibrium fractionation factor between carbonyl oxygen and water. The value of ε_o has been estimated near 27‰ (Sternberg & DeNiro 1983; Sternberg *et al*. 1986), with an – (at least) partly temperature-related (Sternberg & Ellsworth 2011) – uncertainty of a few ‰ (DeNiro & Epstein 1981; Waterhouse *et al*. 2002; Ellsworth & Sternberg 2014). Equation 1 is based on the assumption that oxygen in CO_2 or in metabolic precursors of leaf sucrose – a primary photosynthetic product, common transport sugar and substrate for cellulose synthesis – exchanges with water in such a way that all oxygen in sucrose synthesized by photosynthesis in leaves is in full equilibrium with average leaf water (Cernusak *et al*. 2003a; Barbour 2007, but see also Gessler *et al*. 2013). The exchange of oxygen beween CO_2 and water is greatly accelerated by carbonic anhydrase (Gillon & Yakir 2001a, 2001b; Cousins *et al*. 2006a, 2006b). Together, these conditions imply that $\Delta^{18}O_{Cel}$ is fully independent of the oxygen isotope composition of CO_2 ($\delta^{18}O_{CO2}$). So far, direct empirical support for the absence of a significant $\delta^{18}O_{CO2}$ effect on $\Delta^{18}O_{Cel}$ was provided by a single experiment with wheat (DeNiro & Epstein 1979).

Water uptake by plants occurs with no ^{18}O fractionation, so that source water reflects the $\delta^{18}O$ at the location/soil depth of uptake (White *et al*. 1985; Ehleringer & Dawson 1992; Dawson *et al*. 1993). But leaf water becomes enriched in ^{18}O at the evaporative site in stomatal cavities (Dongmann *et al*.

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1974; Flanagan *et al.* 1991), and this effect propagates through leaves by diffusion of enriched water away from the evaporative sites, against the convective flux of water to the stomata (Farquhar & Lloyd 1993; Farquhar & Gan 2003). Accordingly, evaporative enrichment elevates ^{18}O in leaf water above that of source water ($\Delta^{18}O_{LW} > 0$).

The greatest uncertainty in the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ concerns $(1 - p_{ex}p_x)$, the extent to which the enrichment of leaf water propagates to $\Delta^{18}O_{Cel}$. Knowledge of $p_{ex}p_x$, the attenuation of that propagation, is essential for inferring evaporative conditions (which affect $\Delta^{18}O_{LW}$) from $\Delta^{18}O_{Cel}$, particularly in palaeoclimatic and -physiological reconstructions from fossil cellulose samples (e.g. Gessler *et al.* 2009; Ellsworth & Sternberg 2014). Song *et al.* (2014a) expected a potential range for $p_{ex}p_x$ between 0.1 and 0.9, based on empirical estimations of p_x in a number of species including grasses and trees (range 0.5 to 0.9), and theoretical expectations of p_{ex} (range 0.2 to 1). It is thought that p_x is close to 1 in non-transpiring tissue that is spatially separate from photosynthesizing tissue, such as stems of trees (Roden *et al.* 2000; Kahmen *et al.* 2011), while it can be much smaller than 1 inside concurrently expanding and transpiring dicot leaves (Cernusak *et al.* 2003a, 2005; Gessler *et al.* 2007; Kahmen *et al.* 2011; Song *et al.* 2014a). In C_3 and C_4 grass leaves, growth and differentiation, and associated cellulose synthesis, occur in a non-transpiring zone, which is tightly enclosed by the sheaths of the next-older leaves (Sharman 1942; Begg & Wright 1962; MacAdam & Nelson 1987, 2002; Schnyder *et al.* 1987, 1988, 1990; Bernstein *et al.* 1993; Tardieu *et al.* 2000). By using Eqn 1 and measurements of $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$, Helliker & Ehleringer (2002a) estimated p_x at 0.5 to 0.62 in 10 different grasses (including five C_3 and C_4 grasses), when p_{ex} was constrained to range between 0.4 and 0.5 and ε_o was set constant at 27‰. Concerning p_{ex}, carbohydrate metabolism along the path from source to sink, during storage/mobilization and cellulose synthesis can all lead to the formation of carbonyl groups in a certain proportion of carbon atoms, leading to exchange with local waters (Barbour & Farquhar 2000; Barbour 2007; Gessler *et al.* 2014). In particular, futile cycling of hexose through triose phosphates or turnover of non-structural carbohydrate pools may affect p_{ex} (Hill *et al.* 1995; Barbour & Farquhar 2000; Song *et al.* 2014a). Yet, in the absence of direct evidence, a value ~0.4 has often been taken as a default (e.g. Cernusak *et al.* 2005; Kahmen *et al.* 2011).

Importantly, the parameters of Eqn 1 may be controlled by different biological and environmental factors. Of all environmental factors, vapour pressure deficit (VPD) has the strongest effect on $\Delta^{18}O_{Cel}$ (Lipp *et al.* 1996; Barbour *et al.* 2002; Kahmen *et al.* 2011). This effect seems to derive virtually entirely from the VPD effect on $\Delta^{18}O_{LW}$ (Flanagan *et al.* 1991; Sheshshayee *et al.* 2005; Farquhar *et al.* 2007), as experiments with different VPDs have not reported variation of the attenuation factor in leaves of dicots and grasses (Barbour & Farquhar 2000; Helliker & Ehleringer 2002a; Song *et al.* 2014a). Temperature and relative humidity (RH) cause changes in $\Delta^{18}O_{LW}$ (Flanagan & Ehleringer 1991; Barbour & Farquhar 2000; Barbour *et al.* 2004; Song *et al.* 2014a), which are largely related to their relationship with VPD (Dongmann *et al.* 1974; Barbour 2007; Ripullone *et al.* 2008; Kahmen *et al.* 2011; Cernusak *et al.* 2016). It has also been suggested that temperature (and perhaps other factors) may affect ε_o (Sternberg & Ellsworth 2011). Different light levels did not lead to differences in $\Delta^{18}O_{LW}$ of *Ricinus communis* when VPD was controlled, but affected $\Delta^{18}O_{Cel}$ *via* the variation of $p_{ex}p_x$ (Song *et al.* 2014a). That variation was related to turnover time of non-structural carbohydrate pools and interpreted as an effect on p_{ex}. Kahmen *et al.* (2011) observed a tight relationship between modelled $p_{ex}p_x$ and specific leaf area (the inverse of leaf mass per area, LMA) of *Metrosideros polymorpha* sampled along climatic gradients. These relationships may be taken to suggest, that one should not expect simple relationships between environmental drivers and morpho-physiological controls on the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$, and, hence, the attenuation factor.

In that context, it is remarkable that effects of nitrogen (N) nutrition on the relationship between $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$ have not been examined to date. N fertilizer supply may affect leaf thickness, leaf mass per area and interveinal distances in C_3 and C_4 grasses and other species (Bolton & Brown 1980; Jinwen *et al.* 2009; Lattanzi *et al.* 2012), parameters that were found to correlate with ^{18}O enrichment of leaf water (Helliker & Ehleringer 2000, 2002a; Farquhar & Gan 2003; Barbour *et al.* 2004) and phloem water and sugars (Barbour *et al.* 2000; Cernusak *et al.* 2003a, 2003b; Gessler *et al.* 2013). Brooks & Mitchell (2011) observed variation of $\delta^{18}O_{Cel}$ that appeared to be related to canopy microclimate effects (associated with temperature and RH) resulting from thinning and N fertilizer treatments. Further, N supply affects plant growth rate, carbohydrate pool sizes and futile cycling of carbohydrates (Stitt & Krapp 1999; Lattanzi *et al.* 2012), which might affect the attenuation factor *via* p_{ex} (Barbour & Farquhar 2000; Song *et al.* 2014a).

Given the lack of knowledge on the effects of N fertilizer supply on $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$, and possible interrelationships with VPD, we asked the following questions in experiments with a perennial C_4 grass, *C. squarrosa* (Trin.) Keng.: (1) How do N fertilizer supply and VPD affect – directly or interactively – the $\delta^{18}O_{Cel}$ and $\Delta^{18}O_{Cel}$ of leaf blades, and their relationships with $\Delta^{18}O_{LW}$ and the attenuation factor ($p_{ex}p_x$), as obtained with the Barbour & Farquhar (2000) model (Eqn 1)? (2) Can we confirm with *C. squarrosa* the observation of DeNiro & Epstein (1979) that $\delta^{18}O_{CO2}$ has no effect on the $\delta^{18}O$ of cellulose? *C. squarrosa* is a perennial C_4 grass that is endemic to the Central Asia steppe, distributed over a wide latitudinal and longitudinal (climatic) range (Clayton *et al.* 2006), co-dominant member of the 'typical steppe' of Inner Mongolia (Kang *et al.* 2007) and the most abundant member of the C_4 community that has expanded significantly in the last decades (Wittmer *et al.* 2010; Yang 2010). Question (2) seemed particularly pertinent, as *C. squarrosa* exhibited remarkably high and variable ^{13}C discrimination among leaves of different age in its natural habitats (Yang *et al.* 2011), a trait that might be related to limiting or variable carbonic anhydrase activity (Gillon & Yakir 2001b; Cousins *et al.* 2006a), potentially causing incomplete oxygen exchange between leaf water and CO_2. We also

compared the observed $\Delta^{18}O_{LW}$ of bulk leaf blade water with the Craig–Gordon predicted ^{18}O enrichment at evaporative sites ($\Delta^{18}O_e$), to assess eventual treatment effects on gradients in $\delta^{18}O$ of water at the whole leaf blade level. All experiments were performed in controlled conditions with constant air temperature (25 °C) and constant relative humidities (RH: 50% or 80%) throughout diurnal cycles. These conditions provided constant VPDs of either 1.58 kPa or 0.63 kPa.

MATERIALS AND METHODS

Experimental design

The study had a 2 × 2 factorial design, with VPD and N fertilizer supply as factors, two levels (low and high, see below) for each factor, and four replicates. Combinations of VPD and N levels were termed 'treatments': low N × low VPD (designated N1 V1), low N × high VPD (N1 V2), high N × low VPD (N2 V1) or high N × high VPD (N2 V2). Each replicate consisted of one plant stand of a certain VPD × N combination in a growth chamber. Of the four replicates of these treatments, two received CO_2 that was relatively enriched in ^{18}O, while the other two received CO_2 that was relatively depleted in ^{18}O (Table 1). The CO_2 gases were obtained from CARBO Kohlensäurewerke (Bad Hönningen, Germany) and Linde AG (Unterschleissheim, Germany).

The experiment was performed in plant growth chambers (Conviron PGR15, Conviron, Winnipeg, Canada) that formed part of (a modernized version of) the controlled environment mesocosm system described by Schnyder *et al.* (2003). That system has four chambers; thus, the treatments were distributed between four experimental runs (Table S1), so that pairs of a given treatment were supplied with either high or low $\delta^{18}O_{CO2}$ and run simultaneously. Each run accommodated treatments with high and low VPD, at a given N fertilizer supply level. High N supply treatments were assigned to runs 1 and 4, low N treatments to runs 2 and 3. Besides the treatments factors, all environmental conditions and experimental protocols were kept the same. For details of treatments and growth conditions, including environmental control, see below.

Table 1. Oxygen isotope composition of CO_2 measured at the inlet ($\delta^{18}O_{CO2\ inlet}$) and outlet of the growth chambers ($\delta^{18}O_{CO2\ outlet}$) during light periods. $\delta^{18}O_{CO2\ outlet}$ reflects the $\delta^{18}O$ of CO_2 in the well-mixed atmosphere of the growth chamber. ^{18}O-enriched and ^{18}O-depleted CO_2 were used in the different experimental runs (see experimental plan, Table S1 and Materials and Methods). Data are shown as averages of 10-day-long continuous measurements on two chambers receiving the same source CO_2 (mean ± standard error, $n = 20$). These measurements preceded the sampling of plants for leaf water and cellulose.

Exp. run	$\delta^{18}O_{CO2\ inlet}$ (‰)		$\delta^{18}O_{CO2\ outlet}$ (‰)	
	^{18}O-enriched	^{18}O-depleted	^{18}O-enriched	^{18}O-depleted
1st	−14.18 ± 0.05	−36.80 ± 0.04	−11.25 ± 0.12	−31.30 ± 0.34
2nd	−14.14 ± 0.05	−36.21 ± 0.08	−11.37 ± 0.13	−30.52 ± 0.16
3rd	−1.67 ± 0.08	−16.45 ± 0.13	−0.58 ± 0.12	−13.71 ± 0.11
4th	0.11 ± 0.29	−14.67 ± 0.20	2.21 ± 0.26	−11.69 ± 0.26

Plant material and growth conditions

Seed lots of *C. squarrosa* were collected in 2010 and 2012 in typical steppe grasslands near the Inner Mongolia Grassland Ecosystem Research Station (IMGERS, 43° 38′ N, 116° 42′ E), China. Seed lots were well mixed before seeding.

Four seeds were sown in individual tubes (4.5 cm diameter, 35 cm deep) filled with quartz sand (0.3–0.8 mm diameter). Tubes were placed in free-draining plastic boxes (length: 77 cm, width: 57 cm, depth: 30 cm) with 164 tubes in each box. Two boxes were placed in each growth chamber. The time of first watering was referred to as imbibition of seeds. Before germination, the conditions in all chambers and runs were kept the same with a VPD of 0.63 kPa. About one week after imbibition, plants were thinned to one per tube, and the designated VPD and N treatments were implemented (see below).

Light was supplied by cool white fluorescent tubes with a photosynthetic photon flux density (PPFD) of 800 μmol m^{-2} s^{-1} at canopy height during the 16 h photoperiod. During the development of canopies, irradiance at the top of the canopy was kept constant by periodic measurements with a quantum sensor (LI-190R, Li-Cor, Lincoln, Nebraska, USA) and adjustment of the distance (height) between the fluorescent tubes and the top of the canopy. Air temperature in all chambers was maintained constant at 25 °C throughout the diurnal cycles (Fig. S1).

CO_2-free, dry air (dew point < −70 °C), generated by use of a screw compressor and adsorption dryer system, was mixed with ^{18}O-enriched or ^{18}O-depleted CO_2 and water vapour, and distributed between the four chambers (Schnyder *et al.* 2003). CO_2 and water vapour concentrations in chamber air were measured by an infrared gas analyser (LI-6262, Li-Cor Inc. Lincoln, USA). CO_2 concentration in the chamber was controlled at 390 μmol mol^{-1} during the light period. Water vapour was generated from deionized water using a high-pressure air humidification system (FF4Z, Finestfog, Ottobrunn, Germany). The operation of the humidifier was controlled by humidity sensors in the growth chambers, to keep RH constant throughout the diurnal cycle at either 50% or 80%, thus providing constant VPDs of 1.58 kPa (high VPD) or 0.63 kPa (low VPD), respectively, at 25 °C (Fig. S1).

Nutrient solution was supplied three times per day by an automatic irrigation system throughout the whole experiment similar to Lehmeier *et al.* (2008). The solution contained 7.5 mM N (termed 'low N', as it limited plant growth, see below) or 22.5 mM N (high N) in the form of equimolar concentrations of calcium nitrate and potassium nitrate ($Ca(NO_3)_2$ and KNO_3). The concentration of other nutrients was the same in both nutrient solutions: 1.0 mM $MgSO_4$, 0.5 mM KH_2PO_4, 1 mM NaCl, 125 μM Fe-EDTA, 46 μM H_3BO_3, 9 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 μM Na_2MoO_4.

Plant sampling

Separate sets of plants were sampled at intervals for the assessment of morphological parameters, joint leaf water and cellulose extraction, and determination of developmental gradients (see below). In each experiment sampling activities occurred over a period of three weeks. An average of <3% of all plants were collected from random positions in canopies on any given sampling date. On the whole, <20% of all plants were sampled until the end of an experiment.

Morphological parameters of C. squarrosa

In each experimental run, plants were sampled four or five times at 2 or 3 d intervals after canopy closure (leaf area index, LAI > 2) to determine morphological parameters. On each sampling date, four plants in each chamber were removed from random positions. From each plant, we excised two major tillers (the main tiller and another tiller, which had approximately the same length as the main tiller). Leaf blades were clipped off, counted, photographed (to measure leaf area using the Image J software, National Institutes of Health, Bethesda, Maryland, USA) and combined by position (i.e. leaf age) in one sample (Fig. S2). The remaining shoot material and roots were collected as separate samples. After weighing the fresh mass, all samples were dried in an oven at 60 °C for 48 h and weighed again. For each plant, the following parameters were obtained: number of leaf blades per major tiller, number of tillers per plant (total shoot mass divided by mean major tiller mass), mean leaf area per leaf, leaf thickness estimated as leaf fresh mass per leaf area (Arredondo & Schnyder 2003), leaf blade water content per area, leaf blade dry mass per area (LMA), tiller mass, total shoot mass and LAI (leaf area per plant times number of plants per unit ground surface area, with leaf area per plant = shoot mass × leaf area per tiller/tiller mass). Morphological parameters were calculated from those 2 d of sampling which were closest to the days of sampling for leaf water and cellulose.

Joint sampling of leaf water and cellulose

Samples for leaf blade water and cellulose extraction were collected on the 54th, 55th, 37th or 46th day after imbibition in experimental runs 1 to 4, respectively. Three plants were chosen in each chamber, successively removed from the chambers at approx. 2 h after the beginning of the light period and sampled quickly using a scalpel to minimize changes of the content and $\delta^{18}O$ of leaf water. For each plant, mature (fully expanded) blades in the upper part of the canopy were sampled (including leaf ages 1 to 5 in Fig. S2). In addition we also sampled the lower leaves. For each sample of upper or lower leaves, about 40 blades were pooled and immediately sealed in a 12 mL Exetainer vial (*Labco* Ltd, High Wycombe, UK), capped and then wrapped with Parafilm (PM996, Bemis Company, Wisconsin, USA). All vials were weighed before and after filling with leaf blades. Samples were stored in a freezer at −20 °C until cryogenic extraction of leaf water. Following leaf water extraction, the same samples were used to extract leaf blade cellulose.

Cellulose in leaf blades of different age categories

Additional samples were collected to assess potential developmental gradients, possibly related to changes in canopy microclimate, by sampling separately the different leaf age categories along tillers. These samplings occurred in two experimental runs, on the 53rd day after imbibition in the second experimental run (N1 V1, N1 V2) and the 44th day in the fourth run (N2 V1, N2 V2) (cf. Table S1). For this, eight plants were randomly chosen from each chamber, and five to eight major tillers per plant excised at the base of the shoot. Leaf age categories were numbered from the tip of the tiller (the youngest leaf) to the base (oldest) (cf. Fig. S2). To obtain sufficient material for cellulose extraction, 16 to 20 leaf blades per age category were pooled in one sample. This procedure provided three samples for each plant fraction (individual age categories of leaves) for each growth chamber.

Using knowledge of the leaf appearance interval (time interval between appearance of successive leaves: 2.4 d; Fang Yang *et al.*, unpublished data), the different leaf age categories were assigned to the respective periods of leaf production during stand growth.

Leaf water and cellulose extraction

Leaf water was extracted for 3 h using a custom-built cryogenic vacuum distillation apparatus (see description in Fig. S3). Water extraction was virtually complete, as was confirmed by the absence of weight change of the cryogenically vacuum-extracted samples in a drying oven at 40 °C for 24 h. Water samples were placed in 2 mL Eppendorf tubes and stored at −20 °C until isotope analysis.

α-cellulose was extracted from 50 mg or 25 mg of dry sample material using the procedure of Brendel *et al.* (2000) as modified by Gaudinski *et al.* (2005).

Measurement of $\delta^{18}O$ in CO_2 and vapour

The $\delta^{18}O$ of CO_2 at the inlet and outlet of the well-ventilated growth chambers was measured in a quasi-continuous manner throughout the whole experiment by on-line $^{13}C/^{12}C$- and $^{18}O/^{16}O$-CO_2 mass spectrometry, as in Schnyder *et al.* (2003, 2004).

In experimental runs 1, 3 and 4, the $\delta^{18}O$ of vapour in the growth chambers ($\delta^{18}O_v$) was measured on-line by Cavity Ring-Down Spectroscopy (CRDS, L2120-I, Picarro, California, USA) one day before sampling leaves for leaf water ^{18}O analysis. Measurement started 2 h after the beginning of the light period. Values of measured $\delta^{18}O_v$ were stored when the reading of the vapour concentration measured by the CRDS became stable. In the second experimental run, water vapour in growth chambers was sampled above the canopy by drawing air through a glass trap submerged in a mixture of ethanol and dry ice for 2 h using a pump. Vapour collection started 2 h after the beginning of the light period. $\delta^{18}O_v$ did not differ significantly between growth chambers and treatments, and averaged −13.0‰ (±0.6‰ standard error, $n = 16$). The flux of vapour from transpiration was, under our

experimental conditions, proportionally minor compared to the flux of inlet vapour.

Oxygen isotope analysis of leaf water, nutrient solution and biomass samples

Oxygen isotope composition was expressed in per mil (‰) as

$$\delta^{18}O = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \quad (2)$$

where R_{sample} and $R_{standard}$ are the $^{18}O/^{16}O$ ratios of the sample and the Vienna Standard Mean Ocean Water standard (V-SMOW), respectively.

$\delta^{18}O$ of water samples (leaf water, collected vapour or nutrient solution) was analysed on 300 μL aliquots using the CRDS analyser coupled to an A0211 high precision vaporizer set at 110 °C (Picarro Inc., Sunnyvale, Ca, USA). Each sample was measured repeatedly (five to twelve injections of each 1 μL, depending on memory effects of successive samples differing in isotopic composition) and the results of the last two measurements averaged. Post-processing correction was applied by running the ChemCorrect™ v1.2.0 software (Picarro Inc.) to eliminate the influence of volatiles according to Martín-Gómez *et al.* (2015). However, no sample was flagged as 'possibly contaminated' or 'bad'. After every 20 to 25 samples, two laboratory water standards that spanned the range of the isotopic compositions of samples ($\delta^{18}O$ +13.1‰ and −21.2‰, respectively) were run for possible drift correction and normalizing results to the SMOW-scale. The laboratory standards were previously calibrated against V-SMOW, V-GISP and V-SLAP (from IAEA) using the same analytical procedure as used in sample analysis. Analytical uncertainty (the SD for repeated measurements) for $\delta^{18}O$ was ±0.1‰.

Cellulose samples were re-dried at 40 °C for 24 h, 700 μg aliquots packed in silver cups (size: 3.3 × 5 mm, LüdiSwiss, Flawil, Switzerland) and stored above Silica Gel orange (2–5 mm, ThoMar OHG, Lütau, Germany) in exsiccator vessels. For ^{18}O analysis, samples were pyrolysed at 1400 °C in a pyrolysis oven (HTO, HEKAtech, Wegberg, Germany), equipped with a helium-flushed zero blank auto-sampler (Costech Analytical technologies, Valencia, CA, USA) and interfaced (ConFlo III, Finnigan MAT, Bremen, Germany) to a continuous-flow isotope ratio mass spectrometer (Delta Plus, Finnigan MAT). Solid internal laboratory standards (SILS, cotton powder) were run as a control after every fifth sample. All samples and SILS were measured against a laboratory working standard carbon monoxide gas, which was previously calibrated against a secondary isotope standard (IAEA-601). The long-term precision for the internal laboratory standards was better than 0.3‰ (SD for repeated measurements).

^{18}O enrichment of leaf blade water above source water ($\Delta^{18}O_{LW}$) was calculated as:

$$\Delta^{18}O_{LW} = \frac{\delta^{18}O_{LW} - \delta^{18}O_{SW}}{1 + \delta^{18}O_{SW}/1000}, \quad (3)$$

with $\delta^{18}O_{LW}$, the $\delta^{18}O$ of leaf blade water and $\delta^{18}O_{SW}$ that of the nutrient solution. ^{18}O enrichment of leaf blade cellulose above source water ($\Delta^{18}O_{Cel}$) was calculated accordingly, using $\delta^{18}O_{SW}$ and $\delta^{18}O_{Cel}$ data. The $\delta^{18}O$ of the nutrient solution – the sole source of water for uptake by roots – increased from −8.8 to −8.2‰, at a rate of 0.028‰ d^{-1} ($R^2 = 0.84$) during the experimental period, that is the period of growth and differentiation of all leaves collected in an experimental run. This effect was accounted for in the estimation of source water isotope composition at various times using the regression of the $\delta^{18}O$ of the nutrient solution versus time in the experiment.

The enrichment of evaporative site water above source water ($\Delta^{18}O_e$) was calculated using the precise version of the Craig–Gordon model as provided by Farquhar *et al.* (2007):

$$\Delta^{18}O_e = (1 + \varepsilon^+)[(1 + \varepsilon_k)(1 - w_a/w_i) + w_a/w_i(1 + \Delta_v)] - 1 \quad (4)$$

where w_a/w_i is the ratio of water vapour mole fraction in the air to that in the intercellular air space, ε^+ is the equilibrium ^{18}O fractionation between liquid water and vapour, ε_k is the kinetic ^{18}O fractionation for combined diffusion through the stomata and the boundary layer and Δ_v is the ^{18}O enrichment of vapour (the measurement of $\delta^{18}O_v$ was described above) compared to source water. ε^+ and ε_k were calculated using equations in Cernusak *et al.* (2016). w_a/w_i was calculated from air temperature and RH in the growth chamber and the leaf temperature, measured by six thermocouples placed in the top 10 cm of the canopy evenly distributed across each chamber.

Data analysis

For analysis of variance (ANOVA), we used the general linear model of SAS (SAS 9.1, SAS Institute, USA). Firstly, three-way ANOVA was used to test the effects of $\delta^{18}O_{CO2}$, N supply, VPD and their interactions on $\Delta^{18}O_{Cel}$ of leaf blades in the upper canopy. In those tests means of subsamples from each chamber were used as replicates ($n = 2$). Because of the non-significant effect of $\delta^{18}O_{CO2}$ on $\Delta^{18}O_{Cel}$, we combined replicates with ^{18}O-enriched and ^{18}O-depleted CO_2 by VPD × N treatment for the subsequent statistical analysis by a two-way ANOVA. This tested the effects of N supply, VPD and their interactions on morphological parameters, $\Delta^{18}O_{LW}$, $\Delta^{18}O_e$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$. This ANOVA used means of subsamples from the same chamber, meaning that the number of true independent replicates was four. $p_{ex}p_x$ of each treatment was calculated according to Eqn 1, using the $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ data of upper canopy leaves and assuming a ε_o of 27‰ (unless indicated otherwise). Absolute growth rate of *C. squarrosa* was determined as the slope of a linear regression between plant dry mass and days after imbibition. Effects of N supply and VPD on growth rate were assessed by comparing confidence intervals of the slope estimates. In addition, we performed sensitivity tests of N and VPD and interactive effects of N and VPD on the $p_{ex}p_x$ by varying ε_o between 24‰ and 30‰ or $\delta^{18}O_{LW}$ in a range of ±1.3‰ around the measured value. In the latter sensitivity tests, ε_o was fixed at 27‰.

RESULTS

The effect of oxygen isotope composition of CO_2 on $\delta^{18}O$ of cellulose

Mesocosm-scale on-line $C^{18}OO/C^{16}O_2$ gas exchange measurements (performed on established plant stands between 5 and 8 weeks after imbibition of seed) demonstrated that the $\delta^{18}O_{CO2}$ of the ^{18}O-enriched and ^{18}O-depleted CO_2 sources differed consistently in each experimental run (Table 1). That difference in $\delta^{18}O_{CO2}$ amounted to approx. 20‰ in the first and second experimental run and 13‰ in the third and fourth experimental run, when comparing measurements taken at the outlet of the rapidly ventilated chambers (Table 1). When measured at the inlet of the chamber, those differences were even larger (Table 1, also see Materials and Methods), but exchange with (plant or other) water pools in the chambers caused partial convergence of the $\delta^{18}O_{CO2}$ of the ^{18}O-enriched and ^{18}O-depleted CO_2 sources.

Three-way ANOVA of the effects of $\delta^{18}O_{CO2}$, N fertilizer supply, VPD and their interactions revealed no statistically significant direct or interactive effect of $\delta^{18}O_{CO2}$ on $\Delta^{18}O_{Cel}$ of leaf blades (Table 2). Along the same line, the $\delta^{18}O_{Cel}$ in the parallel growth chambers with different $\delta^{18}O_{CO2}$ in the same VPD and N treatment were virtually indistinguishable. There was a mean, but non-significant, offset of +0.37‰ for $\delta^{18}O_{Cel}$ of leaves grown in the presence of the ^{18}O-enriched CO_2 source relative to that of the depleted source, and a mean absolute difference of 0.76‰ for the match of $\delta^{18}O_{Cel}$ produced in the presence of ^{18}O-enriched or ^{18}O-depleted CO_2 (Fig. 1). The offset of +0.37‰ would have meant a 2.4% contribution of the original oxygen in CO_2 to the total oxygen in cellulose, given that the leaf water $\delta^{18}O$ was unchanged by the CO_2. Indeed, $\Delta^{18}O_{LW}$ did not differ significantly between chambers receiving CO_2 with different $\delta^{18}O_{CO2}$ ($\Delta^{18}O_{LW}$ differed by $+0.35 \pm 1.2$‰ SE between chambers receiving ^{18}O-enriched and -depleted CO_2). The absence of a significant divergence of leaf water isotope composition in chambers receiving CO_2 from the different sources was also expected from isotopic mass balances considering oxygen fluxes connected with CO_2 assimilation, transpiration and invasion/retrodiffusion fluxes of CO_2 and water vapour in leaves. So, as $\delta^{18}O_{CO2}$ had no effect on any of the relationships analysed in this work, the replicates of the ^{18}O-enriched and ^{18}O-depleted CO_2 environments were combined by VPD and N treatment for the subsequent analyses.

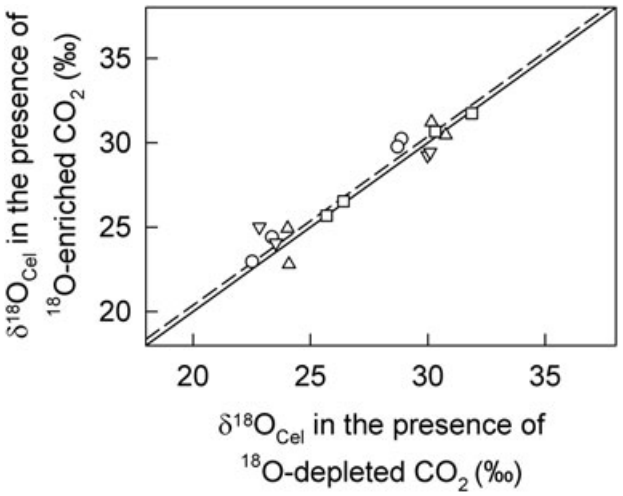

Figure 1. $\delta^{18}O_{Cel}$ of leaf blades grown in the presence of ^{18}O-enriched CO_2 (y-axis) *versus* ^{18}O-depleted CO_2 (x-axis). The two CO_2 sources were used in parallel that is in two growth chambers of the same treatment in the same experimental run (Table S1), with $\delta^{18}O$ of CO_2 as shown in Table 1. The solid line gives the 1:1 relationship; the dashed line is a linear regression line: $\delta^{18}O_{Cel\_E} = \delta^{18}O_{Cel\_D} + 0.37$, with subscript E denoting ^{18}O-enriched and D denoting ^{18}O-depleted CO_2, which did not differ significantly from the 1:1 relationship. The mean absolute difference for the match of $\delta^{18}O_{Cel\_E}$ and $\delta^{18}O_{Cel\_D}$ was 0.76‰ (±0.15‰ standard error) and is based on the comparison of three samples collected both in the upper and lower canopy section in each chamber. Variation of $\delta^{18}O_{Cel}$ was associated with different VPD treatments. Samples from the different runs are designated as: first run, up-pointing triangles; second run, circles; third run, down-pointing triangles, and fourth run, squares.

Table 2. Results of a three-way ANOVA testing the effect of $\delta^{18}O_{CO2}$, N supply, VPD and their interactions on $\Delta^{18}O_{Cel}$ of leaf blades in the upper canopy. Significance levels: ns, not significant ($P > 0.05$); **, $P < 0.01$. For each combination of treatments, the number of replicates was two with each chamber as one replicate. Cellulose was obtained from the same materials as used to extract leaf water.

Treatment	DF	Significance
$\delta^{18}O_{CO2}$	1	ns
N	1	ns
VPD	1	**
$\delta^{18}O_{CO2} \times N$	1	ns
$\delta^{18}O_{CO2} \times VPD$	1	ns
$N \times VPD$	1	ns
$\delta^{18}O_{CO2} \times N \times VPD$	1	ns
Total	18	

N fertilizer and VPD effects on plant growth, morphology and $\delta^{18}O$ of leaf blade cellulose

In all treatments, plant dry mass increased near-linearly with time after canopy closure at around 35 d after imbibition (Fig. 2a,b). This indicated that plants exhibited an approx. constant growth rate, in agreement with the 'grand period' of plant growth in a closed canopy (Loomis & Connor 1992).

As expected, N supply had a significant effect on growth, with growth rates of 0.11 (±0.02 confidence interval) g d^{-1} $plant^{-1}$ at low N and 0.16 ± 0.03 g d^{-1} $plant^{-1}$ at high N, demonstrating a clear N limitation for the low N plants. Accordingly, plants grown at high N had higher nitrogen nutrition index (determined from N content and aboveground standing biomass of each stand according to Lemaire *et al.* 2008) of 1.3 ± 0.04 than that of plants grown at low N (0.80 ± 0.02, $n = 8$, averaged over VPD levels). Furthermore, N content of fully expanded young leaves of plants grown at high N (3.0 ± 0.1% DM) was significantly higher than that of low N plants (2.4 ± 0.1% DM), while VPD and its interaction with N supply had no significant effect on leaf N content per DM ($P > 0.05$).

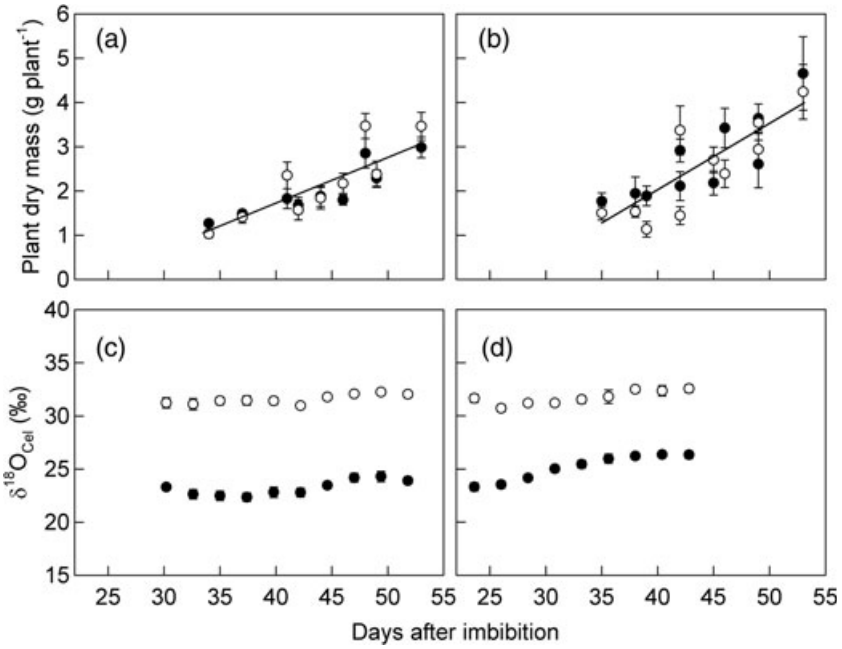

Figure 2. Treatment effects on (a, b) plant growth and (c, d) $\delta^{18}O_{Cel}$ of successively produced leaves in *C. squarrosa*. (a, c), low N; (b, d), high N fertilizer supply. Closed circles: low VPD; open circles: high VPD. Growth rate was determined as the slope of a linear regression in (a) and (b). VPD had no effect on plant growth; therefore, VPD treatments were combined by N supply level for subsequent regression analysis. The linear regression in (a): $y = 0.11\ x - 2.51$, $R^2 = 0.74$; in (b): $y = 0.16\ x - 3.96$, $R^2 = 0.70$. The confidence interval for the slope of the regression line in (a) was 0.11 ± 0.02 (low N), in (b) was 0.16 ± 0.03 g day^{-1} (high N). Each data point and error bar in (a) and (b) represent the mean and standard error of eight plants harvested on the same day. Data in (c) and (d) were obtained from two replicate chambers of each treatment in the second (low N) and fourth experimental run (high N). Major tillers were sampled on the 53rd and 44th day after imbibition of seeds in the second and fourth run, dissected by leaf age category (cf. Materials and Methods, and Fig. S2) and $\delta^{18}O_{Cel}$ determined for each leaf age class. In (c, d), the $\delta^{18}O_{Cel}$ data are reported for the time when the different leaf age categories reached full expansion, using knowledge of leaf appearance interval. Each data point and error bar in (c) and (d) represent the mean ± standard error of six individual plants ($n = 6$).

N content per leaf area was not significantly different between treatments ($P > 0.05$, 1.4 ± 0.1 g m^{-2}, averaged over treatments). High N stimulated plant growth by 45% (relative to low N), increased shoot weight per plant by 42% and enhanced LAI by 53% (Table 3). Greater LAI resulted from greater tiller production (+33%) and, to a lesser extent, individual leaf area (+16%). In addition, high N supply caused a small decrease of LMA (−9%). Apart from these, N supply had no significant effect on morphological parameters: leaf number per tiller, leaf thickness and individual tiller weight. On the other hand, growth rates under low and high VPD did not differ significantly ($P > 0.05$), as indicated by the 95% confidence intervals of the growth rates of the high and low VPD treatments. In addition, VPD (and its interaction

Table 3. Leaf, tiller, plant and canopy parameters of *C. squarrosa* stands grown at low or high N fertilizer supply (N1 or N2) combined with low or high VPD (V1 or V2) in growth chambers. All treatments (N × V combinations) had four true replications and were arranged in four growth chambers in four successive experimental runs (see Table S1). The data of a given replicate is the mean of the data collected in the last two sampling events (see Materials and Methods) of each experimental run: day 49 and 53 after imbibition of seeds in the first (N2 V1 and N2 V2) and second run (N1V1 and N1V2), day 44 and 48 after imbibition in the third run (N1 V1 and N1 V2), and day 45 and 49 after imbibition in the fourth run (N2 V1 and N2 V2). The effects of N and VPD on the different parameters were tested by a two-way ANOVA. Significance levels: ns, not significant; *, $P < 0.05$; **, $P < 0.01$. Values are means ± standard errors ($n = 4$, with each chamber as one replicate).

	Treatment				Significance		
Parameter	N1 V1	N1 V2	N2 V1	N2 V2	N	VPD	N × VPD
Number of leaves tiller^{-1}	11.9 ± 0.4	12.1 ± 0.2	12.0 ± 0.3	12.2 ± 0.2	ns	ns	ns
Number of tillers plant^{-1}	5.8 ± 0.3	7.0 ± 0.3	8.4 ± 0.7	8.6 ± 0.5	**	ns	ns
Individual leaf area (cm^2 leaf^{-1})	2.5 ± 0.1	2.6 ± 0.1	3.1 ± 0.1	2.8 ± 0.1	**	ns	ns
Leaf thickness (μm)	108 ± 3	111 ± 3	110 ± 3	111 ± 1	ns	ns	ns
Leaf blade dry mass per area (LMA, mg cm^{-2})	4.4 ± 0.2	4.2 ± 0.2	3.8 ± 0.2	4.1 ± 0.1	*	ns	ns
Leaf area index (LAI, m^2 m^{-2})	3.6 ± 0.3	4.7 ± 0.2	6.5 ± 0.7	6.2 ± 0.4	**	ns	ns
Tiller dry weight (mg tiller^{-1})	299 ± 9	306 ± 7	317 ± 19	313 ± 13	ns	ns	ns
Shoot dry weight (g plant^{-1})	1.7 ± 0.1	2.1 ± 0.0	2.7 ± 0.2	2.7 ± 0.0	**	ns	ns

with N supply) had no significant effect on any of the morphological variables in Table 3.

The effects of N supply and VPD level on growth and morphology were contrasted by their effects on $\delta^{18}O_{Cel}$ of the successively formed leaf blades: $\delta^{18}O_{Cel}$ was unaffected by N supply, while VPD had a strong effect. Within a treatment, $\delta^{18}O_{Cel}$ of the successively formed leaf blades was nearly constant, except for the low VPD treatment at high N (Fig. 2c,d), for which $\delta^{18}O_{Cel}$ increased somewhat in the period before canopy closure (35 d after imbibition of seed).

The relative constancy of $\delta^{18}O_{Cel}$ after canopy closure was likely related to (1) the near-constant environmental conditions in the growth chambers; (2) the fact that successive leaf growth and associated cellulose synthesis occur at the tip of the grass tiller, at the top of the canopy, where environmental conditions (such as humidity and light) are unaltered by canopy effects; and (3) the substrate for cellulose synthesis is mainly assimilated in the young and (mostly) unshaded leaves at the top of the canopy (Ryle & Powell 1974; Dale 1985, 1988).

The effects of nitrogen supply and VPD on $\Delta^{18}O_{LW}$, $\Delta^{18}O_e$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$

In line with the absence of an effect on $\delta^{18}O_{Cel}$, N supply had no effect on $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ or $p_{ex}p_x$ (Fig. 3). In the same way, N supply did not affect $\Delta^{18}O_e$ ($P > 0.05$), which was 2.4‰ higher than $\Delta^{18}O_{LW}$ on average of all treatments (see Fig. 4). Meanwhile, high VPD increased $\Delta^{18}O_{LW}$ by 6.5‰ and $\Delta^{18}O_{Cel}$ by 5.8‰ in comparison with low VPD (average of both N treatments) (Fig. 3). Moreover, high VPD increased $\Delta^{18}O_e$ by 10.0‰ in comparison with low VPD ($P < 0.05$), and interaction between VPD and N supply had no effect on $\Delta^{18}O_e$ (Fig. 4). Further, the difference between $\Delta^{18}O_e$ and $\Delta^{18}O_{LW}$ was increased strongly by VPD (+0.6‰ at low VPD and 4.1‰ at high VPD, on average of the N treatments; Fig. 4).

The $p_{ex}p_x$, calculated with Eqn 1 in assuming an ε_o of 27‰, ranged between 0.34 and 0.53 in the different treatments, and was significantly lower at high than at low VPD (Fig. 3c). This VPD effect on $p_{ex}p_x$ was also evident with the cellulose data from the youngest leaves that were growing at the time of leaf water sampling (cf. Fig. 2c,d). Moreover, the significance of the VPD effect on $p_{ex}p_x$ was supported by a sensitivity analysis with ε_o values ranging between 25‰ and 30‰ (Fig. S4), encompassing largely the range of suggested plausible variation of ε_o (Ellsworth & Sternberg 2014; Song *et al.* 2014a). The VPD effect on $p_{ex}p_x$ only became non-significant for $\varepsilon_o \leq 24$‰. Additionally, a sensitivity analysis that varied $\Delta^{18}O_{LW}$ by ±1.3‰ (that is ± 20% of the measured difference between $\Delta^{18}O_{LW}$ at high and low VPD) also generally supported the significance of the VPD effect on $p_{ex}p_x$ (Fig. S5).

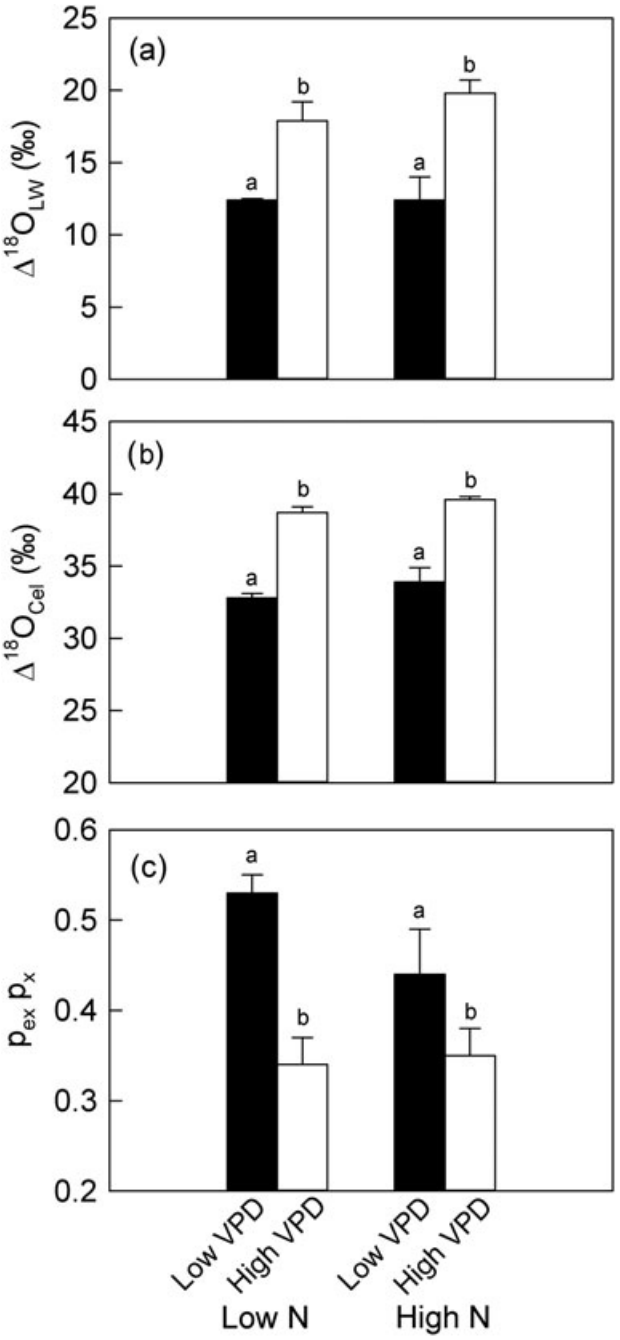

Figure 3. The effects of VPD and nitrogen fertilizer supply (N) on (a) ^{18}O-enrichment of leaf water ($\Delta^{18}O_{LW}$, ‰), (b) ^{18}O-enrichment of leaf blade cellulose ($\Delta^{18}O_{Cel}$, ‰) and (c) $p_{ex}p_x$, estimated using $\varepsilon_o = 27$‰ (cf. Eqn 1) in *C. squarrosa* stands under low VPD (black bars) and high VPD (empty bars) with low and high N supply levels. Samples were collected on the 54th, 55th, 37th and 46th day following imbibition of seeds in the first, second, third and fourth experimental run, respectively. Values are means ± standard error ($n = 4$, with each chamber as one replicate). Letters above bars indicate the results of a two-way ANOVA: effects of VPD on $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$ were highly significant ($P < 0.01$); effects of N supply (and of its interaction with VPD) on $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$ were not significant ($P > 0.05$).

DISCUSSION

Oxygen isotope composition of CO_2 has no effect on $\delta^{18}O$ of cellulose

The $\delta^{18}O$ of CO_2 had no effect on the $\delta^{18}O_{Cel}$ in this C_4 grass, validating the finding of DeNiro & Epstein (1979) with wheat,

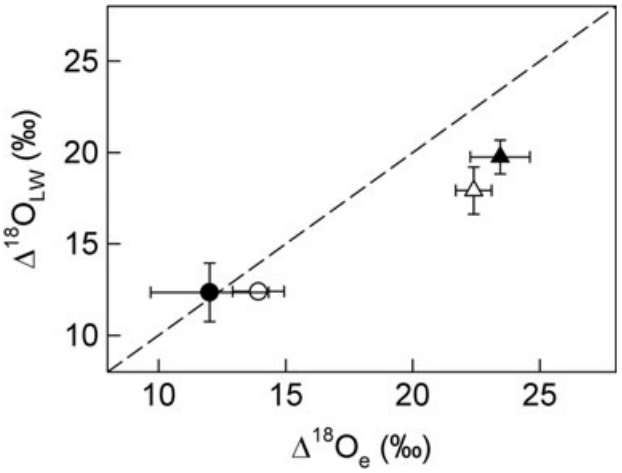

Figure 4. Relationship between observed values of $\Delta^{18}O_{LW}$, measured for bulk leaf blade water, and Craig–Gordon modelled evaporative site ^{18}O-enrichment, $\Delta^{18}O_e$, under low VPD with low nitrogen (empty circle) and high nitrogen supply (filled circle), and under high VPD with low nitrogen (empty triangle) and high nitrogen supply (filled triangle). Each value is presented as the mean ± standard error ($n = 4$). The dashed line represents the 1:1 relationship. Results of two-way ANOVA showed that high VPD significantly increased $\Delta^{18}O_e$ ($P < 0.05$), while N supply and its interaction with VPD had no effect ($P > 0.05$).

a C_3 grass, and providing support for Eqn 1 as a valid mechanistic representation of the factors determining $\Delta^{18}O_{Cel}$. Although biochemical and physiological reasoning has long supported a (near-)complete exchange of oxygen between water and CO_2 or carbonyl oxygen of metabolic intermediates of sugars in leaves, during transport and storage metabolism, and in sink tissue (Sternberg *et al.* 1986; Farquhar *et al.* 1998; Schmidt *et al.* 2001; Song *et al.* 2014b), this is the first experimental assessment of a $\delta^{18}O_{CO2}$ effect on $\delta^{18}O$ of cellulose in a C_4 plant species, and the first verification of the reported absence of such an effect by DeNiro & Epstein (1979). The absence of a significant effect of $\delta^{18}O_{CO2}$ on $\delta^{18}O_{Cel}$ means that carbonic anhydrase activity of *C. squarrosa* was non-limiting or that any limitation in catalysing the exchange of oxygen between leaf water and CO_2/HCO_3^- was overcome by subsequent exchange between water and carbonyl oxygen groups formed during (photosynthetic) reductive pentose phosphate cycle, metabolism of carbohydrates in source leaves, transport and metabolism at the sites of cellulose synthesis (DeNiro & Epstein 1979; Sternberg *et al.* 1986; Hill *et al.* 1995; Farquhar *et al.* 1998; Schmidt *et al.* 2001) in the leaf growth and differentiation zones.

N fertilizer supply had no effect on $\delta^{18}O$ of leaf blade cellulose

N fertilizer supply – and its interaction with VPD – had no significant effect on $\delta^{18}O_{Cel}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_e$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$. This is not a trivial result, given that the effects of N fertilizer supply on the growth and morphology of *C. squarrosa* were typical for effects of N fertilizer supply on growth and development of grass plants and canopies (Cruz & Boval 2000; Gastal & Lemaire 2002), and produced strong effects on plant growth, tillering/branching and LAI. Importantly, however, N fertilizer effects on individual leaf parameters were relatively small (individual leaf area, LMA and N content per DM) or non-existent (leaf thickness, N content per unit area).

A similar analysis of N fertilizer effects on $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$ has not been performed previously, restricting somewhat opportunities for discussion of mechanisms. Absence of a significant N supply effect on $\Delta^{18}O_{LW}$ was likely related to weak (or non-existent) effects on leaf morphological parameters and N content per unit area, and lack of a N effect on stomatal conductance and transpiration rates (Xiao Ying Gong *et al.*, unpublished data). Importantly as well, $\Delta^{18}O_e$, estimated by the Craig–Gordon model (Craig & Gordon 1965; Dongmann *et al.* 1974; Flanagan *et al.* 1991; Farquhar & Lloyd 1993; Cernusak *et al.* 2016), did not differ significantly between the N levels and (on average of the two VPDs) was 2.4‰ more enriched than bulk leaf blade water (Fig. 4). Certainly, our finding of the absence of a N effect on $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$ should be verified with other species and greater contrasts of N fertilizer supply.

A similar overestimation of $\Delta^{18}O_{LW}$ by $\Delta^{18}O_e$ in C_4 grasses was noted by Webb & Longstaffe (2003) and Gan *et al.* (2003) and in a wide range of (other) taxa (Cernusak *et al.* 2016), but differs from the findings of Helliker & Ehleringer (2000), perhaps because of species differences and variation of transpiration along the leaf (Gan *et al.* 2003). Absence of a N effect on $\Delta^{18}O_{LW}$ and $\Delta^{18}O_e$ is consistent with similar fluxes of xylem water through the (enclosed) sheath towards the exposed leaf blade, similar gradients of leaf water isotope composition between the xylem and evaporative sites (although we did not assess the spatial gradients of leaf $\Delta^{18}O_{LW}$ and $\Delta^{18}O_e$ along the length of the leaf; cf. Helliker & Ehleringer 2000), and analogous turnover of leaf water pools, in plants grown with different supplies of N fertilizer. Assuming the same relationships existed in growing leaves, one would thus expect that these processes bring about a similar p_x in the leaf growth and differentiation zone where cellulose is synthesized, and – as $p_{ex}p_x$ was unaltered by N – a similar p_{ex}, supporting the parsimonious hypothesis.

VPD affected $p_{ex}p_x$, the extent to which the effect of leaf water on $^{18}O_{Cel}$ is attenuated by source water

The VPD effect on $\delta^{18}O_{Cel}$, $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ seen here was comparable with that reported by Helliker & Ehleringer (2002a) with a range of C_3 and C_4 grasses. In addition, in close agreement with a synthesis of previous works in many, mainly woody taxa (Cernusak *et al.* 2016), the proportional difference between $\Delta^{18}O_e$ and $\Delta^{18}O_{LW}$ (i.e. $1 - \Delta^{18}O_{LW}/\Delta^{18}O_e$) averaged 0.09 in our works. VPD affected the proportional difference, which was −0.01 at low VPD and 0.18 at high VPD. Unfortunately, we were unable to measure transpiration of the leaves that we sampled for water extraction, but calculations using transpiration measurements on a smaller set of leaves measured in parallel with a clamp-on leaf chamber (Xiao Ying

Gong *et al.*, unpublished data) and the leaf water isotope data suggested that high VPD elevated the Péclet number (0.38, relative to 0.27 at low VPD; $P < 0.05$), but had no significant impact on effective path length (41 mm on average of all treatments), consistent with observations and reasoning by Loucos *et al.* (2015). The observed effects of VPD on $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$ were also similar to those observed in other species and taxonomic groups (Barbour & Farquhar 2000; Helliker & Ehleringer 2002a, 2002b; Song *et al.* 2014a; Cernusak *et al.* 2016) and independent of N fertilizer supply.

Remarkably, we noted a significant effect of VPD on $p_{ex}p_x$. This effect meant that the slope of the relationship between $\Delta^{18}O_{Cel}$ and $\Delta^{18}O_{LW}$ became steeper (that is less attenuated) with increasing VPD, implying that VPD fluctuations estimated from fluctuations of $\Delta^{18}O_{Cel}$ would underestimate the amplitude of the fluctuation if the VPD effect on $p_{ex}p_x$ was not accounted for. To our best knowledge, such an effect has not been discussed previously. However, analysis of the original data of Helliker & Ehleringer (2002a) – who reported on five C_3 and five C_4 grasses exposed to different RHs – also provides some indications for a similar VPD effect on the attenuation factor for the range of RH explored in our work. In their work, the calculated attenuation factor was lower at high VPD (low RH) than at low VPD (medium RH in their study) in 8 out of 10 cases, when the data were evaluated with the Barbour & Farquhar (2000) model using a ε_o of 27‰. If ε_o was set at 28‰, all 10 species had a lower attenuation factor at the high VPD level, similar to our work.

Conclusions and open questions

A certain limitation of this work is that the components of the attenuation factor, the factors p_{ex} and p_x, could not be determined directly. Estimation of p_x, the proportion of unenriched water at the site of cellulose synthesis, requires knowledge of $\Delta^{18}O$ of water in the leaf growth and differentiation zone ($\Delta^{18}O_{LGDZ}$) where primary and secondary cell wall deposition and associated cellulose synthesis occur. During leaf growth and development, that zone (LGDZ) extends between the base of the growing leaf (near the point of attachment to the tiller axis), up to the point where tissue emerges from the surrounding sheath of the next-older, most-recently expanded leaf (Schnyder *et al.* 1990; MacAdam & Nelson 1987, 2002; see also Fig. 5.14 in MacAdam 2009). Such measurements of $\Delta^{18}O_{LGDZ}$ have not been performed to date. Of note, leaf expansion, assimilate import into the LGDZ and structural biomass synthesis within the LGDZ of grass leaves proceed through day–night cycles, and can occur at similar rates in darkness and light (Schnyder & Nelson 1988; Schnyder *et al.* 1988). These relationships are complicated further by (e.g. diurnal) VPD transients, which can provoke strong changes in leaf elongation rate of grasses (Parrish & Wolf 1983), potentially changing the relative rates of daytime versus nighttime cellulose synthesis. These features call for joint analyses of the spatial and temporal dynamics of cellulose synthesis rates and $\Delta^{18}O_{LGDZ}$ during diurnal cycles in scenarios with different VPDs. Execution of such work was not feasible in this experiment.

Conversely, given the absence of VPD effects on plant growth and morphology of *C. squarrosa*, but strong effects on water fluxes in plants, we would expect that VPD-related changes of $p_{ex}p_x$ are mainly determined by the p_x component or by factors emanating from gradients of $\delta^{18}O$ in leaf water on $\delta^{18}O$ of sucrose in source leaves. However, if sucrose was actually in equilibrium with average bulk leaf blade water, then the observation of a mean $p_{ex}p_x$ of 0.48 at low VPD would suggest that p_x should be close to 1 in that scenario, if we accept the notion that p_{ex} is bounded between 0.4 and 0.5 (Helliker & Ehleringer 2002a, 2002b; Cernusak *et al.* 2005; Barbour 2007; Gessler *et al.* 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Typical diurnal time course for (a) air temperature and (b) relative humidity.

Figure S2. A photograph of a major tiller of *Cleistogenes squarrosa*.

Figure S3. Cryogenic vacuum distillation unit with a) overview of the vacuum generating and distributing parts and b) zoom view of the cold trap unit.

Figure S4. Sensitivity analysis showing the effect of uncertainty in ε_o on calculated $p_{ex}p_x$ for leaf blade cellulose of upper leaves at low (circles) and high VPD (triangles).

Figure S5. Sensitivity analysis testing the effect of varying oxygen isotope enrichment of leaf water ($\Delta^{18}O_{LW}$) on the significance of the VPD effect on $p_{ex}p_x$ of upper canopy leaves.

Table S1. Experimental plan that gives the assignment of treatments to the different growth chambers (no. 1 – 4) in the successive experimental runs (first to fourth).